The regulation of the leptin receptor in the liver

A role for PPAR-alpha?

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Abbreviations

ob	obese gene
db	diabetes gene
lepr	leptin receptor
Jak	janus kinase
STAT	signal transducer and activator of transcription
APOE2ki	apolipoprotein E2 knock in
SOCS	cytosolic suppressors of cytokine signalling
POMC	proopiomelanocortin
NPY	neuropeptide Y
AgRP	agouti-related protein
PTP	protein tyrosine phosphatase
FF	fenofibrate
PPAR	peroxisome proliferator-activated receptor
DBD	DNA binding domain
LBD	ligand binding domain
RXR	retinoid X receptor
PPRE	peroxisome proliferator response element
TZD	thiazolidinediones
PPARαko	peroxisome proliferatior-activated receptor α knock out
Acyl-CoA	acyl-coenzyme A
ACOX1	acyl-coenzyme A oxidase
HFD	High-fat diet
Cyclo	cyclophillin A
FFA	free fatty acids
TG	triglycerides
Chol	cholesterol

Preface

During my 6 month internship at the department of Molecular Genetics, I realized that research isn't something you do on your own. Therefore I would like to thank all the people who helped me in any way with my project.

First of all I would like to give a special thanks to 'the leading man' Kristiaan Wouters, without you none of this would have been possible. I can't imagine that I could have encountered a person who would have teached me more in these couple of months than you did! You gave me the freedom I needed to become more confident and aware of my capabilities. I couldn't be more grateful for that, because that isn't a gift you get everyday. Not only did I learn to think in a scientific manner, you were always there for me when I needed advise. Besides this, I will never forget the moments when you tried to cheer me up, with your great sense of humour.

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Of course I can't forget to thank my friends who supported me a lot during the writing of my thesis: Hilde Jacobs, Marijke Lemmens, Eveline Strackx, Dorien Merken, Tim Vanmierlo, Kris Bouchet and Olivier Segers.

And last but not least I also would like to take this opportunity to thank my parents. You are the ones that gave me the chance to study and become the person I am today. You were always there for me, believing in me. I probably didn't always show it, but I am truly grateful for this.

Abstract

Introduction: A recent study, investigating hepatic gene expression in the hyperlipidemic APOE2 knock in (APOE2ki) mouse model, revealed an up-regulation of leptin receptor upon high-fat diet enriched with fenofibrate, an agonist of peroxisome proliferator activated-receptor α (PPARα). It was shown that the synthethic hypolipidemic drug fenofibrate induced a marked decrease in cholesterol and triglyceride levels of these mice. These observations indicate that the nuclear receptor PPARα is possibly involved in the regulation of the expression of the leptin receptor in the liver. Accordingly, our hypothesis is that the combination of high-fat diet with fenofibrate leads to an increased expression of the leptin receptor in the liver, possibly mediated via PPARα.

Materials and methods: To determine whether hepatic leptin receptor up-regulation is dependent on PPARα, gene expression was assessed in both APOE2ki mice and mice with a liver-specific deletion of PPARα, that were fed a high-fat diet with or without co-administration of fenofibrate. Subsequently, liver slices were cultured with fenofibrate to asses whether regulation of the leptin receptor is the result of a cellular or a systemic effect. To investigate whether the increased mRNA production of leptin receptor in the liver is coupled to an increase in protein levels, immunostaining of hepatic leptin receptor was performed. The expression of leptin receptor was also validated in other tissues.

Results: In response to the combination of high-fat diet with fenofibrate, APOE2ki mice show an up-regulation of the leptin receptor in the liver. PPARα knock out mice, did not reveal an increased expression of the leptin receptor upon fenofibrate-treatment. Incubation of liver slices with fenofibrate was not very successful, as the RNA concentration in the slices rapidly diminished. Furthermore, immostaining of liver slides resulted in a brown smear, therefore it was not possible to detect the leptin receptor on protein level. Adipose tissue also revealed expression of the leptin receptor.

Conclusions: These results are in line with the previous study and indicate the importance of PPARα for the regulation of the leptin receptor in the liver. How these two factors are coupled to each other, and what the exact role of fenofibrate is in this whole process, still remains to be elucidated.

1 Introduction

1.1 Leptin

Leptin is the product of the obese (ob) gene, initially discovered by Zhang et al. (1994), using the positional cloning technique. The gene is localized on chromosome 6 in mice and chromosome 7 in humans, and the encoded protein shows a high degree of homology between species [1]. For example, human leptin is 84% identical to mouse leptin and 83% identical to rat leptin [2]. Mutations in this ob gene revealed the key role of leptin in energy balance [1]. Mice carrying a homozygous loss of function in the ob genes show early onset obesity, hyperphagia, hypothermia, hyperinsulinemia, hyperglycaemia and other metabolic and neuroendocrine disorders (fig 1) [1-3].

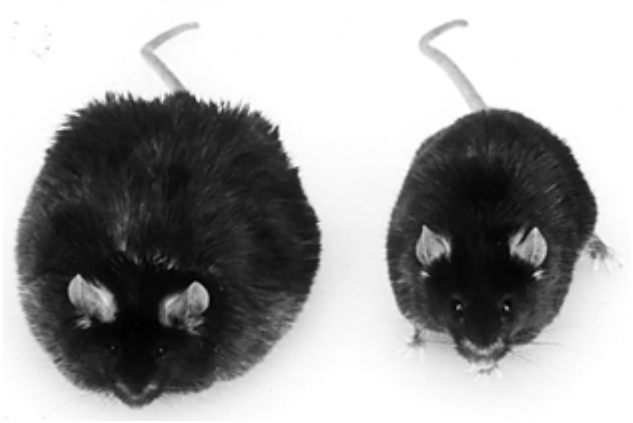

Figure 1. control mice (right) and ob/ob mice (left). Ob/ob mice are massively obese and weigh three times more than normal mice (retrieved Fromwww.wellesley.edu[58]).

In humans, ob mutations are also characterized by morbid obesity and hyperphagia. Unlike mutant mice, hypothermia, hyperinsulinemia and hyperglycaemia were not detected in humans [4].

However, human ob gene mutations are relatively rare, with only a few cases reported to date [2].

Leptin is a 16kDa non-glycosylated molecule that communicates the status of body energy stores to the central nervous system, regulating appetite, metabolic rate, and neuroendocrine function [3,5]. It is primarily secreted by white adipose tissue and can thus be considered an adipocytokine. Both in humans and in other mammals, circulating levels of leptin are correlated with the body-mass index and the amount of total body fat (fig 2) [6-7].

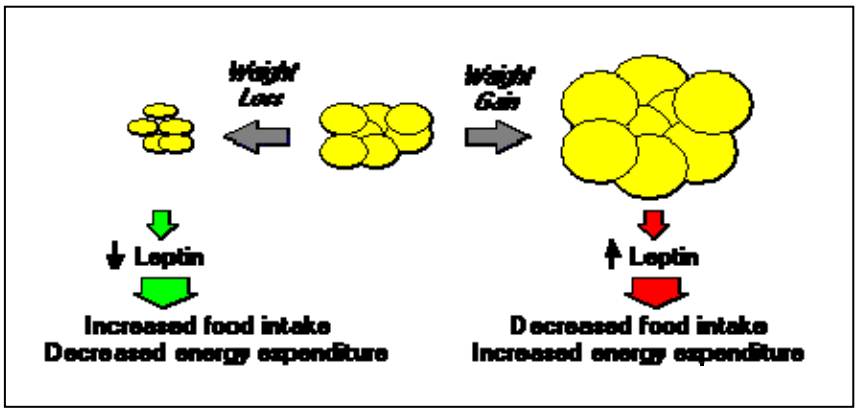

Figure 2. The amount of leptin expressed by adipocytes correlates well with the lipid content of the cells. Once synthesized, leptin is secreted through a constitutive pathway and not stored in the cell. (retrieved from http://arbl.cvmbs.colostate.edu[59])

However white adipose tissue is not the only source of leptin production. Other cell types: gastric mucosa, skeletal muscle, mammary epithelium, placenta, bone marrow, pituitary, hypothalamus [8-10], and also primary cultures of osteoblasts (to promote bone mineralization) produce substantial amounts [11].

Leptin is secreted into the blood where it partially binds to plasma proteins [2]. By binding to specific receptors, expressed on the cell surface of various tissues, leptin can exert its hormonal effects [2].

Today leptin is viewed as an anti-steatotic peptide, playing a key role in the regulation of lipid metabolism [12]. Unger (2000) referred to it as an antilipogenic hormone since it prevents the storage of free fatty acids in non-adipose tissue. By this redistribution of free fatty acids to adipose tissue, leptin protects vulnerable

tissues from the deleterious effects of fatty acid overloading and the consequences of non-oxidative metabolism [12-13].

In summary, leptin plays an important role in energy homeostasis by decreasing food intake and increasing energy expenditure via hypothalamic centres, affecting feeding behaviour and activating the sympathic nervous system.

1.2 The leptin receptor

Leptin exerts is effects through a member of the Class I cytokine receptor family, which also includes the receptor for interleukin 6. Members of this family all share characteristic extracellular motifs of four cysteine residues and a 4-aminoacid motif Trp-Ser-X-Trp-Ser, but differ in the number of the fibronectin type III domains [14].
The extracellular region of the leptin receptor contains four fibronectin type III domains and two cytokine receptor domains [15-16].

Encoded by the diabetes (db) gene, the leptin receptor (lepr) has several alternatively spliced isoforms (leprA, B, C, D and E), all sharing an identical extracellular leptin-binding domain but differing in the length of their intracellular parts (fig 3) [9,15]. The isoforms can be classified into three classes: long, short and secreted. Besides the extracellular and the transmembrane domain, the short and the long isoforms also share the proximal 29 intracellular amino acid residues, including the box 1 motif (important for signalling, see below) [15]. The additional cytoplasmic region differs in length. The leprB contains 301 intracellular amino acids, whereas the short isoforms, leprA, leprC and leprD have 34, 32 and 40, respectively. The leprE isoform lacks the transmembrane and cytoplasmic parts and functions as a soluble receptor [5,15].

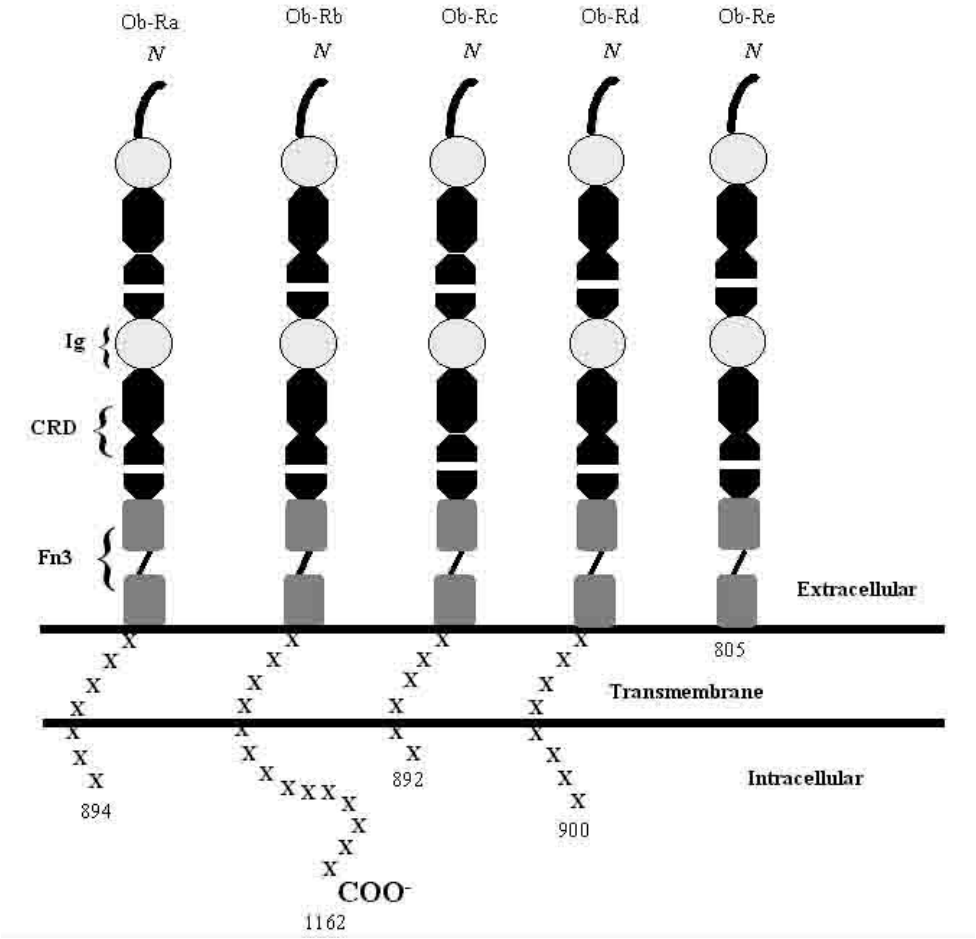

Figure 3. Leptin receptor isoforms in mouse. There are five different isoforms of the leptin receptor in mouse Ob-Ra – Ob-Re (leprA-leprE). All share an identical extracellular, ligand-binding domain but they differ at the C-terminus. Four of the five have transmembrane domains, but only the leprB encodes all protein motifs capable of activating the Jak/STAT signal transduction pathway (retrieved from www.bioscience.org[60]).

The long form of the leptin receptor (leprB) distinguishes itself from other isoforms in that it is the only isoform with full length signalling capabilities and contains various motifs necessary for interaction with other proteins and subsequent pathway activation [8-9]. Since this form was initially detected in the hypothalamus, a brain region which is known to be involved in regulation of feeding behaviour and energy balance, it was believed that leptin signal transduction is primarily regulated in the hypothalamic centres [17-18]. However, discovery of leprB in other tissue types, such as the lung, kidney, adipocytes, endothelial cells, mononuclear blood cells, stomach, muscle, liver, pancreatic islets, osteoblasts, endometrium, placenta and umbilical cord [19-24], led to the believe that these tissues are also capable of activating the

leptin signal transduction pathway, independent of the central nervous system [16,25].

The leprA and leprC isoforms are highly expressed in the choroid plexus and microvessels, where they function in the uptake and efflux of leptin from the cerebrospinal fluid as well as in receptor-mediated transport of leptin across the blood-brain barrier [3,15]. According to Hoggard et al. (1997) the leprC isoform plays also another role. Together with the leprD, they are implicated to function in the clearance of leptin from the circulation. The leprE variant, having no intracellular domain, is a putative soluble receptor [15,26]. The soluble lepr circulates in the blood and is capable of binding leptin with a high affinity [29-30]. So, this splice variant plays a key role in the regulation of the plasma levels of free leptin, the biologically active form [31].

Mutations in the lepr result in an obese phenotype identical to that of mice harbouring a leptin mutation [2-3]. In db/db mice, a premature stop codon is inserted in the 3' end of the leprB mRNA transcript, leading to synthesis of a truncated receptor that replaces the leprB isoform with the leprA isoform, which is incapable of mediating janus kinase and signal transducer and activator of transduction (Jak/STAT) signalling [4,26]. As a result of this, the lepr lacks functional activity and leads to obesity and diabetes. Mice carrying lepr mutations display elevated leptin levels and are not capable of responding to leptin. In humans, mutations in the lepr gene are infrequent. Both humans and rodents lacking functional lepr show early onset obesity and hyperphagia [2,3].

1.3 Hyperlipidemic mouse models

As already mentioned above, both the ob/ob and db/db models have been investigated to date, hoping to bring clarity to the increasing problems associated with perturbations in lipid metabolism, in which leptin and its receptor are key players. However, since both these mouse models lack the capacity to activate the signal transduction pathway [3], which normally is triggered upon binding of leptin to its receptor, it seems that these models are not ideal to study the mechanisms underlying these phenomena.

Apolipoprotein E2 (APOE2) is a relatively common recessive allele, which is the main cause of type III hyperlipidemia in humans. In the APOE2 knock in (APOE2ki) mouse, the endogenous mouse APOE gene has been replaced by the human APOE2 gene, and it is shown that this mouse develops severe diet-induced hyperlipidemia. In contrast with the other models, this mouse model shows normal expression of leptin and its receptor, therefore the signal transduction pathway will not be disturbed [32].
So, this model is not only more arranged to study the role of possible contributing factors to hyperlipidemia, but also to examine factors that might improve these perturbations in lipid metabolism, since it still displays leptin-induced signal transduction.

1.4 The primary signal transduction pathway of leptin: Jak/STAT signalling

The Jak (Janus kinase)/ STAT (signal transducer and activator of transcription) pathway is the main leptin receptor signalling pathway [9,33]. Upon leptin stimulation, the leptin-bound receptor undergoes ligand-induced conformational changes which lead to the recruitment of Jak2 proteins to an important protein sequence in the membrane-proximal domain of the receptor, the box1 motif [5,9,16,33]. Bahrenberg et al., (2002) and Kloek et al. (2002) demonstrated that this box1 motif and its adjoining amino acids are essential for Jak activation [5,9]. Subsequently, the Jak proteins transphosphorylate each other, as well as certain tyrosine residues of the receptor. In the case of the leprB, these residues are Tyr985 and Tyr1138 [16,33]. By this they provide a docking site for downstream signalling molecules such as the STAT3 proteins. These docking sites are located on another crucial protein sequence, the box2 motif [9,16,33]. As a next step, recruited STAT3 molecules become tyrosine-phosphorylated by Jaks, which leads to their dissociation from the receptor [16,33]. After dimerisation, the STAT3 proteins translocate into the nucleus where they act as transcription factors by binding to the promoter sequence of target genes (Fig 4) [16,33]. In the brain, these target genes are neuropeptide Y (NPY), agouti-related protein (AgRP), proopiomelanocortin (POMC), and suppressor of cytokine signalling 3 (SOCS3) [34]. However, the target genes activated in the liver are still unknown at the moment.

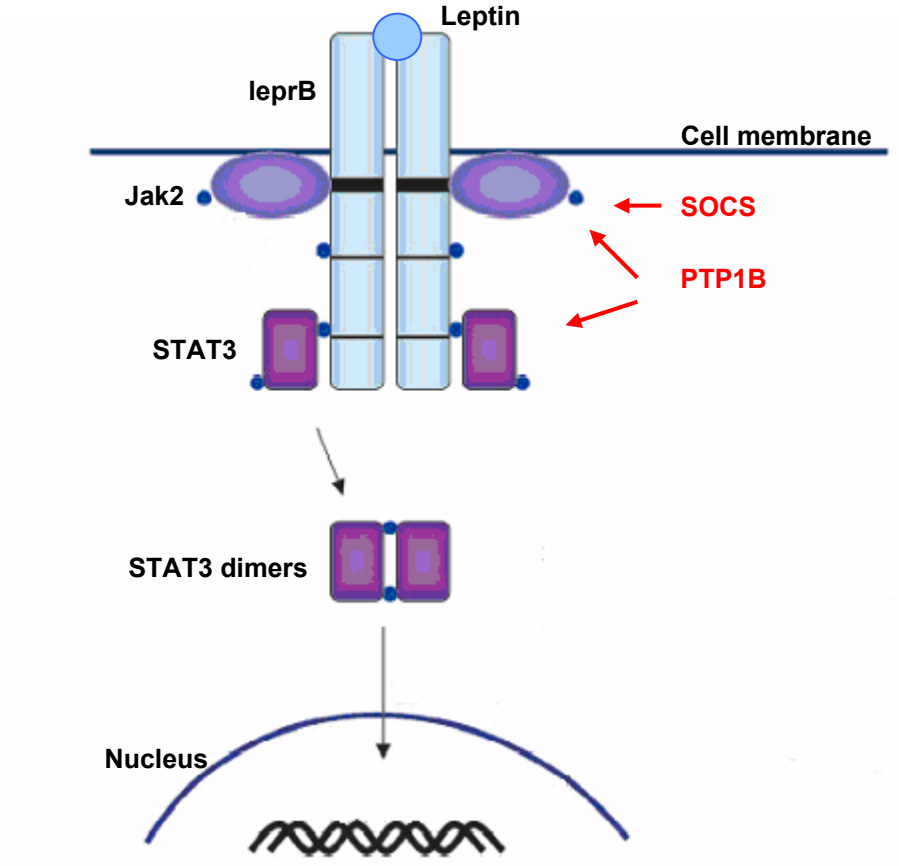

Figure 4. Leptin induced Jak/STAT signal transduction pathway. Upon leptin binding, a conformational change takes place that allows juxtaposition of Jaks, which then become activated and are able to tyrosine-phosporylate other Jaks and tyrosine residues on the receptor. Phosphorylation of Tyr^{138} allows association of STATs, which then become substrates of receptor-associated Jaks. Phosphorylation of STATs lead to their dissociation from the receptor and the formation of active dimers, which translocate to the nucleus to regulate gene expression, binding to the promoter regions of target genes.. Negative regulators are shown in red (adapted from Hegyi et al. (2004)[61]).

There is clearly a need for tight control of the leptin signal transduction pathway, but the precise molecular events involved in the regulation of transmission, duration and termination of the leptin signal transduction pathway remain to be fully elucidated.

It is believed that the leptin signalling pathway is under control and can be terminated by a number of feedback mechanisms, including (i) internalization and degradation of the leptin receptor-ligand complex (ii) dephosphorylation and inactivation of signalling proteins mediated by protein tyrosine phosphatases (PTP) and (iii) feedback

inhibition by negative regulators such as the cytosolic suppressors of cytokine signalling (SOCS) [35].

The first feedback mechanism, capable of terminating the signalling pathway, leads to internalization of the receptor-ligand complex, possibly via the ubiquitin-proteasome pathway [35].
Furthermore, it is also assumed that protein tyrosine phosphatases, such as protein tyrosine phosphatase 1B (PTP1B) are capable of controlling cellular cytokine responses [36]. Results from studies of PTP1B-deficient mice and cell lines derived from these mice suggest a key role of PTP1B in the control of the leptin-induced signal transduction pathway, since these animals are resistant to diet-induced obesity and experience increased leptin signalling [37-38]. These studies pointed to JAk2 as possible substrate of this PTP. Lund et al. (2005) investigated if PTP1B mediates the cessation of leptin signal transduction by direct targeting of signalling molecules such as JAK2 and STAT3. They provided evidence that PTP1B induces a direct and selective dephosphorylation of both JAK2 and STAT3 proteins, resulting in an approximately 90% reduction level after 20 min [35].

The SOCS proteins appear to act in a negative feedback loop to suppress signal transduction from cytokine receptors [39,40]. In the absence of stimulation, little or no expression of any of the SOCS genes was detectable by PCR. SOCS gene expression is induced by cytokines both in vitro and in vivo, and, once produced, they act directly on components of the cytokine signalling pathways to switch them of. Starr et al. (1997) demonstrated that the effect of SOCS1 appeared to be specific for the JAK-STAT pathway because there was no reduction in the overall level of tyrosine-phosphorylated proteins noticed upon stimulation of the signalling pathway in SOCS1 expressing cells.
But, the expression of other SOCS genes was also increased in the liver upon stimulation of the signalling pathway, including SOCS3, SOCS2. It has to be further elucidated whether these genes also function in the termination of the leptin signal transduction pathway in the liver.
Once expressed, it is proposed that the SOCS proteins inhibit the activity of Jaks and so reduce the phosphorylation of STATs, thereby suppressing the signal transduction. Importantly, inhibition of STAT signalling will, over time, lead to a

reduction in SOCS gene expression, which allows the cells to regain there responsiveness to cytokines [41-42].

1.5 Peroxisome proliferator- activated receptors

A previous study using the APOE2ki mouse model, has shown an up-regulation of the lepr in the liver upon high-fat feeding enriched with fenofibrate (FF), an agonist of peroxisome proliferator-activated receptor alpha (PPARα). Also, it was seen that FF was capable of causing a marked decrease in cholesterol and triglyceride levels of these mice [43]. These observations indicate that the transcription factor PPARα plays a role in the diet-induced perturbations in lipid metabolism, and that this receptor possibly plays a role in regulating the expression of the lepr in the liver.

Up to now, three PPAR subtypes have been identified: PPARα, PPARγ, and PPARβ/δ, constituting a subfamily of nuclear receptors. These receptors function as lipid sensors and are important regulators of nutrient metabolism and energy homeostasis by modulating the expression of genes involved in metabolic events [44-46].

The PPAR proteins consist of five different domains: a NH_2- terminal region termed A/B domain, a DNA binding domain (DBD) (C domain), a hinge region (domain D), a ligand binding domain (LBD) (domain E), and a F domain [45,47].
Following agonist binding to the LBD in the cytoplasm, PPARs become activated and bind to another transcription factor, the retinoid X receptor (RXR). The formation of heterodimeric complexes induces the transport of this complex into the nucleus, where it binds via its DBD to a specific sequence: the peroxisome proliferator response element (PPREs). This sequence is localized in the promoter region of the target gene, leading to activation of transcription of the regulated gene [45,47-48].

Several endogenous ligands of PPAR have been identified. In fact, all three PPAR subtypes are activated by fatty acids, especially polyunsaturated fatty acids, as well as various eicosanoids (derivates of FFA).

Furthermore these receptors are also targets of synthetic drugs, designed for treatment of metabolic disorders such as type 2 diabetes mellitus and atherosclerosis. The anti-diabetic thiazolidinediones (TZD) such as rosiglitazone and pioglitazone are PPARγ agonists implicated in improving the insulin resistance in diabetes. But also the synthetic ligands of PPARα have therapeutic actions. The fibrate hypolipidemic agents such as FF and gemfibrozil have anti-atherosclerotic actions [48-50].

PPARα is highly expressed in liver, heart, skeletal muscle and kidney, tissues that extract most of their energy from lipids. Fatty acids are released from fat depots and find their way to the liver where they subsequently are taken up and oxidized and metabolized into ketone bodies, providing energy for the peripheral tissues. The important role of PPARα in energy homeostasis has been confirmed by the PPARα knock out (PPARαko) mouse, which displays a phenotype characterized by hyperlipidemia, liver steatosis, hypoglycaemia and hypoketonemia [49].
Dislipidemia, characterized by elevated blood levels of triglycerides, together with a decreased amount of high-density lipoprotein cholesterol, often predicts a high chance for development of cardiovascular disease [49-50].
Therefore synthetic agonists are designed, decreasing the plasma triglyceride levels and increasing the high-density lipoprotein cholesterol content [49].
Decreasing the plasma triglyceride levels is mediated by increasing the amount of lipid uptake, activation, and catabolism through transcriptional modulation of genes controlling these processes: acyl-coenzyme A (acyl-CoA) synthetase, acyl-CoA oxidase (Acox1), acyl-CoA dehydrogenase and carnitine palmitoyltransferase I.
The increase in high-density lipoprotein cholesterol content is partially mediated by augmentation of the hepatic apolipoprotein A-I and A-II production, important components of high-density lipoprotein cholesterol [49-50].
PPARγ is present in high concentrations in adipose tissue, and to a lesser extent in spleen, cells of the hemopoietic system, liver and skeletal muscle [49].
This receptor subtype not only functions in adipocyte differentiation, but also promotes the storage of lipids in adipocytes. For that reason it is believed that the PPARγ ligands exert their effect primarily through adipose tissue. It has been demonstrated that these ligands alter the expression of genes involved in lipid uptake, lipid metabolism, and insulin action in adipocytes. The result of ligand binding

to these receptors is improved adipocyte insulin signalling, lipid uptake and anabolic lipid metabolism, and attenuated lipolysis and free fatty acid (FFA) release. As a consequence of this, the lipid content in the adipose tissue rises while the circulating FFAs diminish. By redistributing the lipids away from the liver and the skeletal muscle, the two tissues that are primarily responsible for insulin-mediated glucose disposal and metabolism, PPARγ agonists improve hyperglycaemia by reversing lipotoxicity-induced insulin resistance [48-50].
In addition to the alteration of fat disposal, PPARγ ligands are also known for modulating the endocrine activity of adipose tissue by regulating the synthesis and secretion of adipokines, influencing insulin signalling in hepatic and peripheral tissue. For example, PPARγ activation leads to an up-regulation of adiponectin, which ameliorates insulin sensitivity in the liver and skeletal muscle [48-49].

PPARβ/δ is ubiquitously expressed and the small number of ligands makes this PPAR subtype the least understood. It is believed that PPARβ/δ activation leads to an increased expression of genes in skeletal muscle that promote lipid catabolism and mitochondrial uncoupling, thereby increasing β-oxidation of fatty acids in skeletal muscle [49].

1.6 Experimental setup

Since imbalance between energy intake and expenditure, likely resulting in malfunctioning of several organs, is an ever increasing problem in our western society, there can not be enough research done concerning this subject.
However, initial discovery of leptin led to the believe that the signal transduction of this hormone was centrally mediated, for the reason that its receptor was detected in hypothalamic centres controlling food intake and energy balance [3]. Discovery of lepr in other tissues implicated that this receptor could also mediate important actions in peripheral tissues. This thought was further confirmed by a study showing an up-regulation of the lepr in the liver of the hyperlipidemic APOE2ki mouse model upon high-fat diet enriched with FF, an agonist of PPARα [43]. It was shown that the synthetic hypolipidemic drug FF induced a marked decrease in cholesterol and triglyceride levels in these mice [43]. These observations indicate that the nuclear

receptor PPARα is possibly involved in the regulation of the expression of the lepr in the liver. Accordingly, our hypothesis is that the combination of high-fat diet with FF leads to an increased expression of lepr in the liver, possibly mediated via PPARα.

In the present project we will use the APOE2ki model, to validate the findings of the study mentioned above and to examine which isoforms of the lepr show up-regulation in the liver upon high-fat feeding, supplemented with FF.

Next, we want to determine whether hepatic lepr up-regulation is indeed dependent on PPARα. Therefore we will make use of another mouse model, the PPARαko model, which exhibits a liver-specific deletion of PPARα [51].

Next, we will determine whether the regulation of the lerp is the result of a cellular effect or a systemic effect. To verify this, we will make use of a new in vitro model. Slices of APOE2ki liver tissue will be cut and incubated with a varying concentration of FF. In case of a systemic effect, we expect to see no difference in regulation of the lepr, since this organ is no longer in contact with its normal environment.

As a next step, we want to investigate whether the increased mRNA expression of the lepr in the liver, is linked to an increase in protein levels. This will be done, by immunostaining of the lepr in APOE2ki liver tissue.

Finally, we will examine whether other tissues of the APOE2ki mice also display the same pattern of lepr expression, or that it is specific for liver tissue.

2 Materials and methods

2.1 Animal handling and organ isolation

APOE2ki mice and PPARαko mice, both with a C57BL/6 background strain, were housed under standard conditions and given access to food and water ad libitum. All animal procedures for this study were carried out according to the Dutch laws, approved by the Committee for Animal Welfare of Maastricht University.

90 homozygote female APOE2ki mice of 13 weeks old were divided into 9 groups with a sample size of 10 per group. A first group was kept on standard chow during the whole experiment. 4 groups received a high-fat diet (HFD), containing 17% casein, 0.3% DL-methionine, 34% sucrose, 14,5% cornstarch, 0,2% cholesterol, 5% cellulose, 7% CM 205B, 1% vit 200, 21% butter (diet 1635, Scientific Animal Food and Engineering, Villemoisson-sur-orge, France) for 2, 4, 7 and 21 days. The last 4 groups were put on a HFD that was supplemented with 0.2% FF (F6020, Sigma Aldrich, Zwijndrecht, the Netherlands) for 2, 4, 7 and 21 days.

18 homozygote PPARαko mice of 13 weeks old were divided into 3 groups. A first group of 6 mice received standard chow during the entire experiment. A second group was given a HFD (17% casein, 0.3% DL-methionine, 34% sucrose, 14,5% cornstarch, 0,2% cholesterol, 5% cellulose, 7% CM 205B, 1% vit 200, 21% butter) (diet 1635, Scientific Animal Food and Engineering, Villemoisson-sur-orge, France) for 2 days. The last group received a HFD to which 0.2% FF was added (F6020, Sigma Aldrich, Zwijndrecht, the Netherlands) for 2 days.

Blood samples were withdrawn from the tail after a 4 hour fast and collected in glass capillaries, coated with heparin (to prevent blood from coagulating to quickly) and diethyl p-nitro phenyl phosphate (to prevent the catabolism of free fatty acids (FFA)) (D9286, Sigma Aldrich, Zwijndrecht, the Netherlands). Mice were sacrificed by cervical dislocation. Tissues were isolated and snap-frozen in liquid nitrogen and stored at -80°C. A portion of each liver was fixed in formalin for histology.

2.2 Real time quantitative PCR

Total RNA was extracted from the liver, red and white muscle and visceral and uteral adipose tissue. The frozen tissue sections were homogenized in TRI Reagent (T9424, Sigma Aldrich, Zwijndrecht, the Netherlands) with the MiniBeadBeater (Biospec products, Bartlesville, OK, USA). Visceral and uteral adipose tissue needed an additional centrifugation step to remove the excess insoluble fat. Subsequently 200 µl of chloroform was added per 1 ml of TRI Reagent used. After incubation and high-speed centrifugation (13k rpm) at 4°C for 15 minutes, the mixture is separated into three layers: a red organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing the RNA). The aqueous phase was recovered and the RNA was precipitated in 0.5 ml of isopropanol. After removing the supernatant, the RNA pellet underwent a washing step in 0.5 ml 70% ethanol. Thereafter, the supernatant was removed completely and the pellet was air dried for a short period before being dissolved in 50 µl of miliQ. RNA was quantified by measuring the absorption at 260 nm on a Nanodrop ND-1000 Spectrophotometer.

Approximately 1 µg of total RNA was reverse transcribed into cDNA with the iScript^{TM}cDNA Synthesis Kit (170-8891, Bio-Rad, Veenendaal, the Netherlands). The reaction protocol for this cDNA Synthesis Kit included primer annealing at 25ºC for 5 min, amplification at 42ºC for 30 min and inactivation of amplification at 85ºC for 5 min. The mix contained both random hexamer primers and oligo dt primers. The reverse transcription products were stored at -20°C for real time fluorescence quantification PCR of the target genes.

The quantitative analysis of the gene expression was carried out on an ABI Prism 7700 (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). The PCR protocol used in these amplified reactions included: enzyme incubation at 50°C for 2 min, denaturing at 95°C for 10 min, followed by 40 cycles of annealing at 95°C for 15 s, primer extension at 95°C for 15 s, and denaturing at 95°C for 2 s.

The 25 µl reaction system contained 12.5 µl of Mastermix containing the double-stranded-specific fluorescent dye SYBR Green (RT-SN2X-03+, Eurogentec, Seraing, Belgium), 1.0 µl of primer solution which contained 0.5 µl of the forward and the reverse primers, 6.5 µl miliQ and 5 µl of cDNA template. Oligonucleotide sequences of sense and antisense primers are seen in table 1.

A standard curve was generated for each gene. Data were analysed with SDS 1.9.1 (Applied Biosystems). The final result was expressed as relative expression by comparing the amount of target gene to the cyclophillin A gene (Cyclo).

Table 1. Primer sequences for Real-time quantitative PCR analysis

Gene Symbol	Gene name	Forward primer	Reversed primer
leprA	Leptin receptor A	5'-GGCACAAGGACTGAATTTCCAA-3'	5'-GGTCATGAGAGACTTCAAAGAGTGTC-3'
leprB	Leptin receptor B	5'-AAC CCCAAGAATTGTTCCTGG-3'	5'-TCTGCATGCTTGGTAAAAAGATG-3'
leprC	Leptin receptor C	5'-TCTCAAAAGCTGGGTTTGGG-3'	5'-TAAGGGAGCGAACTTTGTTTTCTCT-3'
leprD	Leptin receptor D	5-'CCTGGGCACAAGGACTGAATT-3'	5'-AATGAT GGTGAAAGAGACATTGACAG-3'
leprE	Leptin receptor E	5'-CATGGATTA GTATGACATGTAGACTGG-3'	5'-TGTCATTAAATG ATTTATTATCAG-3'
Cyclo	Cyclophillin A	5'-CAAATGCTGGACCAA ACACAA-3'	5'-GCCATCCAGCCATTCAGTCT-3'
Acox1	Acyl-coenzyme A oxidase1	5'-CTTGAGGGGAACATCATCACA-3'	5'-GCCAAGGGTCACATCCTTAAAGT-3'

2.3 Plasma parameters

Plasma was obtained from the blood after high-speed centrifugation for 5 minutes. Total plasma Cholesterol, Triglyceride and Free Fatty acids were measured (1489232, Cholesterol CHOD-PAP, Roche, Almere, the Netherlands; 994-75409, NEFA-C, Wako, Neuss, Germany) according to manufacture's protocols on a Benchmark 550 Micro-plate Reader (170-6750XTU, Bio-Rad, Veenendaal, the Netherlands).

2.4 Liver parameters

Frozen liver samples were homogenized in 1 ml SET buffer (Sucrose 250mM, EDTA 2mM and Tris 10mM) with the MiniBeadBeater. For cell destruction the tissue samples underwent two freeze-thaw cycles. To assure complete cell destruction, suction up and down through a very fine needle was carried out. Cholesterol, Triglyceride and Protein content was determined in the liver homogenates (1489232, Cholesterol CHOD-PAP, Roche, Almere, the Netherlands; 1488872, Triglyceride GPO-PAP, Almere, the Netherlands; Protein BCA, Pearson) according to manufacture's protocol on a Benchmark 550 Micro-plate Reader (170-6750XTU, Bio-Rad, Veenendaal, the Netherlands).

2.5 Histology and immunohistochemistry

Formalin-fixed liver tissues were dehydrated and imbedded in paraffin. Tissue specimens were cut with a thickness of 4 µm and stained with hematoxylin and eosin (H&E) for histological analysis. A pathologist who was blinded to the experimental conditions, examined all sections for inflammation and fat accumulation. Inflammation was evaluated by giving a score from 0 to 3 as follows: 0, no inflammation; 1, mild; 2, moderate; 3, severe. The degree of fat accumulation was scored in the same matter as the inflammation: 0, no fat accumulation; 1, mild; 2, moderate; 3, severe.

2.6 Cutting liver slices

After sacrificing mice, livers were isolated and placed in ice cold PBS. With a coring tool, tissue cores of 5 mm diameter were obtained. These tissue cores were placed in the Krumdieck Tissue Slicer, immersed with cold medium (500 ml Dmem (4500 mg/Lglucose, L-glutamine, 25mM hepes, private), 1ml glucagon (3,5 µg/ ml), 375 µl hydrocortisone (10 mg/ ml) and 5 ml pen/strep), to bring about live slices. A motor moves the tissue core past the oscillating blade, generating slices of a thickness varying between 100 and 200 µm.

Liver slices were placed in a 24 wells plate that contained the same medium as mentioned above, supplemented with insulin. For culturing, the plate is positioned in a stove of 37°C and under continuous movement.

Each well contained one tissue slice and was incubated with a concentration of FF of 50 µM or 250 µM. Control samples were treated with DMSO only (vehicle). As a negative control, some liver slices were incubated with medium only.

The slices were incubated for 2h, 4h or 8h. Thereafter, RNA was isolated from the liver samples and gene expression of the leprB isoform and Acox1 was measured with qPCR. A portion of the liver slices was fixed in formalin for histology.

2.7 lepr immunoreactivity

Frozen liver sections at 7 µm thick were cut and placed on poly-L-lysine-coated glass slides. Sections were fixated with 4% paraformadehyde in 0.1 M Phosphate buffered saline (PBS) for 2 min. Thereafter the slides were washed thoroughly with tris buffered saline (TBS) (Tris 60.6 g + NaCl 88.0 g to 1000 ml distilled water, pH 7.6). Endogenous peroxidase activity was blocked by incubating the slides for 5 min with 0.1% H_2O_2 dissolved in TBS. An avidin and biotin blocking step was executed to block the high endogenous avidin and biotin expression. Detection of the lepr was performed using three different goat polyclonal antibodies, directed against the N-terminal, the P983 phosphorylated form, and the P1138 phosphorylated form

respectively. To detect the leprB isoform, a mouse monoclonal antibody directed against the C-terminal was employed. All primary antibodies were applied at a dilution of 1:500 and incubated for one hour in a humid chamber. Control samples were incubated in 0.05 M TBS-T (TBS10x 10.0 ml + tritonx100 0.33 ml to 90 ml distilled water), without the primary antibodies. After extensive washing (three times for 5 min) with 0.05 M TBS and TBS-T, slides were incubated with 1:2000 biotinylated donkey anti-goat IgG antibody or a goat anti-mouse IgG antibody for 1 hour in a humid chamber. Slides were washed with TBS and subsequently incubated with preformed avidin- horseradish peroxidase complex for 15 min. Immunostaining was developed by adding DAB substrate to the slides, which resulted in a brown precipitate. To optimize the colour development, the liver slides were washed with Tris-HCl before the administration of the DAB solution. Washing the slides thoroughly with TBS and dehydrating them in an ethanol line finished the staining procedure. A cover slip was mounted with entallan. Throughout the procedure, care was taken to prevent tissue sections from drying to avoid artefacts and false positives.

2.8 Statistical analysis

Data were analysed using Graphpad Prism 4.0. Groups were compared using Mann-Whitney 2-tailed non-paired t-tests. Data is expressed as means ± SEM and considered significant at $p < 0.05$.

3 Results

3.1 Expression of different lepr isoforms in liver tissue

A previous study, investigating gene expression analysis in liver of APOE2ki mice, demonstrated an increased expression of many lipid metabolism genes, such as genes involved in fatty acid β-oxidation and lipid transport upon HFD, enriched with FF [43].

Among these genes, lepr showed a 3.6 fold up-regulation in expression level after only 2 days of FF treatment [43]. To clarify which lepr isoform corresponded to this increase, it was checked what the probe on the array detected. These qPCR results revealed the expression of three splice forms of the lepr, respectively leprA, leprB and leprC (data not shown). The two other splice forms, leprD and leprE, could not be detected (data not shown).

3.2 Regulation of lepr mRNA production in the liver

To validate the expression pattern that was found in the microarray data, liver RNA of individual APOE2ki mice that were fed HFD with FF, was used to check lepr expression. In these mice, leprB and C splice variants (fig 5B and 5C) showed a similar expression pattern, while leprA (fig 5A) showed a slightly less pronounced up-regulation upon FF administration (3 fold vs 8 fold). These results clearly verify the micro-array data.

Since FF is an agonist of PPARα, we wanted to asses whether the up-regulation of the lepr upon 2 days administration of FF is dependent on PPARα. Therefore we examined the expression of the lepr both in APOE2ki mice and in mice with a liver-specific deletion of PPARα.

In the PPARαko mice FF- treatment did not induce an increase in the expression level of all three lepr isoforms. In stead, there was a substantial decrease in the expression of all three isoforms. Furthermore, addition of a HFD lowered the

expression of the leprB and C isoforms even further. This was not the case for the leprA.

Furthermore, Acox1, a well-known target gene of PPARα involved in lipid catabolism, was not up-regulated in the PPARko mice, indicating that these mice have no PPARα expression in their liver (data not shown).

Remarkably, in PPARαko mice on chow all three lepr splice variants show a much higher expression level then APOE2ki mice on chow.

Fig 5A

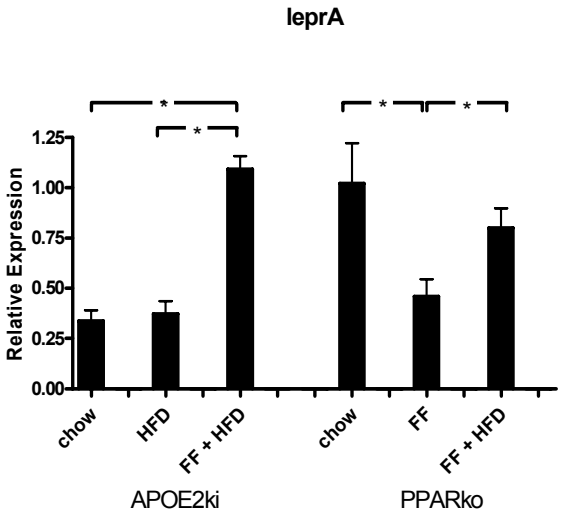

Fig 5B

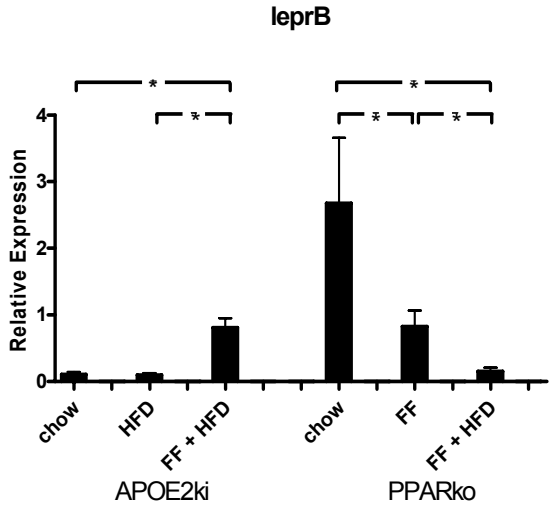

Fig 5C

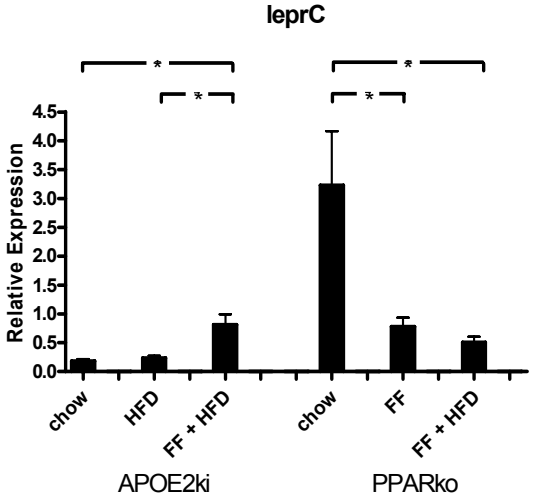

Figure 5. qPCR expression analysis of alternatively spliced leptin receptors in liver tissue of APOE2ki mice and PPARαko mice. Using lepr isoform-specific primers, the expression of the lepr isoforms was determined in liver tissue of APOE2ki and PPARαko mice. The expression of each splice variant was compared between the two mouse strains. Significant differences were shown with *.

3.3 Plasma and liver parameters of PPARαko mice

Plasma and liver parameters were examined in the PPARαko mice, to see if these parameters displayed features that could explain the substantially higher expression of lepr on chow, compared to the APOE2ki mice. HFD (containing 21% fat and 0.2 % cholesterol) supplemented with 0.2% FF, induced a nearly 2-fold increase in plasma TG levels after 2 days, while FF treatment or regular chow kept the TG at a basal level (fig 6A).

Similarly, the HFD enriched with FF induced a rise in plasma Chol levels, whereas FF completely abolished this increase and displayed Chol concentrations in the same range as regular chow (fig 6B).

Plasma FFA levels decreased significantly upon high-fat feeding with addition of FF. FF treatment lowered the concentration of FFA also, but did not reach significant levels (fig 6C).

HFD supplemented with FF increased liver TG levels markedly after 2 days, while FF treatment tended to decrease the TG levels, but not significantly (fig 6D).

Liver Chol levels displayed a significant rise upon high-fat feeding enriched with FF. FF treatment had no lowering effect on liver cholesterol levels (fig 6E).

Overall, no abnormalities could be detected. The data also confirm the absence of PPARα in the mice.

Fig 6A

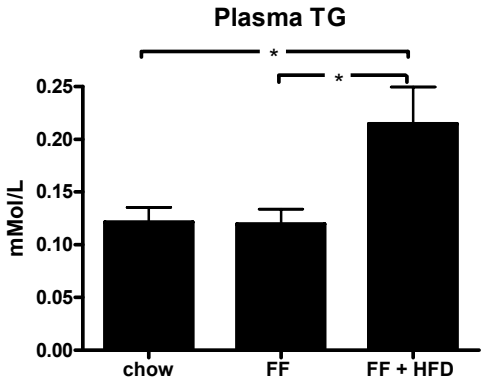

Fig 6B

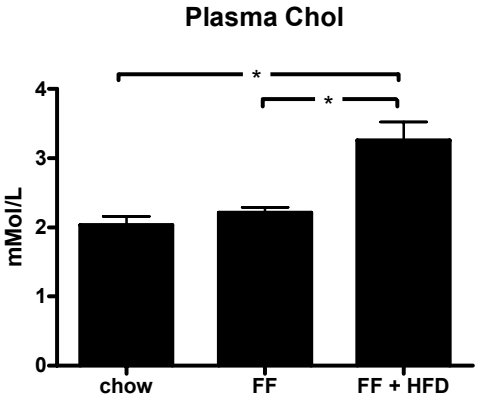

Fig 6C

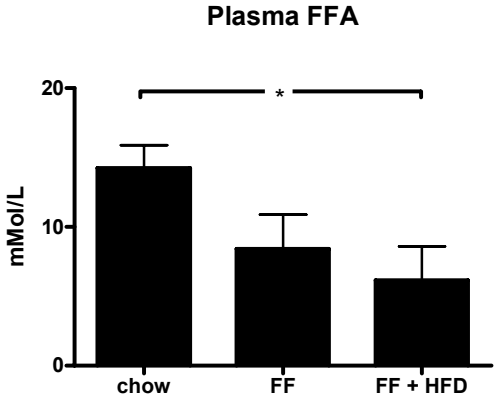

Fig 6D

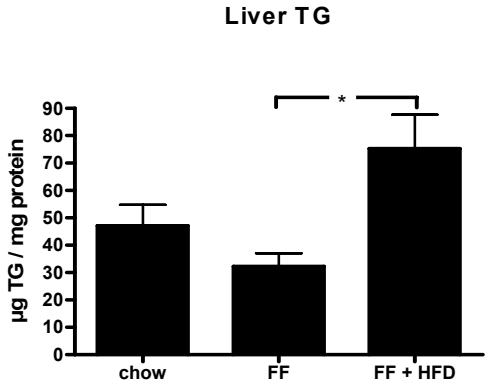

Fig 6E

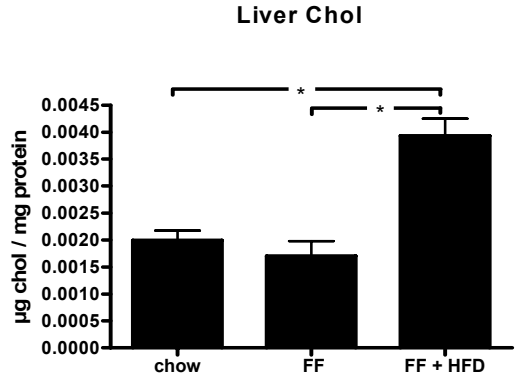

Figure 6. Lipid parameters of PPARαko mice. Plasma values are shown in fig 6A (TG), 6B (Chol) and 6C (FFA). Liver values are shown in fig 6D (TG) and 6E (Chol). Significant differences are indicated by *.

3.4 Liver histology of PPARαko mice

Since plasma and liver parameters could not explain the difference in lepr expression on chow between PPARαko mice and APOE2ki mice, H&E staining was performed to investigate whether the PPARαko mice displayed abnormal histological features. Tissue sections showed a gradual fat accumulation in the liver cells upon 2 days high-fat feeding supplemented with FF. No increase in the hepatic TG content was noticed in mice that received only FF.

No aggregates of inflammatory cells were visible in the H&E-stained liver tissues of mice fed the HFD compared to animals on the chow diet or mice receiving the FF-treatment.

Overall, no abnormal histological features could be detected that, to our knowledge, could lead to an increased expression of the lepr or altered PPARα activity.

3.5 Effects of administration of FF to liver slices

Liver slices of APOE2ki mice were cut with a thickness between 100 and 200 µm and cultured for a period of 2, 4, or 8 hours in the presence of FF or DMSO (as negative control). This method seemed ideal to investigate if the increased leprB mRNA production in the liver, upon addition of FF, is a systemic effect or a cellular effect. In the case of a systemic effect we expected to see no up-regulation of leprB after administration of FF to the medium in which the tissue slices were cultured. If the increased expression of leprB upon FF treatment was due to a cellular effect, there would be an up-regulation of leprB noticed.

Gene expression analysis of leprB was performed with qPCR. The expression of Acox1, the PPARα target gene, was also analysed as a control. Unfortunately, it seems that the quality of the RNA diminishes really quickly. The RNA concentration of untreated slices on beforehand ranged around 350 ng/µl. Upon 2 hours of culturing, the RNA concentration was already diminished to an average of 220 ng/ µl. The concentration decreased even further, reaching a relative expression of 130 ng/µl after 4 hours and 80 ng/µl after 8 hours.

However, the large decrease in expression levels of the leprB (fig 7A) is clearly depending on the duration of culturing, despite adding equal amounts of cDNA to the qPCR reaction. The same expression pattern is seen for Acox1 (fig 7B). Furthermore, even the expression of Cyclo, used as reference gene, which reaches relatively high expression levels in liver tissue, shows a marked reduction in expression upon culturing (fig 7C). Therefore, we were unable to use the data of the slices treated with FF or DMSO.

Fig 7A

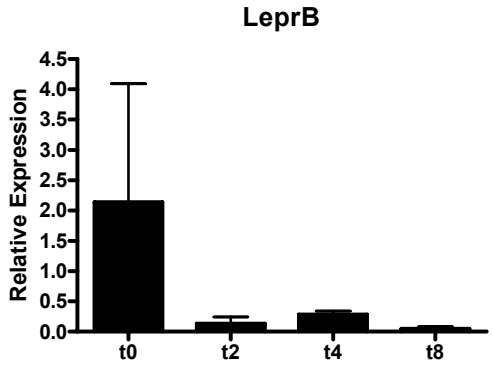

Fig 7B

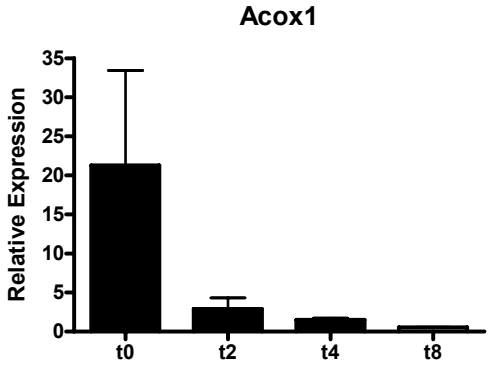

Fig 7C

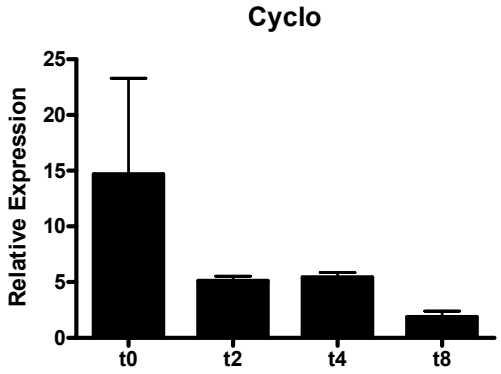

Figure 7. qPCR expression analysis of liver slices of APOE2ki mice. At each time point t0, t2, t4, and t8 the relative expression of leprB, Acox1, and Cyclo is shown in fig 7A, 7B and 7C, respectively.

3.6 Immunostaining of lepr in the liver

Whereas HFD supplemented with FF induced an increase in leprB mRNA production in the liver of APOE2ki mice, we wanted to investigate whether this up-regulation of leprB on RNA level was also related to an increase in protein. Therefore, immunohistochemistry emphasized the localization of the lepr in the liver. Using an N-terminal lepr antibody that recognizes the long and the short forms of the lepr, we were unable to detect specific hepatic immunoreactivity; the slides were characterized by a brown smear (data not shown). Furthermore, a C-terminal antibody, thought to detect only the signalling leprB isoform, revealed the same brown smear (data not shown). Incubation of the liver slides with antibodies against the phosphorylated form of the lepr, as occurs during signalling, also showed this smear (data not shown). Therefore, we were unable to relate the increase in leprB mRNA production to an increase in protein levels.

3.7 Lepr mRNA production in other tissues

The expression of the leprB was evaluated by qPCR in visceral and uteral adipose tissue and red and white skeletal muscle of APOE2ki mice.

In visceral adipose tissue, the relative expression of leprB showed a nearly 3-fold decrease upon 2 days high-fat feeding. This same trend was also noticed in the group receiving the HFD for 2 days enriched with FF (fig 8A).

On the other hand, uteral fat tissue displayed a completely different expression pattern of leprB. 2 days high-fat feeding caused a slight increase in the expression level of leprB. Addition of FF to the HFD raised the expression even further (fig 8B).

In red and white skeletal muscle leprB reached insufficient expression levels and could therefore not be detected (data not shown).

Fig 8A **Fig 8B**

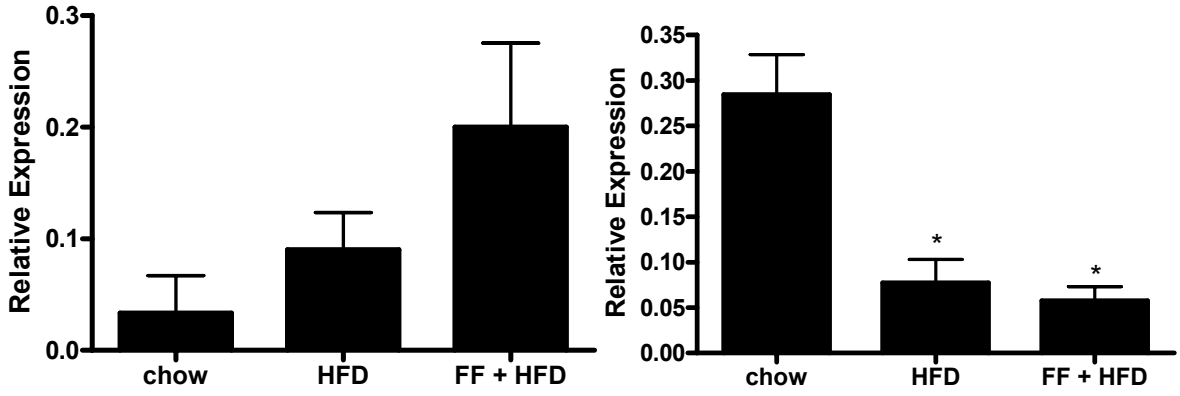

Figure 8. qPCR expression analysis of leprB in fat tissue of APOEki mice. Fig 8A shows the relative expression of the leprB in visceral adipose tissue. Fig 8B shows the relative expression of the leprB in uteral adipose tissue. Significant differences are indicated by *.

4 Discussion

The discovery of leptin has provided insight into the interaction between energy stores and hypothalamic centres that regulate feeding behaviour and energy balance. Therefore, it was believed that primary leptin signal transduction is regulated centrally in the hypothalamus [3]. While initial focus was on the central effects of leptin, important actions have been discovered in peripheral tissues expressing the lepr. Also, studies have found that neuronal deletion of lepr did not result in body weight gain, achieved by the ob/ob and db/db mice, indicating that leptin could act directly on peripheral tissues expressing the lepr, without interference of the central nervous system [16, 25]. A previous study revealed a marked increase in the expression of the lepr in the liver of APOE2ki mice upon high-fat feeding, enriched with FF [43]. Therefore, we analyzed the functionality and the mechanisms responsible for regulation of the lepr in the liver. Our results provide some interesting observations.

Previous microarray analysis revealed that addition of FF to HFD increased hepatic expression of lepr 3.6 fold [43]. Our qPCR analyses validate these results. We show a significant up-regulation of leprA, leprB, and LeprC isoforms in liver tissue of APOE2ki mice after 2 days high-fat feeding, enriched with FF.
According to Cohen et al. (2005) liver is a major source of lepr mRNA expression under conditions of negative energy balance, as occurs during food deprivation. During this period the organism experiences a calorie intake that is insufficiently high to meet the requirements of the body. They found an increase in the mRNAs encoding the long form (leprB) and the short forms (leprA and leprC) of the lepr in the liver upon fasting [25]. It is feasible that the other end of the metabolic spectrum, a diet rich in fat and cholesterol, leading towards a positive energy balance, could also cause an up-regulation of the three lepr isoforms. This proposition has found to be true for the brain. Ziotopoulou et al. (2000) showed that both low- and high-fat-fed mice displayed no difference in expression of the hypothalamic peptides NPY, AgRP, POMC and SOCS3, genes that are transcribed during activation of the leptin signal transduction pathway [34].

However, in the current study, after 2 days HFD, qPCR results did not detect an elevated expression level of either leprA, leprB or leprC in the liver of APOE2ki mice, suggesting that the up-regulation of the isoforms is coupled to the administration of FF. This is of particular interest since previous studies have shown that FF is a synthetic agonist of PPARα, a nuclear receptor reaching high expression levels in liver tissue [44,46]. The present data indicate that the up-regulation of lepr, upon administration of FF could be mediated via PPARα. Interestingly, we observed no up-regulation of the three lepr isoforms in mice with a liver-specific deletion of PPARα, confirming our hypothesis. Remarkably, chow-fed PPARαko mice display lepr mRNA levels that reach a more than 10-fold higher expression than their APOE2ki counterparts on chow. Patsouris et al. (2005) provided evidence that mice with hepatic deletion of PPARα on HFD, exhibit PPARγ mRNA levels reaching a 20-fold elevation compared with wild-type mice fed a low fat diet, showing normal PPARα expression levels. They concluded that PPARα-signalling is activated in the liver upon high-fat feeding and that PPARγ compensates for PPARα in mice lacking hepatic expression of PPARα. Not only did PPARγ up-regulate it own target genes involved in lipogenesis, characteristic PPARα target genes involved in fatty acid oxidation were also up-regulated [44]. Furthermore, another study also provided evidence that in three mouse models of obesity, namely, ob/ob (leptin-deficient), db/db (leptin-receptor deficient), and serotonin 5-HT2cR mutant mice hepatic PPARα mRNA levels are increased by 2- to 3-fold in all three obese models whereas hepatic PPARγ mRNA levels are increased by 7- to 9-fold in ob/ob and db/db mice and 2-fold in obese 5-HT2cR mice [46]. Thus, it could be possible that increased activation of PPARγ in the PPARαko mice, up-regulates the basal expression level of the lepr.

Plasma and liver values of the PPARαko confirm these results. Upon high-fat feeding, supplemented with FF, plasma TG and Chol levels are significantly elevated, whereas plasma FFA are significantly decreased. This could indicate an enlarged storage of FFA in the liver. Indeed, the elevation in liver TG and Chol support these indications. Additionally, both in plasma and liver, FF treatment kept the lipid parameters at the basal level, as seen in the regular chow group, except the plasma FFA tended to decrease upon FF. Not only does this observation provide evidence that the mice truly lack PPARα in their liver, these data might also verify the increased hepatic activity of PPARγ in PPARαko mice, since storage of lipids is increased in the liver.

Examination of the liver histology of these PPARαko mice detected no abnormal features; we only saw an accumulation of fat in the hepatic cells. Alternatively, referring back to Patsouris et al. (2005), this accumulation could possibly also be explained by increased activity of PPARγ, leading to expression of genes that promote storage of TG [44].

Since the studies of Patsouris et al. (2005) and Menon et al. (2000), mentioned above, indicated that PPARα expression levels are increased upon high-fat feeding, and our hypothesis implies that the hepatic lepr up-regulation dependent is on PPARα, we would expect to see an increase in lepr expression of APOE2ki mice fed only a HFD. However, this is not the case as expression of this receptor in mice on a HFD reaches the same level as mice on chow. Thus, it seems likely that the up-regulation is indirectly dependent on PPARα, and that ligand binding to this nuclear receptor is an important determinant.

To identify whether the up-regulation of lepr is mainly regulated on tissue or cellular level, or more a systemic effect of FF, we made use of an in vitro model. To our knowledge, cutting liver slices and incubating them with varying concentrations of FF, has never been performed in mice. Therefore, we had no assurance that this technique would actually be a good model to test these objectives. The results indeed confirm the latter. Incubation of liver slices with FF was not very successful. It seems that the tissue slices are not able to survive culturing, as their RNA concentration rapidly declines after 2 hours of culturing. Possible explanations could be that cutting the slices causes substantial damage, leading to apoptosis of the cells. Also, the medium in which the slices were cultured could be non-ideal. Overall, this technique needs to be further optimized for future experiments. Another possibility would be to make use of another in vitro model, culturing of primary mouse hepatocytes to which FF is added comes to mind. But here again, it is doubtful that this model is representative, since the assumption is that hepatic lepr is expressed in the hepatocytes and less or even not expressed in other cell types compromising this tissue. However, hepatic stellate cells have been shown to express lepr upon activation [52].

In our study we wanted to examine whether the up-regulation of hepatic lepr mRNA is directly linked to an increase in lepr protein levels. Therefore we chose to perform an immunostaining to detect lepr in liver tissue of APOE2ki mice. Unfortunately, our staining was not successful since our negative controls displayed the same brown

smear as the other tissue slides. We tried to optimize the staining procedure by adding extra blocking steps that would minimize the endogenous avidin and biotin activity in the liver. Also we included brain sections, which included the hypothalamus, as a positive control, since lepr expression has been proved to be present in this part of the brain [3]. These changes did not improve our staining, and remarkably the brain slides also showed the same smear, seen in the liver slides. Therefore it is likely that something was wrong with the antibodies, also since these are relatively old, and there was a precipitate visible. A four-fold more dilution of the secondary antibody was also unable to improve the results of the staining. Because the negative slides did also still show the smear, we are likely to think that the secondary antibody might be a-specific. To be sure of this, other secondary antibodies should be used for future experiments.

In our study we also wanted to focus on the fact whether the increase in hepatic lepr expression upon high-fat feeding, supplemented with FF is tissue specific, or that this expression pattern is also observed in other tissues, such as the adipose tissue and skeletal muscle. The qPCR results for adipose tissue are contradictory, in that the leprB expression pattern in uteral and visceral adipose tissue of APOE2ki mice reveals an opposite pattern. Since fat tissue does not express PPARα, but high expression of PPARγ [44], we expected to find no up-regulation of the lepr after administration of FF. The findings of visceral fat tissue were in agreement with our hypothesis, we did not detect an up-regulation of the leprB upon high-fat feeding, supplemented with FF. Contrasting with this were the results of the uteral fat tissue, which showed a tendency towards an increased leprB expression. We only looked at the main signalling isoform of the lepr, leprA and leprC expression in uteral fat tissue might support our hypothesis. However, it may not be neglected that there was a relatively large variation within the groups of the uteral fat tissue. This could point to the fact that something went wrong with the analysis of the gene expression, like contamination during RNA isolation, unequal distribution of the qPCR mix to the samples. Another explanation could be that since leptin has been shown to be of critical importance for normal function of the female reproductive system in rodents [53], and expression of PPARα has been detected in the ovary of rats [54], it could be that there is little expression of PPARα in the immediate surrounding fat tissue of the ovary. It is also likely that if the up-regulation of the lepr is indeed coupled to the binding of FF to PPARα, this increased expression is not only linked to the direct

localization of PPARα, but that this is also noticed in the adjacent region. Another contributing factor to the increase in lepr expression might be other hormones like estrogens, present in uteral fat tissue. Also, basal expression levels (chow) of the lepr differed a lot between the two fat tissues. Interestingly, visceral fat tissue is known to have less leptin expressing adipocytes than other fat tissue [55]. Since leptin production is directly linked to feedback mechanisms, inhibiting the signalling pathway [41,56], it seems plausible that visceral fat tissue displays a substantially higher leprB expression level on chow. When comparing the lepr expression pattern in the liver with that of the adipose tissue, it is seen that visceral fat tissue displays a similar pattern that observed in the liver of PPARαko mice, indicating that there is higher activity of PPARγ in visceral than uteral tissue. According to Rodriguez et al. (2004), this seems to be the case; visceral fat was associated with a higher expression of the key adipogenic transcription factor PPARγ [57].
Whereas skeletal muscle has been shown to express both lepr and PPARα [26,49], our results demonstrated otherwise. In stead of seeing an up-regulation of the leprB upon HFD, enriched with FF, we hardly detected any expression of the leprB in both red and white skeletal muscle. As with the uteral fat tissue, this could be due to inaccurate RNA isolation or incorrect gene expression analysis. However, more likely is the explanation of Hoggard et al. (1997); they only detect the expression of leprA isoform in skeletal muscle of mice [26].

In summary, PPARα seems to be of importance for the up-regulation of lepr expression in the liver. How these two factors are coupled to each other and what the exact role of FF is in this whole process, still remains to be elucidated.

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