Modulation of VLDL triglyceride metabolism

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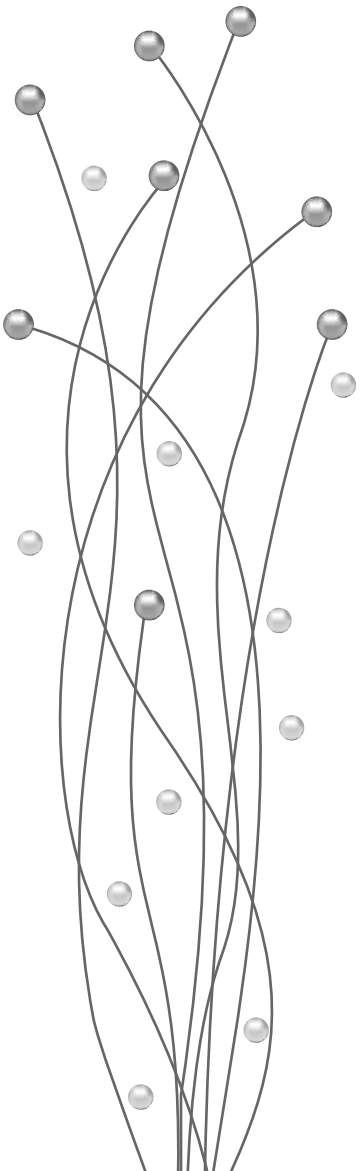
Chapter 6

Gene expression profiles distinguish fasting and high-fat diet induced steatosis



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High-fat diet-induced hepatic triglyceride accumulation or steatosis is associated with hepatic insulin resistance and hypothesized to play a role in the development of the pathology associated with the metabolic syndrome. However, steatosis is also induced by prolonged fasting, which is not associated with hepatic insulin resistance. To investigate whether different pathways lead to steatosis, we performed microarray analysis on livers of mice either fasted for 16 hours or fed a high fat diet for 2 weeks. We analysed expression of 7,500 genes and subsequently performed a pathway analysis to identify changes in hepatic gene expression in both models. Fasting induced a high number of differentially expressed hepatic genes, resulting in a change towards an energy saving phenotype. In contrast, only a small number of genes were differentially expressed after high fat diet. Fasting promoted gluconeogenesis and β-oxidation, strongly suppressed cholesterol synthesis and activated pathways to preserve hepatic function. High fat diet induced steatosis was accompanied by the activation of *Scd1* and the lipogenic transcription factor *Srebp-1c*, both implicated in the development of hepatic insulin resistance. Thus, hepatic lipid overload caused by either prolonged fasting or a high-fat diet activates significantly different gene expression programs.

Introduction

The metabolic syndrome represents a cluster of well-documented risk factors for the development of type 2 diabetes and cardiovascular disease. In addition to visceral obesity, dyslipidemia and insulin resistance (IR), excessive triglyceride (TG) accumulation in the liver has been implicated to play a role in the development of the metabolic syndrome[227]. Whether hepatic steatosis is primary or secondary to the development of insulin resistance may depend on specific pathology[228]. Numerous studies have shown that high fat diet induces hepatic steatosis, insulin resistance and obesity in rodents. Fasting, on the other hand, also increases hepatic TG content and this is due to the uptake of of adipocyte derived free fatty acids (FA's). This form of hepatic steatosis, in response to a physiological stimulus most living beings are exposed to on a regular basis, develops in the absence of hepatic insulin resistance[229].

This raises the question what the difference is between the development of hepatic steatosis due to increased dietary intake of fat and an increase of available FA's due to release from adipose tissue. Since the liver plays a central role in coordinating the metabolic effects in response to alterations in nutrient availability, we speculated that the control of the expression of key genes involved in metabolism is differently regulated in these distinct forms of hepatic steatosis.

To examine hepatic gene expression in response to hepatic steatosis of different aetiology, we performed a microarray analysis on steatotic livers from mice fed a high fat diet for 2 weeks or fasted for 16 hours. Fasting-induced steatosis is not associated with hepatic insulin resistance[229] while 1 week of high fat diet is enough to induce hepatic insulin resistance[230]. Here, we show that a number of genes that have been proposed to be involved in the development of hepatic insulin resistance are differently regulated in fasting compared to high fat induced steatosis. Furthermore, we show that a number of genes involved in detoxification and regulation of hepatic regeneration are induced in fasted livers while no such activation takes place in high fat diet treated animals. High fat diet on the other hand induces the activation of *Scd1* and *Srebp-1c*, two genes that have been implicated as key factors in the development of hepatic insulin resistance.

Materials and Methods

Animals

Male C57Bl6/J, 12-16 week old mice (n=30) were housed in a 12h light/dark cycle under standard conditions. Animals had free access to food and water. Control mice were fed standard chow. Animals of the high fat group received a diet with 21.5 wt% saturated bovine fat (Hope Farms, Woerden, The Netherlands). Animals were sacrificed at 09:00 h AM after a 16 h fast for the fasted group or 4 h fast for the high fat and control group. Two separate experiments were performed. For each experiment 15 mice were randomly assigned to the different experimental groups (n=5 per group). Livers from experiment 1 were extracted at day 15 and used for RNA isolation and subsequent microarray and RT-PCR analysis. From animals of experiment 2, blood was collected at day 0, day 10 and day 15. All experiments were approved by the Animal Ethics Committee of the Leiden University Medical Center.

Plasma parameters

Plasma was obtained via tail vein bleeding in chilled paraxonized capillary tubes to prevent *ex vivo* lypolysis[231], and assayed for glucose, insulin, TG, and total cholesterol using commercially available Kits (glucose hexokinase method (Instruchemie, Delfzijl, The Netherlands), ultra sensitive mouse insulin ELISA (Mercodia, Uppsala, Sweden), 1488872 and 236691 Roche Molecular Biochemicals, (Indianapolis, IN, USA), respectively).

Isolation, amplification and labelling of RNA

Total RNA was isolated from livers of mice fed a high fat diet or chow for 2 weeks or an overnight fast (16 h). RNA was isolated with the NucleoSpin RNA® II-kit (Machery-Nagel, Düren, Germany). The RNA concentration was determined by absorbency at 260 nm with a NanoDrop (Isogen Life Science, Ijsselstein, The Netherlands), and RNA integrity was verified by use of the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). RNA was reverse transcribed with incorporation of amino-allyl-UTP (aa-UTP) using the MessageAmp™ aRNA kit, according to the manufacturer's instructions, then column purified and eluted in nuclease-free

water. A total of 3.75 mg column-purified aRNA of each liver was labelled with both monofunctional dyes, cyanine-3 (Cy3) and cyanine-5 (Cy5) (Amersham Biosciences, GE Healthcare, Diegem, Belgium), as previously described[232] to allow all experiments to be performed as dye-swaps. Cy3 labelled aRNA of one experimental group was combined with Cy5 labelled aRNA of the other experimental group and vice versa. Combined probes were concentrated with Montage PCR columns (Millipore, Amsterdam, The Netherlands), recovered in TE and dye incorporation efficiency was then determined by wave-scan with the NanoDrop.

Hybridization of glass oligonucleotide microarrays

Murine oligonucleotide microarrays were produced in the Leiden Genome Technology Center by spotting the Sigma-Genosys mouse 7.5K oligonucleotide library (v. 1.0) (65mer, 20 µl in 50% DMSO) in duplicate on poly-L-lysine-coated slides[232]. Hybridization was done as described previously[232] with minor modifications. To the combined aRNA's hybridization mix was added (final concentrations, 0.15 mg/ml yeast tRNA, 0.15 mg/ml poly(A)+ RNA, 3 x SSC and 0.3% SDS), probes were denatured at 95°C for 2 min and centrifuged for 10 min at 13 000g. Hybridizations were performed overnight in Corning Hybridization chambers at 55°C in a water bath. Slides were washed with increasing stringency and then dehydrated by short washes in 70, 90 and 100% ethanol. After drying arrays were scanned with the Agilent G2565BA microarray scanner. Feature analysis was done with GenePix Pro 5.0 (Axon, Molecular Devices, Sunnyvale, U.S.A). Fluorescence intensities were normalized to median array densities to generate normalized measurements for each gene across all samples.

Quantitative real-time PCR

One microgram of total RNA of individual animals was reverse transcribed into cDNA with the RevertAid™ H Minus first strand Kit (Fermentas, St.Leon-Rot, Germany) primed with random hexamers. Thirty nanogram cDNA were amplified with gene specific primers in a 15 µl reaction containing 1 x SYBR-green mix (BioRad, Veenendaal, The Netherlands). All primers were tested to have efficiency between 90 and 110%. All samples were amplified in duplicates on a MyiQ machine (BioRad). Values were normalized to the amount of two

endogenous control genes (Cyclophilin B and β-2-microglobulin) and relative changes in gene expression were then calculated with the iQ5-software version 2.0 (BioRad). Three animals of each experimental group were analysed.

Statistical Analysis

All data are expressed as mean ± SD. Statistical analysis was performed by one-way ANOVA with post hoc using Newmann-Keuls test. Microarray data were analysed with the limma-package[233] from the Bioconductor software project (http://bioconductor.org) in the R environment for statistical computing (http://www.R-project.org). Only genes with an average expression level exceeding 1.5 times the background over all arrays and channels and a P-value < 0.05 were included in the pathway analysis. Associations with GO biological process, molecular function, cellular component groups, and biological pathways were obtained with the Web-based integrated data mining system WebGestalt[234]. A hypergeometric test was performed with the whole list of genes as the reference.

Results

Plasma parameters

Plasma TG, total cholesterol, insulin and glucose were determined in 16 hour fasted, high fat fed and control animals. Table 1 summarizes plasma parameters at day 0, day 10 and day 15. Cholesterol was elevated in animals receiving the high fat diet at day 10 and increased further until day 15. TG was slightly decreased in 16 hour fasted mice at day 15 compared to controls. Glucose levels did not differ between controls and high fat diet fed mice at any time point. As expected, glucose was significantly reduced after 16 hours of fasting compared to controls at day 15. Insulin levels were not altered by high fat feeding and dropped bellow detection limits after 16 hours of fasting (data not shown).

Gene expression changes induced by 16 hours of fasting or high fat diet

We performed three comparisons by microarray analysis. A comparison was made between individual mice of the control and high fat fed or 16 hour fasted groups of mice. In addition, a direct comparison was made between high fat fed and 16 hour fasted animals. Based on the performed microarray experiments,

we could identify 390 differentially expressed genes comparing livers from high fat fed animals with livers from 16 hour fasted mice, 407 differentially expressed genes comparing 16 hour fasted animals to control animals and 65 differentially expressed genes comparing high fat fed animals to control mice. Only 23 genes showed overlap in gene expression between the 16 hours fasted animals compared to control and high fat fed animals compared to control mice as is shown in figure 1.

Table 1. Plasma values measured in control, high fat fed and 16 hour fasted mice.

	control	high fat	16 hour fasted
Triglycerides (mmol/L)			
day 0	0.8 ± 0.2	0.7 ± 0.1	n.d.
day 10	0.8 ± 0.1	0.7 ± 0.1	n.d.
day 15	0.9 ± 0.1	0.8 ± 0.2	0.5 ± 0.1
Cholesterol (mmol/L)			
day 0	2.8 ± 0.2	2.2 ± 0.2	n.d.
day 10	2.9 ± 0.3	4.0 ± 0.8 ***/a	n.d.
day 15	2.5 ± 0.3	4.8 ± 0.5 ***/a	2.4 ± 0.1
Glucose (mmol/L)			
day 0	6.4 ± 0.9	6.3 ± 0.6	n.d.
day 10	6.2 ± 0.8	7.1 ± 1.0	n.d.
day 15	6.2 ± 1.0	6.1 ± 1.1	4.1 ± 1.0 **

Values are expressed as means ± SD (n=5 in each group). *p<0.05, **p<0.01, ***p<0.001 compared to controls and a <0.001 compared to day 0 of the same treatment group. Significance was determined by ANOVA followed by Newman-Keuls post-hoc test. n.d. not determined.

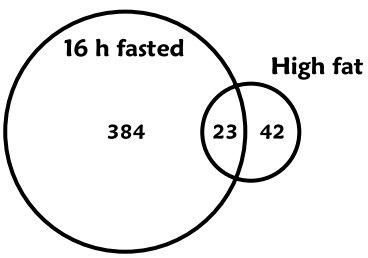

Figure 1 Venn diagram of differentially expressed genes
Hepatic expression of genes in 16 hour fasted mice compared to control and high fat fed mice compared to control and the overlap in gene expression between both groups.

To get a better understanding of coordinated changes in their biological context we performed an analysis with the web-based tool "WebGestalt" to group genes according to their Gene Ontology (GO) categories. Table 2 summarizes pathways enriched with differentially expressed genes of the comparisons of high fat fed versus fasted, 16 hour fasted versus controls and high fat fed versus control. 16 hours of fasting induced the majority of differentially expressed genes detected as is implied by the overlap of affected pathways in the comparisons of high fat fed versus 16 hour fasted and 16 hour fasted versus controls. Only a relative small number of genes were differentially expressed in high fat fed animals compared to control. This small number of differentially expressed genes clearly hampered the pathway analysis. This is reflected by the higher P-values calculated by the hypergeometric test as well as the fact that only very general GO terms were relatively enriched (Table 2).

Table 2. Gene expression pathway analysis

	16 fasted vs control		**16h fasted vs high fat**		**high fat vs control**	
GO-term	# genes observed	P-value	# genes observed	P-value	# genes observed	P-value
alcohol catabolism					3	4.20E-03
alcohol metabolism	27	4.99E-09	20	2.67E-05	5	5.96E-03
carbohydrate catabolism					3	7.50E-03
carbohydrate metabolism	31	1.25E-06				
carbon-oxygen lyase activity	11	1.86E-06				
carboxylic acid metabolism	40	6.75E-11	34	3.37E-08		
cellular carbohydrate metabolism	22	8.54E-05			3	7.50E-03
cellular catabolism					7	3.13E-03
cellular lipid metabolism	49	2.58E-16	38	4.23E-10		
cholesterol biosynthesis	5	9.01E-05				
cholesterol metabolism	10	1.36E-05				
cofactor metabolism			16	1.53E-05		
complement activation	9	6.81E-06				
complement activation, classical pathway	8	5.00E-06				
electron transport	30	7.97E-09	22	5.97E-05	9	9.91E-06

	16 fasted vs control		16h fasted vs high fat		high fat vs control	
GO-term	# genes observed	P-value	# genes observed	P-value	# genes observed	P-value
endopeptidase inhibitor activity					4	3.81E-03
endoplasmic reticulum	42	8.72E-10			9	5.31E-04
energy derivation by oxidation of organic compounds	17	1.71E-06				
fatty acid metabolism	19	6.20E-08	14	7.50E-05		
generation of precursor metabolites and energy	47	4.15E-13	34	9.15E-07	12	1.60E-06
glucose catabolism					3	3.86E-03
glucose metabolism					4	1.93E-03
glutathione transferase activity					3	2.61E-04
glycolysis					3	2.96E-03
hexose catabolism					3	3.86E-03
hexose metabolism					4	4.31E-03
humoral defense mechanism (sensu Vertebrata)	9	9.38E-05				
hydro-lyase activity	10	3.90E-06				
lipid biosynthesis	26	3.43E-11	18	5.94E-06		
lipid binding					7	3.28E-03
lipid metabolism	53	1.08E-15	42	5.19E-10		
lipid transport					4	1.41E-04
lipid transporter activity					3	3.61E-03
lipoprotein metabolism					3	1.77E-03
mitochondrial envelope	23	1.80E-07	19	2.99E-05	23	1.97E-03
mitochondrial inner membrane	18	1.18E-05				
mitochondrial membrane	18	2.67E-05				
mitochondrion	58	2.75E-16	46	1.03E-09		
monooxygenase activity	17	1.37E-08			6	1.82E-05
monosaccharide catabolism					3	3.86E-03
monosaccharide metabolism					4	4.31E-03
organelle envelope	24	4.32E-06				

	16 fasted vs control		16h fasted vs high fat		high fat vs control	
GO-term	# genes observed	P-value	# genes observed	P-value	# genes observed	P-value
organelle inner membrane	19	7.09E-06	17	7.51E-05		
organelle membrane	28	7.23E-05				
organic acid metabolism	40	6.75E-11	34	3.37E-08		
peroxisome	14	2.29E-07	14	1.83E-07		
protease inhibitor activity					4	3.81E-03
steroid biosynthesis	16	1.07E-12	10	2.95E-06		
steroid dehydrogenase activity	6	7.75E-06	6	6.81E-06		
steroid metabolism	22	1.41E-11	20	3.03E-10		
sterol biosynthesis	7	1.97E-06				
sterol metabolism	12	4.12E-07				

Pathways with a P-value <0.01 determined by hypergeometric test in the comparison of 16 hour fasted versus controls, 16 hour fasted versus high fat fed and high fat fed versus controls.

Expression of key genes involved in energy homeostasis by RT-PCR

We confirmed the changes detected with the microarray analysis by performing RT-PCR on a number of differentially expressed genes. We selected five up- (*Pcx, Cpt1a, Crat1, Cyp8b1* and *Cyp3a13*) and four down-regulated genes (*Cyp51*, *Elovl3*, *Gck*, and *Sqle*) from the 16 hour fasted versus control and high fat versus control comparison to confirm our array findings. Table 3 summarizes the results of the RT-PCR experiments. We could confirm the findings of the microarray experiment with the exception that 3 genes reached statistical significance in the RT-PCR experiment in the high fat fed group that had not been significant in the microarray experiment.

A number of genes known from the literature to be involved in the development of obesity and the metabolic syndrome did not pass the technical selection threshold or were not present on the microarray at all. We therefore decided to extend the analysis with additional RT-PCR experiments to analyze the expression of a selected set of transcription factors and genes suspected to play a role in the development of obesity and the metabolic syndrome (Fig. 2). Compared to control, the Farnesoid X receptor (*Fxr*, gene name: *Nr1h4*) was

increased in both groups, but more pronounced in 16 hour fasted animals. The same was true for the Liver X receptor alpha (*Lxrα, Nr1h3*), Pregnane X receptor (*Pxr, Nr1i2*) and the Hepatic Nuclear Factor 4 alpha (*Hnf4α, Nr2a1*) (Fig. 2a). In contrast, we detected an increase in hepatic peroxisome proliferate activated receptor alpha *(Pparα, Nr1c1*) expression in high fat fed mice which was absent in 16 hour fasted livers and was not detected on microarray. The PPAR delta isoform (*Pparδ, Nr1c2*) was not altered in either group while the PPAR gamma isoform (*Pparγ, Nr1c3*) was up-regulated in livers of 16 hour fasted animals but not in those fed a high fat diet. This up-regulation of *Pparγ* in 16 hour fasted animals was accompanied by a concomitant increase in PPAR gamma co-activation factor 1a (*Pparγc1a)* expression which was absent in high fat fed mice while no change in *Pparγc1b* expression was detectable in either group (Fig. 2b). For the sterol regulatory element binding proteins family (*Srebp's*), *Srebp-1a* showed the same pattern as *Pparα* with only an increase in expression in high fat fed mice. *Srebp-1c* on the other hand, was upregulated in high fat fed animals but downregulated in 16 hour fasted animals, compared to control. This same pattern of expression was seen for stearoyl-CoA desaturase (*Scd1*). The constitutive androstane receptor (*Car*) was exclusively up-regulated in livers of 16 hour fasted animals.

Table 3. Validation of microarray gene expression data with RT-PCR

	16 hr fasted vs control		**high fat vs control**	
Gene	Array, Ratio	PCR, relative expression	Array, Ratio	PCR, relative expression
Pcx	+ 1.56**	3.43***	+ 1.16	1.43
Cpt1a	+ 1.46*	3.85***	+ 1.16	1.82*
Crat1	+ 1.53**	5.87***	+ 1.04	1.20
Cyp8b1	+ 2.42***	4.39***	- 1.18	1.14
Cyp3a13	+ 1.66**	3.45***	- 1.12	0.90
Cyp51	- 2.47***	0.33**	+ 1.10	1.06
Elovl3	- 4.10***	0.26**	- 1.19	1.34**
Gck	- 3.60***	0.35**	+ 1.12	2.30**
Sqle	- 1.37*	0.24*	- 1.05	1.18

Relative expression to controls as determined with the ΔΔCt-method. PCR Values are means of 3 animals per group. *p<0.05, **p<0.01, ***p<0.001 as determined by ANOVA followed by Newman-Keuls post-hoc test.

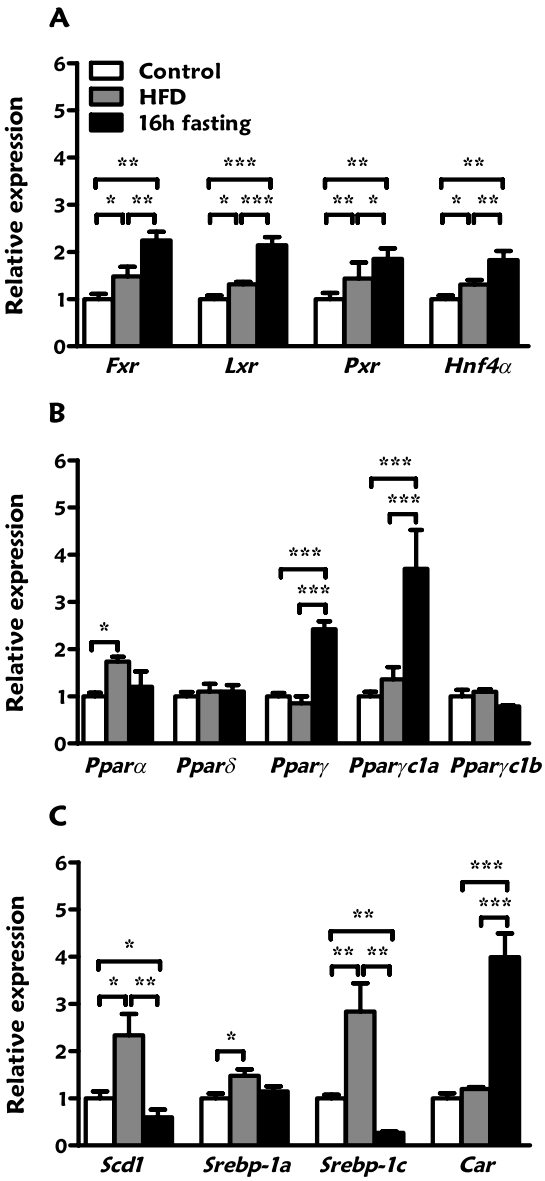

Figure 2 Expression levels of selected transcription factors determined by real-time PCR in livers from high fat fed or fasted mice relative to controls.
Relative expression to controls as determined with the ΔΔCt-method. Data represent means of 3 animals per group. *p<0.05, **p<0.01, ***p<0.001 as determined by ANOVA followed by Newman-Keuls post-hoc test.

Discussion

Hepatic steatosis can be induced in mice by fasting for 16 hours and is accompanied by an increase in hepatic TG content comparable to that induced after two weeks of high fat diet[230]. In contrast to steatosis induced by high fat diet, the fasting response does not result in the development of hepatic insulin resistance. We have investigated the alterations in hepatic gene expression induced by 16 hour fasting or two week high fat diet exposure to identify genes that are differentially expressed in these distinct forms of steatosis. Fasting had a profound effect on pathways involved in lipid metabolism, fatty acid metabolism, steroid metabolism and biosynthesis, carbohydrate metabolism as well as on energy generation related genes. These changes reflected the expected shift from glucose utilization towards energy generation derived from fatty acid oxidation. Interestingly, we also detected the up-regulation of a number of genes involved in hepatic detoxification.

Fasting induces a shift in fuel utilization for energy generation from glucose to FA's. This was reflected by significant changes in enzymes involved in glycolysis: glucokinase (*Gck*) was down-regulated and aldolase A (*AldoA*) was up-regulated in fasted liver, which was detected in the microarray experiments. We further detected the up-regulation of the lactate dehydrogenases A (*Ldha*) and B (*Ldhb*) (microarray) together with pyruvate carboxylase (*Pcx*) (both microarray and RT-PCR (Table 3)). *Pcx* encodes the mitochondrial enzyme that converts pyruvate to oxaloacetate and thus provides intermediates for the citric acid cycle[235].

The shift towards FA utilization upon fasting was further reflected by an up-regulation of several genes involved in mitochondrial FA β-oxidation. With microarray analysis, we detected up-regulation of acyl-CoA synthetase long-chain family member 1 (*Acsl1*), carnitine palmitoyltransferase 1a (*Cpt1a*), solute carrier family 25 (*Slc25a20*), acyl-Coenzyme A dehydrogenase very long chain (*Acadvl*), acyl-Coenzyme A dehydrogenase long-chain (*Acadl*), acyl-Coenzyme A dehydrogenase, medium chain (*Acadm*), dodecenoyl-Coenzyme A delta isomerase (*Dci*) and enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase (*Ehhadh*). Interestingly, we saw a down-regulation of peroxisomal trans-2-enoyl-CoA reductase (*Pecr*), the hepatic microsomal enzyme that catalyses the reduction of trans-2-enoyl-

CoAs of varying chain lengths from 6:1 to 16:1. None of these genes were identified as differentially expressed in livers from mice fed a high fat diet (data not shown). The change in the expression of *Cpt1* was also confirmed with RT-PCR (Table 3).

One of the most prominently affected pathways in fasted livers was cholesterol biosynthesis. The SREBP family of transcription factors is known to represent the master regulators of lipogenesis and cholesterogenesis. They are able to directly activate genes involved in the synthesis and uptake of cholesterol, fatty acids, TG's and phopsholipids[236]. Of the three isoforms SREBP-1a, -1c and -2 it is known that SREBP-2 is the main regulator of cholesterol synthesis if expressed at physiological levels. We did not detect a change in the microarray experiments and only a minor decrease in SREBP-2 transcript with PCR in the fasted livers, but this was not significant. One of the known target genes of SREBP-2, HMG-CoA reductase (*Hmgcr*) was also not changed. However, both SREBP-2 and HMG-CoA reductase have been shown to be subject to post-transcriptional regulation[237, 238]. Since we have not measured activated protein levels of SREBP-2, we can not exclude that these might be diminished in the situation of fasting. Nonetheless, even in the absence of changes in gene expression levels of SREBP-2, we did see reduced expression levels of the SREBP-2 regulated genes *Mevalonate kinase (Mvk), farnesyl diphosphate farnesyl transferase (Fdft1), Squalene epoxidase (Sqle), cytochrome P450 family 51 (Cyp51), sterol C5 desaturase (Sc5d) and 7-dehydrocholesterol reductase (Dhcr7)*[78] in fasted livers compared to control mice fed chow diet (data not shown). The down-regulation of *Cyp51* and *Sqle* was also confirmed by RT-PCR (Table 3). Overall, these data confirm previous results showing that fasting results in downregulation of the cholesterol biosynthesis pathway[239].

Another pathway affected by fasting included the regulators of hepatic detoxification processes, pregnane X receptor (*Pxr*) and constitutive androstane receptor (*Car*). Car was exclusively up-regulated in livers of fasted animals as detected by microarray and RT-PCR while Pxr was up-regulated in livers of fasted animals and also modestly increased in high fat fed mice (Fig. 2). Target genes regulated by these lipid-activated nuclear transcription factors include, among others, the family of cytochrome P-450 enzymes and

cytosolic binding proteins[240]. We could detect up-regulation of 8 Cyp-family members (*Cyp3a13, Cyp3a25, Cyp4a10, Cyp4a14*, *Cyp8b1*, *Cyp17a1*, *Cyp26a1* and *Cyp39a1*) and down-regulation of 3 members (*Cyp2f2*, *Cyp2j6* and *Cyp4f14*) in our microarray experiments when we compared fasted livers with those of controls. In livers of high fat fed mice all 6 detected Cyp-family members (*Cyp2a4, Cyp2f2*, *Cyp3a25, Cyp4a10, Cyp4a14* and *Cyp4a41*) were down-regulated when compared to controls. Two of these, Cyp4a10 and Cyp4a14, appear to be regulated diametrically opposed in fasted and high fat fed mice, which makes them potential target genes of Car. The activation of hepatic detoxification was further supported by the up-regulation of two members of the sulfotransferase family of phase-II conjugation enzymes, *Sult1a1* and *Sult1d1*. These enzymes help to conjugate endo- and xenobiotics with a sulfonate group to render them water soluble and excretable. Interestingly, it has recently been demonstrated that CAR and PXR also play a role in the regulation of hepatic energy metabolism by influencing gluconeogenesis, beta-oxidation, ketogenesis, lipogenesis and thyroid hormone activity via crosstalk with transcription factors such as FOXO1[241].

As can be seen in Figure 2a, changes in the expression of nuclear transcription factors belonging to the PPAR family were mainly seen in fasted livers. High fat diet did induce a mild up-regulation of Pparγ. Pparγ activation stimulates FA transport, FA oxidation, ketogenesis and gluconeogenesis. Pparγ is a regulator of adipogenesis and induces FA uptake and storage when activated. It has therefore been proposed that the up-regulation of Pparγ is a mechanism that allows hepatocytes to facilitate storage of excess FA's in a way that will not cause damage to the cell[242]. There are several mouse models of obesity in which the development of hepatic steatosis was correlated with increased hepatic levels of Pparγ[243, 244]. The transcription of *Pparγc1a* is a co-activator of FOXO-1 and has been shown to be intimately involved in the regulation of energy metabolism in response to fasting[245].

We have not found direct evidence of altered insulin signalling, but two genes, *Scd1* and *Srebp-1c,* which have been associated with the development of hepatic steatosis and IR, were inversely regulated in fasting and high fat diet. We detected a significant down-regulation of *Srebp-1c*, the main regulator of the fatty acid biosynthesis pathway in fasted livers and a significant

up-regulation in livers of high fat fed mice (Fig. 2). There is evidence that hepatic steatosis and IR is associated with an up-regulation of Srebp-1c, which is increased in response to the elevated insulin levels as seen in the leptin deficient *ob/ob* mice[78]. Furthermore, hepatic overexpression of Srebp-1c in transgenic mice leads to an increased *de novo* synthesis of TG which can cause hepatic steatosis [246]. A potential mechanism for the role of SREBPs in the development of IR is a direct interaction with the promoter of the insulin receptor substrate 2 (IRS-2). The IRS-2 promoter contains an insulin response element and is regulated by the forkhead proteins. It has been shown that this binding site can be occupied by all three SREBP isoforms and results in decreased IRS-2 transcription and protein, providing a potential molecular mechanism that explains the switch to increased lipogenesis and decreased hepatic glycogen synthesis seen in obesity and the metabolic syndrome[247].

Scd1 was exclusively regulated in response to high fat diet and has been shown to be modulated in response to dietary, physiological and hormonal stimuli, including insulin and polyunsaturated FA's[248]. Absence of Scd1 has been associated with the resistance to diet induced obesity and IR[248, 249]. Experiments with anti-sense oligo (ASO) treatment confirmed a role of Scd1 in IR. In these experiments, severe hepatic insulin resistance as determined by insulin clamp studies was reversed by 5 day treatment with a ASO against Scd1 which resulted in a 80% reduction of hepatic mRNA levels of Scd1, comparable to the reduction seen in livers of fasted mice (Fig. 2c).

The most striking difference comparing livers from fasted mice with high fat diet fed mice was the number of differentially expressed genes. While fasting induced changes in the expression of more than 400 hepatic genes compared to chow fed mice, only a small number of differentially expressed genes could be detected in response to a two week period of high fat diet feeding. Apparently, the metabolic adaptation induced by high fat feeding does not represent a strong adaptive signal and can be achieved without all too dramatic changes in the hepatic transcriptional program. Since prolonged fasting endangers survival and fertility and thus survival of the species, selective evolutionary pressure for a strong adaptive response upon fasting would have been beneficial.

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