Lipids, inflammation and atherosclerosis

Stitzinger, M.

Chapter 6

MICROARRAY ANALYSIS INDICATES AN IMPORTANT ROLE FOR FABP5 AND PUTATIVE NOVEL FABPS ON A WESTERN-TYPE DIET

Miranda Stitzinger[1]*, Menno Hoekstra[1]*, Eva J.A. van Wanrooij[1], Ingrid N. Michon[1], J. Kar Kruijt[1], J. Kamphorst[2], M. Van Eck[1], E. Vreugdenhil[2], Theo J.C. Van Berkel[1] and Johan Kuiper[1]

[1]Division of Biopharmaceutics, LACDR, Leiden University, The Netherlands

[2]Division of Medical Pharmacology, LACDR, Leiden University, The Netherlands

*M. Stitzinger and M. Hoekstra contributed equally to this study

Journal of Lipid Research (2006) **47(10)**: 2198-2207

ABSTRACT

Liver parenchymal cells play a dominant role in hepatic metabolism and thereby total body cholesterol homeostasis. To gain insight in the specific pathways and genes involved in the response to increased dietary lipid levels under an atherogenic condition, in the current study changes in parenchymal cell gene expression upon feeding a Western-type diet for 0, 2, 4, and 6 weeks were determined using microarray analysis in LDLr deficient mice, an established atherosclerotic animal model. By using ABI Mouse Genome Survey Arrays we were able to detect 7507 genes (28% of total number on array) that were expressed in parenchymal cells isolated from livers of LDLr deficient mice at every time point investigated. Time-dependent gene expression profiling identified FABP5 and four novel FABP5-like transcripts located on chromosomes 2, 8, and 18 as important proteins in the primary response of liver parenchymal cells to Western-type diet feeding, since their expression was 16- to 22-fold increased within the first 2 weeks on the Western-type diet. The rapid substantial increase in gene expression suggests that these FABPs may play an important role in the primary protection against cellular toxicity of cholesterol, free fatty acids and/or lipid oxidants.

INTRODUCTION

High levels of circulating cholesterol due to consumption of Western-type/high fat diets form a major risk factor for atherosclerosis and subsequent cardiovascular diseases (e.g. myocardial infarction, stroke)[1], which are the leading cause of death in the Western world. Several mutations in the low-density lipoprotein (LDL) receptor (LDLr) are associated with familial hypercholesterolemia, a dominantly inherited error of metabolism characterized by raised plasma LDL levels, xanthomas of skin and tendons, and premature heart disease due to atherosclerosis of the coronary arteries[2].
The liver is an essential organ in the regulation of serum cholesterol levels since it is able to clear excess cholesterol from the blood for subsequent excretion into the bile[3,4]. In addition, the liver is responsible for the synthesis and secretion of very low-density lipoprotein (VLDL) and high-density lipoprotein (HDL)[5,6]. Due to the important role of the liver in the control of serum cholesterol levels, several studies have recently been conducted using microarray technology to determine the molecular mechanisms underlying long-term high fat diet induced alterations in total mouse liver[7-9]. However, a common problem of these types of microarray studies is the heterogeneity of the liver, since the liver contains several different cell types, which each have their specific localization and function. Kupffer cells are tissue macrophages strategically located within the liver sinusoids, and their function is the removal of bacteria and the clearance of modified lipoproteins. Hepatic endothelial cells line the sinusoids, where they function in the removal of modified lipoproteins and mediate their natural barrier function. However, the majority of the liver cells consist of parenchymal cells (~60%), which are located between bile canaliculi and sinusoids, where they mediate both the uptake and metabolism of cholesterol for biliary excretion and the synthesis and secretion of VLDL and HDL. Importantly, in earlier studies we have shown that not only the function of parenchymal cells is different from that of other hepatic cells, but that also the expression and regulation of genes involved in lipid metabolism is markedly different between the different hepatic cell types[10,11]. Furthermore, earlier studies by Recinos *et al*[12] using microarray analysis of total liver RNA have indicated that feeding mice a high fat diet results in significant changes in the expression of hepatic genes involved in cholesterol metabolism, but also in the expression of CD68 and CD63. The latter two proteins are expressed in Kupffer and stellate cells[13,14] but not in parenchymal cells, which together with our findings[10,11] suggests that it is difficult to interpret data of microarray studies that are based on total liver mRNA. Therefore, in the current study, using microarray technology, we focused on the specific response of liver parenchymal cells to atherogenic diet feeding in LDLr deficient mice, an established atherosclerosis mouse model.

MATERIALS AND METHODS

Animals

Homozygous LDLr deficient mice[15,16] were obtained from The Jackson Laboratory as mating pairs and bred at the Gorlaeus Laboratories, Leiden, The Netherlands. Female mice were maintained on sterilized regular chow containing 4.3% (wt/wt) fat and no cholesterol (RM3, Special Diet Services, Witham, UK), or were fed a semi-synthetic Western-type diet containing 15% (wt/wt) cacao butter and 0.25% (wt/wt) cholesterol (Diet W, Special Diet Services, Witham, UK) for 2, 4, or 6 weeks. Subsequently, parenchymal liver cells were isolated essentially according to the method of Nagelkerke *et al*[17] as modified for mice by Van Berkel *et al*[18]. The purity and viability of the cells was analysed using trypan blue staining and phase contrast microscopy. Western-type diet feeding had no effect on the viability or purity of the isolated cells; the liver parenchymal cell fractions consisted for >99% of parenchymal cells with a viability >95% under both standard and Western-type diet feeding conditions.

Serum lipid analysis

Serum concentrations of free and total cholesterol were determined using enzymatic colorimetric assays (Roche Diagnostics). The cholesterol distribution over the different lipoproteins in serum was analysed by fractionation of 30 µl serum of each mouse using a Superose 6 column (3.2×30 mm, Smart-system, Pharmacia). Total cholesterol content of the effluent was determined using enzymatic colorimetric assays (Roche Diagnostics).

Microarray analysis

Total RNA from liver parenchymal cells was isolated according to Chomczynski and Sacchi[19]. Double stranded cDNA was prepared from total RNA. An in vitro transcription (IVT) reaction was used to synthesize UTP-digoxigenin-labeled cRNA. Equal amounts of cRNA from 2 pooled RNA samples of 2 mice (total of 4 mice) per time point was hybridized to ABI Mouse Genome Survey Arrays (Applied Biosystems) according to the manufacturer's instructions. The ABI Mouse Genome Survey Arrays used in the study contained 33.012 different probes representing 26.514 genes, which included transcripts from the public domain as well as from the Celera library. Subsequently, an alkalic phosphatase-linked digoxigenin antibody was incubated with the array and the phosphatase activity was initiated to start the chemiluminescent signal. The chemiluminescent (cRNA) and fluorescent (spot background) signals of the cRNA and standard controls spots were scanned for 5 and 25 seconds using an AB1700 Chemiluminescence Analyser (Applied Biosystems). Using the software supplied with the AB1700 apparatus, the spot chemiluminescent signal was normalized over the fluorescent signal of the same spot (using the standard control signals) to obtain the normalized signal value that was used for further analysis. In addition, a signal-to-noise (s/n) ratio for every spot was obtained, which needed to be at least 1 at each time point (>90% spot confidence) in order to use the spot for further analysis. In the analysis, the median value of the normalized signal of two independent arrays for

each time point was calculated as an indication for the relative gene expression number at that time point. To identify genes that are regulated in a similar manner upon Western-type diet feeding, K-means clustering was performed on gene expression profiles (relative expression compared to the chow diet) derived from the primary microarray analysis. In detail, for the K-means clustering initialization a data centroid based search was used with a maximum of 5 clusters, whilst similarity between gene expression profiles was determined using a cosine correlation (Spotfire software).

Gene expression

Quantitative gene expression analysis on isolated liver parenchymal cells was performed as described[10]. In short, total RNA was isolated according to Chomczynski and Sacchi[19] and reverse transcribed using RevertAid™ reverse transcriptase. Gene expression analysis was performed using real-time SYBR Green technology (Eurogentec) with the primers displayed in **Table 1**. Hypoxanthine guanine phosphoribosyl transferase (HPRT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and acidic ribosomal phosphoprotein P0 (36B4) were used as the standard housekeeping genes. Relative gene expression numbers were calculated by subtracting the threshold cycle number (Ct) of the target gene from the average Ct of housekeeping genes and raising 2 to the power of this difference. The average Ct of three housekeeping genes was used to exclude that changes in the relative expression were caused by variations in the separate housekeeping gene expressions. The average Ct values for housekeeping genes did not differ significantly between the groups

Table 1: Primers for quantitative real-time PCR analysis

Gene	GenBank Accession	Forward primer	Reverse Primer
36B4	X15267	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
ACC	XM109883	AGAATCTCCTGGTGACAATGCTTATT	GCTCTGTGAGGATATTTAGCAGCTC
ACLY	NM134037	AGGTACCCTGGGTCCACATTC	CTACGATCATCTTGACTCCTGGAGT
FABP5	BC002008	GGAAGGAGAGCACGATAACAAGA	GGTGGCATTGTTCATGACACA
GAPDH	NM008084	TCCATGACAACTTTGGCATTG	TCACGCCACAGCTTTCCA
HPRT	J00423	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
ME	NM008615	TTAAGGATCCACTGTACATCGGG	GGCGTCATACTCAGGGCCT
PKLR	NM013631	AAGACAGTGTGGGTGGACTACCA	CGTCAATGTAGATGCGGCC
SREBP-1	AB017337	GACCTGGTGGTGGGCACTGA	AAGCGGATGTAGTCGATGGC

Immunoblotting

Pelleted liver parenchymal cells were suspended in 500 µl lysis buffer (10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DDT) in the presence of protease inhibitors (0.02 µg/ml leupeptin, 0.02 µg/ml aprotinin, and 0.02 µg/ml trypsin inhibitor) and allowed to stand for 20 min on ice. Nuclei were pelleted by 10 min of centrifugation (13000 rpm) at 4°C. The pelleted nuclei were resuspended in a hypertonic buffer (20 mM Hepes, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 25% (v/v) glycerol) in the presence of protease inhibitors. Nuclei were incubated for 10 min at 4°C and a clear nuclear extract was obtained by centrifugation at 13000 rpm for 10 min at 4°C. Subsequently, equal amounts of cytoplasmic and nuclear protein (50 µg) were separated on 7.5% SDS-PAGE gels and electrophoretically transferred to Protran nitrocellulose membrane (Schleicher & Schnell). Immunolabeling was performed using either goat polyclonal FABP5 (R&D systems) or rabbit polyclonal SREBP-1 (H160; Santa Cruz) as primary

antibody and donkey-anti-goat IgG and goat-anti-rabbit IgG (Jackson ImmunoResearch), respectively, as secondary antibodies. Finally, immunolabeling was detected by enhanced chemiluminescence (ECL, Amersham Biosciences). Changes in protein expression levels were quantified using ImageQuant software.

RESULTS

Serum lipid levels

Feeding LDLr deficient mice, an established mouse model for atherosclerosis[15,16], a Western-type (atherogenic) diet containing 0.25% cholesterol and 15% fat resulted in a significant rise in free and total serum cholesterol levels as compared to animals on a regular chow diet containing 4.3% fat and no cholesterol (**Fig. 1A**). In agreement with previous atherosclerosis studies using the same diet[20,21], the Western-type diet induced an atherogenic lipoprotein profile in LDLr deficient mice, since the circulating serum cholesterol levels of both LDL and VLDL are markedly induced upon feeding the Western-type diet as compared to the chow diet (**Fig. 1B**). The dramatic rise in serum VLDL cholesterol levels upon Western-type diet feeding suggests that the liver responds to the increase in dietary lipid by stimulating VLDL secretion while the clearance is greatly inhibited by the absence of the LDLr.

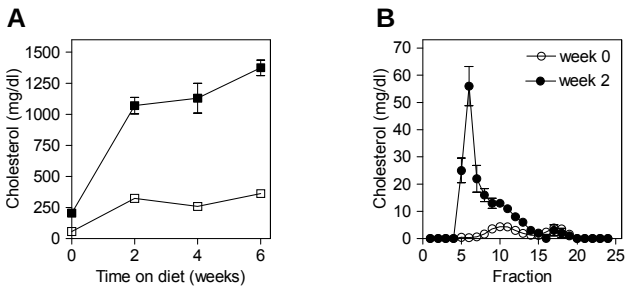


Fig. 1: Effect of Western-type diet on serum lipids. **A** The effect of a Western-type diet on serum free (open squares) and total (filled squares) cholesterol levels in LDLr deficient mice. **B** The effect of a Western-type diet on the serum cholesterol distribution in LDLr deficient mice. Blood samples were drawn on a chow diet (open circles) and two weeks on a Western-type diet (filled circles). Sera from individual mice were loaded onto a Superose 6 column and fractions were collected. Fractions 3-7 represent VLDL; fractions 8-15, LDL; and fractions 15-19, HDL, respectively. Values are expressed as mean±SEM.

Microarray analysis and K-means clustering

To gain insight in the primary and possible secondary response of liver parenchymal cells to a rise in dietary lipids, time-dependent changes in gene expression upon Western-type diet feeding were investigated using large-scale gene expression (microarray) analysis. RNA was isolated from liver parenchymal cells (>99% pure) of LDLr deficient mice on the regular chow or Western-type diet (2, 4, or 6 weeks). Two RNA samples containing pooled RNA of two separate mice per group (total group size: 4 mice) were transformed into digoxigenin-labeled cRNAs, which were simultaneously hybridized to Applied Biosystems Mouse Genome Survey Arrays (33.012

different probes representing 26.514 genes) for subsequent gene expression profiling using ABI1700 software. Whilst using a cut-off minimal signal-to-noise ratio of 1 (>90% spot confidence) for every time point, we were able to detect 7507 genes (28% of total number on array) that were expressed in parenchymal cells isolated from livers of LDLr deficient mice at every time point investigated. Subsequently, by using K-means clustering, 5 groups of genes were identified that are regulated in a similar manner upon feeding the diet enriched in cholesterol and fat (**Fig. 2**). Gene clusters 1 (n=1305), 2 (n=2110), and 3 (n=2408) contain ~78% of the total number of genes detected in liver parenchymal cells. Gene cluster 1 does contain some genes whose expression was >5-fold downregulated after 6 weeks of Western-type diet feeding. However, in general, the expression of the genes in clusters 1-3 does not appear to be highly affected upon Western-type diet feeding at any time point studied, which suggests a minor role for these gene clusters in the response to increased dietary lipid levels. In contrast, gene cluster 4 (n=954) contains genes whose expression is highly (up to 22-fold) upregulated within the first two weeks of diet feeding, after which the gene expression rapidly declines even sometimes to basal level, indicating that these genes play an important role in the *primary* response of liver parenchymal cells to a rise in dietary lipid levels. Furthermore, the genes in gene cluster 5 (n=730) appear to be involved in the *secondary* steady response of liver parenchymal cells to Western-type diet feeding, since their expression is gradually but steadily increasing over time upon Western-type diet feeding for 6 weeks.

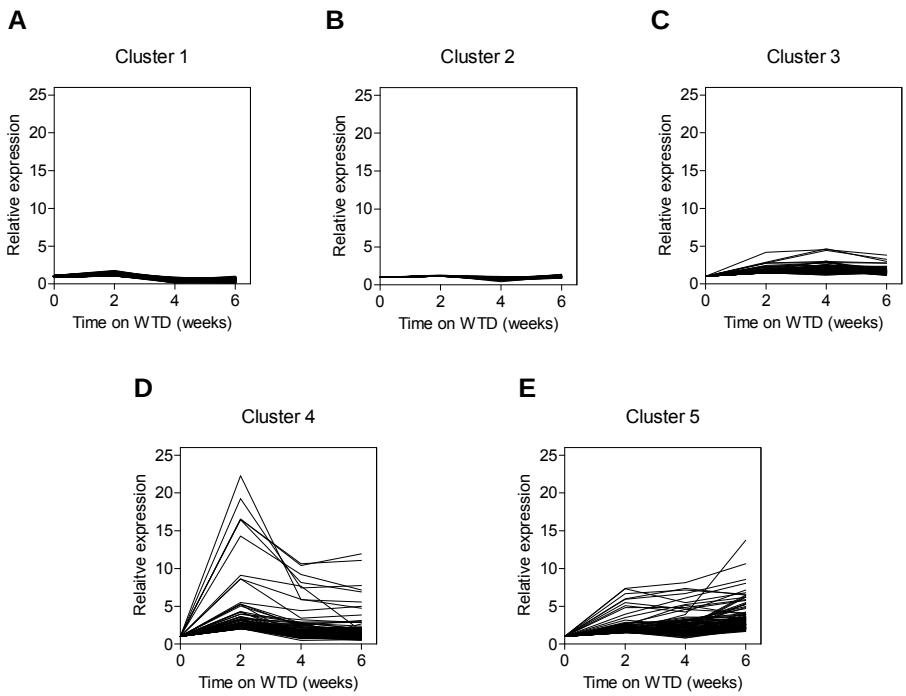


Fig. 2: Gene clusters in liver parenchymal cells of LDLr deficient mice. K-means clustering of genes was performed based upon similarity in their regulation profiles upon Western-type diet (WTD) feeding.

Primary response

All genes on the microarray chips were classified using the PANTHER classification system, which is a database that classifies genes into families and subfamilies of shared function, which are then categorized by molecular function, biological process, and pathway. As mentioned above, the 954 genes located in gene cluster 4 appear to be involved in the *primary* response of liver parenchymal cells to the rise in dietary lipid levels, since their expression is highly increased within the first 2 weeks and returns for most genes to almost basal levels after 4-6 weeks of Western-type diet feeding. **Table 2** summarizes the list of genes whose expression is stimulated >5-fold within the first two weeks of Western-type diet feeding. The known/assigned genes in this list include fatty acid binding protein (FABP)5, thyroid hormone responsive SPOT14 homolog (THRSP), DNA-damage-inducible transcript 4 (DDIT4), fatty acid elongase 6 (ELOVL6), acetyl-CoA carboxylase (ACC), fatty acid synthase (Fasn), acetoacetyl-CoA synthetase (AACS), and CCR4-NOT transcription complex, subunit 7 (CNOT7). To identify significantly affected biological processes that may play an essential role in the *primary* response to increased dietary lipid levels, the highly (>5-fold) regulated gene-list (**Table 2**) was compared to the total list of genes detected in liver parenchymal cells using PANTHER.

Table 2: Genes involved in the *primary* response. Genes in liver parenchymal cells of LDLr deficient mice, whose expression was >5-fold changed after two weeks of Western-type diet (WTD) feeding

Celera	GenBank	WTD / chow			Gene
Gene ID	Accesion	2 w	4 w	6 w	
mCG1638	NM010634	22	5.9	5.5	Fatty acid binding protein 5, epidermal (FABP5)
mCG22278	AC134443	19	7.4	7.7	*Unassigned* Fatty acid binding protein, Chr. 8
mCG5289	AC130218	17	10	12	*Unassigned* Fatty acid binding protein, Chr. 18
mCG22653	AC147992	16	11	11	*Unassigned* Fatty acid binding protein, Chr. 18
mCG7050	NM009381	16	8.1	6.9	Thyroid hormone responsive SPOT14 homolog (THRSP)
mCG9729	AL954662	14	9.2	7.2	*Unassigned* Fatty acid binding protein, Chr. 2
mCG11295	NM029083	9.1	7.7	1.7	DNA-damage-inducible transcript 4 (DDIT4)
mCG125511	NM130450	8.6	3.4	3.9	ELOVL family member 6, elongation of long chain fatty acids (ELOVL6)
mCG3541	AK076301	8.6	5.9	4.8	Acetyl-CoA carboxylase beta
mCG141310	**	5.5	4.4	5.0	*Unassigned* Acetyl-CoA carboxylase, Chr.11
mCG2138	NM007988	5.3	2.3	2.1	Fatty acid synthase (FASN)
mCG141659	AK044017	5.3	2.8	2.0	Acetoacetyl-CoA synthetase (Aacs)
mCG4614	NM011135	5.2	0.7	2.7	CCR4-NOT transcription complex, subunit 7 (CNOT7)
mCG1036474	AC153894	5.0	2.5	3.1	*Unassigned* AIG1, Chr. 6

** *Unassigned* Celera transcript is 99% homologous to AY451393 (Acetyl-CoA carboxylase 1)

Fig. 3 clearly shows that, in general, processes dealing with lipid, fatty acid, and steroid transport and metabolism are markedly stimulated on the Western-type diet in liver parenchymal cells of LDLr deficient mice. More specifically, the biological process classes lipid and fatty acid binding, steroid hormone-mediated signaling, and vitamin/cofactor transport are extremely (>100-fold) over-represented as compared to expected.

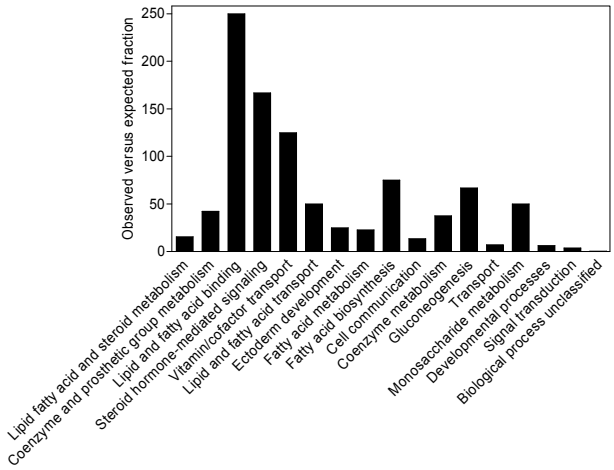
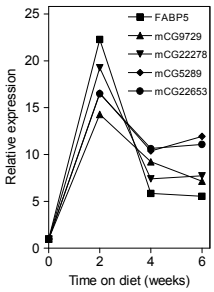
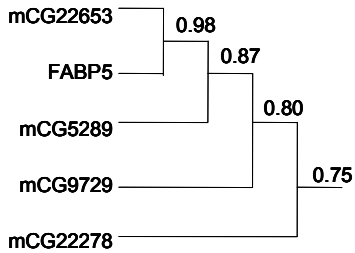


Fig. 3: Biological process classes significantly affected in the *primary* response. Biological processes with an enhanced number of highly regulated genes associated with the *primary* response cells to increased dietary lipid levels of liver parenchymal in LDLr deficient mice (P<0.05; Binomial test; PANTHER software).

A



C



B

```
FABP5      M A S L K D L E G K W R L M E S H G F E E Y M K E L G V G L A L R K M A A M A K P D C I I T C D G N N I T V K T E S T V
mCG22653   M A S L K D L E G K W C L M E S H G F E E Y M K E L R V G L A L R K M A A M A K P D C I I T C D G N N I T V K T E S T V
mCG5289    M A S L K D L E G K W R L  L E S H S F E E Y M K E L G V G L A L R K M A A M A K S D C I I I C D G N N I T F K T E S T V
mCG22278   M A S L K D L E G K W R L M E S H G F E E Y A K E L G V G L A L R K M A A M A K P D S I I T C D G N N I T  I K T E S T V
mCG9729    M A N L K D L K G K W C L M E S H G F E E Y T K E L G V G L A L R K M A A M A K P D C I I T C D G N N I T V K T V S T V

FABP5      K T T V F S C N L G E K F D E T T A D G R K T E T V C T F Q D G A L V Q H Q Q W D G K E S T  I T R K L K D G K M I V E C
mCG22653   K T T V F S C N L G E T F D E T T A D G R K T E T V C T F Q D G A L V Q H Q Q W D G K E S T  I T R K L K D G K M I  V E C
mCG5289    K T T V F S C T L G E K F D E T T A D G R K T E T V C T F Q D G A L  I Q H Q Q W E G K E S  T V T G N L K D A K M T V E R
mCG22278   K T T V F S C T L G E K F D E TM A D G R K T E M V C T F Q D G A L V Q H Q Q W E G E H D N R K T E G W E V D C G A C H
mCG9729    K T T L F S C T L G E K F D E T T A D G R K T E T V C T F K D G A W S S  T S N G K G R R T

FABP5      V M N N A T C T R V Y E K V Q
mCG22653   V M N N A T C T R V Y E K V Q
mCG5289    V T N N A T C  T R L Y E K V Q
mCG22278   E Q C H L H S G L R E G A M R A S S S S W T G V S C Q S E Y A Q F S E R T
mCG9729
```

Fig. 4: Effect of Western-type diet on FABPs A Time dependent effect of a Western-type diet on parenchymal liver cell gene expression of fatty acid binding protein 5 (FABP5) and four putative novel FABPs located on respectively chromosome 2 (mCG9729), 8 (mCG22278), and 18 (mCG5289 and mCG22653), as determined by microarray analysis. Values are expressed as fold induction on the Western-type diet as compared to the regular chow diet (0 weeks). **B** Alignment of the protein amino acid sequences of FABP5 and the four putative novel FABPs. **C** Homology tree of the protein sequences of FABP5 and the four putative novel FABPs. Numbers indicate the fraction homology between the different FABP sequences.

Strikingly, the 5 genes affected in these three classes are FABP5 and four unassigned putative novel FABPs located on chromosomes 2 (mCG9729), 8 (mCG22278), and 18 (mCG5289 and mCG22653), of which the expression was 16- to 19-fold increased within the first 2 weeks of Western-type diet feeding. Interestingly, in addition to a similar regulation profile, these putative FABPs share high sequence homology with FABP5 (**Fig. 4**), suggesting that these four proteins may have a function comparable to FABP5. Recent evidence has indicated that (intra)cellular lipid binding proteins, such as FABPs, play a central role in cellular lipid uptake and metabolism (reviewed by Glatz *et al*[22] and Boord *et al*[23]). Combined, these findings suggest that liver parenchymal cells induce the expression of FABP5 and the four novel FABPs, thereby potentially facilitating lipid uptake, transport and metabolism as a *primary* response to a rise in dietary lipid levels.

Secondary response

Table 3: Genes involved in the *secondary* response. Genes in liver parenchymal cells of LDLr deficient mice, whose expression was >5-fold changed after two weeks of Western-type diet (WTD) feeding

Celera	GenBank	WTD / chow			Gene
Gene ID	Accession	2 wk	4 wk	6 wk	
mCG19285	NM172692	0.9	0.4	14	Glucosidase beta 2 (GBA2)
mCG11623	NM009139	1.8	3.9	14	Chemokine (C-C motif) ligand 6 (CCL6)
mCG131749	NM009127	7.4	8.1	11	Stearoyl-CoA desaturase 1 (SCD1)
mCG4527	NM009274	1.3	1.3	10	Serine/arginine-rich protein specific kinase 2 (SRPK2)
mCG117361	N.F.	1.2	0.7	9.8	*Unassigned* High mobility group protein, Chr. 2
mCG21064	NM019811	5.9	6.7	8.6	Acyl-CoA synthetase short-chain family member 2 (ACSS2)
mCG3047	NM013590	1.2	2.4	8.3	P lysozyme structural (LZP-S)
mCG17532	NM011125	4.0	6.1	8.0	Phospholipid transfer protein (PLTP)
mCG1045095	AC115121	5.1	4.3	7.2	*Unassigned* Olfactory receptor, Chr. 19
mCG11880	XM001004685	7.3	5.5	6.8	*Unassigned* Malic enzyme, Chr. 9
mCG18119	NM008149	6.7	7.2	6.6	Glycerol-3-phosphate acyltransferase, mitochondrial (GPAM)
mCG20527	NM134037	6.0	7.4	6.5	ATP-citrate lyase (ACLY)
mCG133578	NM016751	1.5	2.3	6.5	C-type lectin domain family 4, member f (CLEC4F)
mCG6775	NM008035	1.8	2.2	6.3	Folate receptor 2 (fetal) (FOLR2)
mCG119533	NM013532	2.1	2.0	6.2	Leukocyte immunoglobulin-like receptor, subfamily B, member 4 (LILRB4)
mCG1028439	AK007376	3.5	5.2	6.2	RIKEN cDNA 1810008I18 gene (1810008I18RIK)
mCG21218	NM008062	4.8	4.8	5.9	Glucose-6-phosphate dehydrogenase X-linked (G6PDX)
mCG17567	NM013631	5.5	4.6	5.8	Pyruvate kinase liver and red blood cell (PKLR)
mCG3791	NM023121	1.4	2.1	5.6	Guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 2 (GNGT2)
mCG129478	AL831708	1.1	1.8	5.5	*Unassigned*, Chr. U
mCG117710	X12905	1.5	2.6	5.5	Properdin factor, complement (PFC)
mCG9333	NM009777	1.2	1.8	5.4	Complement component 1, q subcomponent, beta polypeptide (C1QB)
mCG117848	NM028717	2.4	1.8	5.4	Amyotrophic lateral sclerosis 2 (juvenile) homolog (ALS2)
mCG3081	NM009690	1.8	2.3	5.3	CD5 antigen-like (CD5L)
mCG6766	NM010531	2.0	2.9	5.2	Interleukin 18 binding protein (IL18BP)
mCG12963	NM013706	1.4	1.6	5.0	CD52 antigen (CD52)

N.F. No homologous GenBank Accession found for the given *unassigned* Celera transcript sequence

In contrast to gene cluster 4, the parenchymal liver cell expression of the 730 genes in gene cluster 5 gradually increases over time upon feeding LDLr deficient mice the diet enriched in cholesterol. It is therefore assumed that the genes in gene cluster 5 are involved in the *secondary* response of liver parenchymal cells to increased dietary lipid levels. Further investigation into the genes in gene cluster 5 showed that amongst others the expression of glucosidase beta 2 (GBA2), stearoyl-CoA desaturase 1 (SCD1), and serine/arginine-rich protein specific kinase 2 (SRPK2) was markedly (>5-fold) stimulated after 6 weeks of Western-type diet feeding (**Table 3**). In addition, biological process identification using the >5-fold regulated gene list in PANTHER revealed that the tricarboxylic acid pathway was 40-fold overexpressed as compared to the expected fraction after 6 weeks of Western-type diet with less prominent inductions (8- to 20-fold) in carbohydrate, coenzyme, and prosthetic group metabolism (**Fig. 5**).

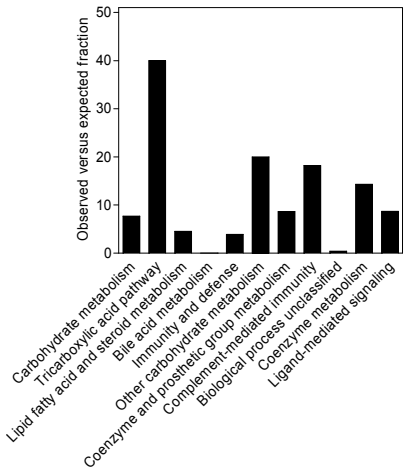


Fig. 5: Biological process classes significantly affected in the *secondary* response. Biological processes with an enhanced number of highly regulated genes associated with the *secondary* response cells to increased dietary lipid levels of liver parenchymal in LDLr deficient mice (P<0.05; Binomial test; PANTHER software).

The genes in the tricarboxylic acid and carbohydrate, coenzyme, and prosthetic group metabolism pathways of which the expression was >5-fold changed upon feeding the Western-type for 6 weeks include ATP-citrate lyase (ACLY; 6.5-fold), liver pyruvate kinase (PKLR; 5.8-fold), glucose-6-phosphate dehydrogenase (G6PDX; 5.9-fold), acyl-CoA synthetase short-chain family member 2 (ACSS2; 8.6-fold), and an unassigned transcript coding for malic enzyme (ME; 6.8-fold). ACLY, PKLR, G6PDX, and ME are key enzymes involved in endogenous cholesterol, fatty acid, triacylglycerol and phospholipid synthesis (lipogenesis) in the liver[24]. Interestingly, sterol regulatory element binding proteins (SREBPs) are a family of transcription factors involved in lipogenesis as they can regulate the gene expression of lipogenic enzymes[25]. Interestingly, the gene expression of SREBP-1 was increased ~2-fold upon Western-type diet feeding.

Confirmation by real-time quantitative PCR

Representative genes of the different responses were selected for validation using real-time quantitative PCR. Time-dependent regulation of the expression of genes involved in the *primary* (i.e. FABP5 and ACC) and *secondary* response (i.e. PKLR, ACLY, ME, and SREBP-1) of liver parenchymal cells to Western-type diet feeding could be confirmed by real-time quantitative PCR using HPRT, 36B4, and GAPDH as housekeeping gene controls (**Fig. 6**). FABP5 mRNA expression is readily detectable under normal feeding conditions (Ct = 23.07, whilst the no-RT control Ct value is 38 (data not shown)) and it is markedly enhanced upon Western-type diet feeding (Ct values 18.91, 18.76, and 18.72 after 2, 4, and 6 weeks, respectively). FABP5 thus seems to play a very important role in the primary response, and therefore it was determined whether Western-type diet induced changes in gene expression levels of FABP5 were translated in similar changes on the protein level. FABP5 protein expression was undetectable under both chow and Western-type diet feeding conditions, indicating that the protein expression level of FABP5 is low in parenchymal cells. This is in agreement with our original microarray data which show that the gene expression of FABP5 and the four putative novel FABPs is relatively low (relative expression level of 9.3 (FABP5), 50.3 (mCG22278), 13.5 (mCG5289), 19.0 (mCG22653), 9.6 (mCG9729), respectively) as compared to, for instance, the expression of FABP1/L-FABP (relative expression 7058), an established liver expressed FABP[22].

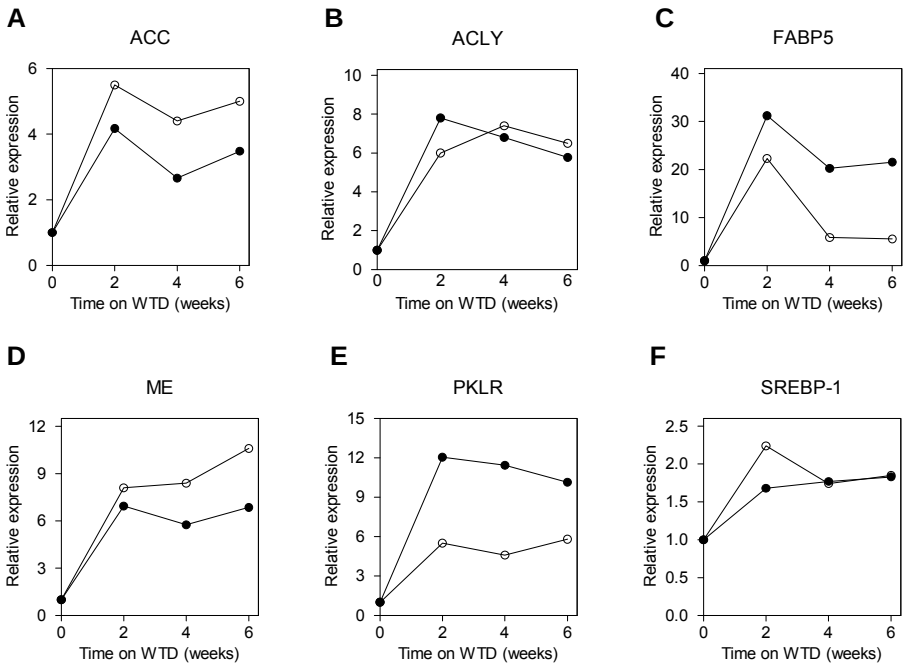


Fig. 6: Real-time quantitative PCR validation. Real-time quantitative PCR (filled circles) and microarray analysis (open circles) of the Western-type diet (WTD) induced changes in the expression of genes involved in the response of liver parenchymal cells in LDLr deficient mice.

Since SREBP-1 is an important transcription factor that can affect the expression of the highly upregulated genes involved in lipogenesis (i.e. SCD-1, PKLR and ACLY) it was also determined whether SREBP-1 protein expression was increased upon Western-type diet feeding. Importantly, SREBP-1 is synthesized as an inactive precursor protein (125-kD), which has to be cleaved into a smaller active mature SREBP-1 protein (66-kD)[26]. Strikingly, in contrast to the observed increase in SREBP-1 mRNA expression, a clear downregulation of the mature SREBP-1 protein was determined upon two weeks of atherogenic diet feeding both in the nuclear and cytoplasmic fraction (**Fig. 7**). Quantification using ImageQuant software revealed that nuclear mature SREBP-1 protein expression was 40% (n=2) decreased on the Western-type diet, while cytoplasmic protein expression was 12% (n=2) decreased. The marked decrease in nuclear SREBP-1 protein expression indicates that SREBP-1 activity was decreased in liver parenchymal cells from LDLr deficient mice upon Western-type diet feeding.

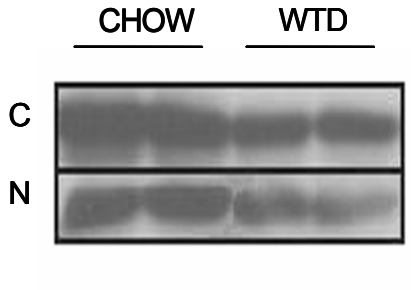


Fig. 7: Protein expression of SREBP-1. Effect of Western-type diet (WTD) feeding for two weeks on the protein expression of mature SREBP-1 in liver parenchymal cell nuclear (N) and cytoplasmic (C) fractions.

DISCUSSION

The purpose of the current study was to gain insight into the response of the liver parenchymal cell, which is mainly responsible for the livers metabolic function, to a rise in dietary lipid levels in an atherosclerosis prone mouse model. More specifically, liver parenchymal cell gene expression profiles in LDLr deficient mice on a Western-type (atherogenic) diet containing 0.25% cholesterol and 15% fat for 2, 4, and 6 weeks were compared to those on a regular chow diet containing 4.3% fat and no cholesterol using microarray technology.

Based upon the microarray expression profiles, we propose that FABP5 and four putative novel FABP members may play an essential role in the *primary* response of liver parenchymal cells to a rise in dietary lipid levels, since their expression highly (>10-fold) increases within the first two weeks of diet feeding with a subsequent decline in the following 2-4 weeks. The FABP family consists of low-molecular-mass, soluble, intracellular lipid carriers that bind fatty acid ligands with high affinity. Importantly, deficiencies in or malfunctioning of FABPs have been associated with the etiology of several lipid-related diseases such as diabetes, hyperlipidemia, and atherosclerosis in both humans and animal disease models[27-31]. FABP1, also named liver FABP (L-FABP), is the key FABP involved in the cellular uptake and metabolism of long-chain fatty acids in the liver and its expression is essential for peroxisomal beta-oxidation of fatty acids[32-34]. In addition to fatty acids, FABP1 is also able to bind or interact with a wide

variety of other ligands, including anionic cholesterol derivatives and bile acids[35,36]. However, Western-type diet feeding did not affect gene expression levels of FABP1 in liver parenchymal cells (data not shown). FABP5, also named endothelial FABP (E-FABP), functions as an antioxidant protein by scavenging reactive lipids (i.e. fatty acids) such as 4-hydroxynonenal (4-HNE)[37] and leukotriene A(4) (LTA(4))[38]. In addition, FABP5 also plays a role in basal and hormone-stimulated lipolysis in adipose tissue[39]. It thus seems that expression of specific fatty acid transporters in liver parenchymal cells is markedly changed as a result of increased dietary lipid levels, thereby potentially facilitating lipid uptake, transport and metabolism. The fact that the expression of FABP5 and the four novel FABP-5-like transcripts is highly induced upon Western-type diet feeding suggests that, in liver parenchymal cells, these proteins may play an important role in the protection against cellular toxicity of reactive lipids like 4-HNE and LTA(4) through transporting them to intracellular compartments for subsequent metabolism. Since the four putative FABPs share a homology with respect to their sequence, expression, and regulation with FABP5, it will be very interesting to further study the possible specific (shared?) functions of FABP5 and these novel FABPs in liver. Interestingly, microarray analysis by Maxwell *et al*[7] revealed that FABP5 may be a novel hepatic SREBP target gene, since its cholesterol diet induced regulation profile in total liver correlated with other known SREBP target genes. The mRNA expression of SREBP-1 was increased in liver parenchymal cells in response to Western-type diet feeding. However, the cytoplasmic and nuclear expression of the mature (active) SREBP-1 protein was decreased by 2 weeks of Western-type diet feeding, indicating that the activity of SREBP-1 was actually lower on the diet. The decrease in mature SREBP-1 protein is likely due to the extensive lipid loading as Wang *et al* have shown that cholesterol or oxysterol loading of cells results in a rapid decay of mature SREBP-1 protein due to an impaired cleavage of the precursor protein[26]. Although data from Maxwell *et al* have suggested that FABP5 is a putative novel SREBP-1 target gene, the ~20-fold increase in parenchymal liver cell FABP5 expression within the first two weeks on the Western-type diet in the current study was thus not due to an enhanced SREBP-1 activity. Loading of cells with fatty acids and cholesterol(-derivatives) has also been associated with changes in the activity of other nuclear receptors such as the liver X receptor (LXR), the retinoic acid receptor (RAR) and peroxisome proliferators-activated receptors (PPARs)[40-44]. It will therefore be interesting to study whether these and possibly other nuclear receptors are involved in the regulation of the hepatic expression of FABP5 and the novel FABPs.

In the *secondary* response of liver parenchymal cells to the Western-type diet the expression of key genes involved in lipogenesis pathways was markedly stimulated. More specifically, in our study a marked consistent upregulation of genes involved in hepatic glucose metabolism (i.e. pyruvate kinase[45]) and subsequent pyruvate metabolism and lipogenesis pathways (i.e. ACLY[46] and ME[46]) was observed in isolated liver parenchymal cells upon Western-type diet feeding, which suggests that the Western-type diet as a *secondary* response induces glycolytic and lipogenesis pathways. This is in agreement with microarray data provided by Fourmestraux *et al*[9],

which indicates that high fat diet feeding stimulates glycolytic pathways in total liver. The apparent increase in liver lipogenesis may also explain the observed increase in serum VLDL cholesterol levels in the LDLr deficient mice on Western-type diet, since Grefhorst *et al* have also shown that stimulation of lipogenesis through pharmacological activation of the nuclear receptor LXR leads to the production of large triglyceride-rich VLDL particles[47]. In accordance, LDLr mice deficient that have an increased hepatic lipogenesis rate due to crossing with SREBP-1 transgenic mice accumulate large lipid-rich lipoproteins (VLDL) due to an increased synthesis and secretion and a blocked degradation via the LDLr[48].
In conclusion, using a microarray based approach, we have identified FABP5 and four putative novel FABP5-like fatty acid binding proteins as important genes involved in the *primary* response of liver parenchymal cells from LDLr deficient mice to Western-type diet feeding, since they may play an important role in the detoxification of (specific) free fatty acids and/or lipid oxidants. Furthermore, as a *secondary* response, liver parenchymal cells stimulate glycolysis and lipogenesis pathways, resulting in a subsequent rise in the serum levels of atherogenic lipoproteins VLDL/LDL.

ACKNOWLEDGEMENTS

The study was supported by the Pharmacogenomics Unit of the Leiden/Amsterdam Center for Drug Research within the Centre for Medical Systems Biology, a Genomics Center of Excellence recognized and financially supported by the Netherlands Genomics Initiative. M. Stitzinger and J. Kuiper were supported by the Netherlands Heart Foundation (grants 2001B164 and 2000T40). R. Dorland and N.H. Binh are thanked for their assistance with the immunoblotting.

REFERENCES

1. Cui Y, Blumenthal RS, Flaws JA, Whiteman MK, Langenberg P, Bachorik PS and Bush TL (2001) Non-high-density lipoprotein cholesterol level as a predictor of cardiovascular disease mortality. *Arch Intern Med*. **161**: 1413-1419.
2. Goldstein JL and Brown MS (1979) The LDL receptor locus and the genetics of familial hypercholesterolemia. *Annu Rev Genet*. **13**: 259-289.
3. Glomset JA (1980) High-density lipoproteins in human health and disease. *Adv Intern Med*. **25**: 91-116.
4. Brown MS and Goldstein JL (1986) A receptor-mediated pathway for cholesterol homeostasis. *Science*. **232**: 34-47.
5. Gibbons GF (1990) Assembly and secretion of hepatic very-low-density lipoprotein. *Biochem J*. **268**: 1-13.
6. Brewer HB, Jr. and Santamarina-Fojo S (2003) New insights into the role of the adenosine triphosphate-binding cassette transporters in high-density lipoprotein metabolism and reverse cholesterol transport. *Am J Cardiol*. **91**: 3E-11E.
7. Maxwell KN, Soccio RE, Duncan EM, Sehayek E and Breslow JL (2003) Novel putative SREBP and LXR target genes identified by microarray analysis in liver of cholesterol-fed mice. *J Lipid Res*. **44**: 2109-2119.
8. Kim S, Sohn I, Ahn JI, Lee KH and Lee YS (2004) Hepatic gene expression profiles in a long-term high-fat diet-induced obesity mouse model. *Gene*. **340**: 99-109.

9. de Fourmestraux V, Neubauer H, Poussin C, Farmer P, Falquet L, Burcelin R, Delorenzi M and Thorens B (2004) Transcript profiling suggests that differential metabolic adaptation of mice to a high fat diet is associated with changes in liver to muscle lipid fluxes. *J Biol Chem*. **279**: 50743-50753.
10. Hoekstra M, Kruijt JK, Van Eck M and Van Berkel TJ (2003) Specific gene expression of ATP-binding cassette transporters and nuclear hormone receptors in rat liver parenchymal, endothelial, and Kupffer cells. *J Biol Chem*. **278**: 25448-25453.
11. Hoekstra M, Out R, Kruijt JK, Van Eck M and Van Berkel TJ (2005) Diet induced regulation of genes involved in cholesterol metabolism in rat liver parenchymal and Kupffer cells. *J Hepatol*. **42**: 400-407.
12. Recinos A, 3rd, Carr BK, Bartos DB, Boldogh I, Carmical JR, Belalcazar LM and Brasier AR (2004) Liver gene expression associated with diet and lesion development in atherosclerosis-prone mice: induction of components of alternative complement pathway. *Physiol Genomics*. **19**: 131-142.
13. Tanaka M, Nakashima O, Wada Y, Kage M and Kojiro M (1996) Pathomorphological study of Kupffer cells in hepatocellular carcinoma and hyperplastic nodular lesions in the liver. *Hepatology*. **24**: 807-812.
14. Mazzocca A, Carloni V, Sciammetta S, Cordella C, Pantaleo P, Caldini A, Gentilini P and Pinzani M (2002) Expression of transmembrane 4 superfamily (TM4SF) proteins and their role in hepatic stellate cell motility and wound healing migration. *J Hepatol*. **37**: 322-330.
15. Ishibashi S, Brown MS, Goldstein JL, Gerard RD, Hammer RE and Herz J (1993) Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J Clin Invest*. **92**: 883-893.
16. Ishibashi S, Goldstein JL, Brown MS, Herz J and Burns DK (1994) Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. *J Clin Invest*. **93**: 1885-1893.
17. Nagelkerke JF, Barto KP and van Berkel TJ (1983) In vivo and in vitro uptake and degradation of acetylated low density lipoprotein by rat liver endothelial, Kupffer, and parenchymal cells. *J Biol Chem*. **258**: 12221-12227.
18. Van Berkel TJ, Van Velzen A, Kruijt JK, Suzuki H and Kodama T (1998) Uptake and catabolism of modified LDL in scavenger-receptor class A type I/II knock-out mice. *Biochem J*. **331 (Pt 1)**: 29-35.
19. Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*. **162**: 156-159.
20. van Eck M, Bos IS, Kaminski WE, Orso E, Rothe G, Twisk J, Bottcher A, Van Amersfoort ES, Christiansen-Weber TA, Fung-Leung WP, Van Berkel TJ and Schmitz G (2002) Leukocyte ABCA1 controls susceptibility to atherosclerosis and macrophage recruitment into tissues. *Proc Natl Acad Sci U S A*. **99**: 6298-6303.
21. Van Eck M, Bos IS, Hildebrand RB, Van Rij BT and Van Berkel TJ (2004) Dual role for scavenger receptor class B, type I on bone marrow-derived cells in atherosclerotic lesion development. *Am J Pathol*. **165**: 785-794.
22. Glatz JF, Luiken JJ, van Bilsen M and van der Vusse GJ (2002) Cellular lipid binding proteins as facilitators and regulators of lipid metabolism. *Mol Cell Biochem*. **239**: 3-7.
23. Boord JB, Fazio S and Linton MF (2002) Cytoplasmic fatty acid-binding proteins: emerging roles in metabolism and atherosclerosis. *Curr Opin Lipidol*. **13**: 141-147.
24. Shimano H, Yahagi N, Amemiya-Kudo M, Hasty AH, Osuga J, Tamura Y, Shionoiri F, Iizuka Y, Ohashi K, Harada K, Gotoda T, Ishibashi S and Yamada N (1999) Sterol regulatory element-binding protein-1 as a key transcription factor for nutritional induction of lipogenic enzyme genes. *J Biol Chem*. **274**: 35832-35839.
25. Eberle D, Hegarty B, Bossard P, Ferre P and Foufelle F (2004) SREBP transcription factors: master regulators of lipid homeostasis. *Biochimie*. **86**: 839-848.
26. Wang X, Sato R, Brown MS, Hua X and Goldstein JL (1994) SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. *Cell*. **77**: 53-62.
27. Binas B, Danneberg H, McWhir J, Mullins L and Clark AJ (1999) Requirement for the heart-type fatty acid binding protein in cardiac fatty acid utilization. *Faseb J*. **13**: 805-812.
28. Vassileva G, Huwyler L, Poirier K, Agellon LB and Toth MJ (2000) The intestinal fatty acid binding protein is not essential for dietary fat absorption in mice. *Faseb J*. **14**: 2040-2046.

29. Pratley RE, Baier L, Pan DA, Salbe AD, Storlien L, Ravussin E and Bogardus C (2000) Effects of an Ala54Thr polymorphism in the intestinal fatty acid-binding protein on responses to dietary fat in humans. *J Lipid Res*. **41**: 2002-2008.
30. Georgopoulos A, Aras O and Tsai MY (2000) Codon-54 polymorphism of the fatty acid-binding protein 2 gene is associated with elevation of fasting and postprandial triglyceride in type 2 diabetes. *J Clin Endocrinol Metab*. **85**: 3155-3160.
31. Carlsson M, Orho-Melander M, Hedenbro J, Almgren P and Groop LC (2000) The T 54 allele of the intestinal fatty acid-binding protein 2 is associated with a parental history of stroke. *J Clin Endocrinol Metab*. **85**: 2801-2804.
32. Brandes R, Kaikaus RM, Lysenko N, Ockner RK and Bass NM (1990) Induction of fatty acid binding protein by peroxisome proliferators in primary hepatocyte cultures and its relationship to the induction of peroxisomal beta-oxidation. *Biochim Biophys Acta*. **1034**: 53-61.
33. Newberry EP, Xie Y, Kennedy S, Han X, Buhman KK, Luo J, Gross RW and Davidson NO (2003) Decreased hepatic triglyceride accumulation and altered fatty acid uptake in mice with deletion of the liver fatty acid-binding protein gene. *J Biol Chem*. **278**: 51664-51672.
34. Hanhoff T, Lucke C and Spener F (2002) Insights into binding of fatty acids by fatty acid binding proteins. *Mol Cell Biochem*. **239**: 45-54.
35. Thumser AE and Wilton DC (1996) The binding of cholesterol and bile salts to recombinant rat liver fatty acid-binding protein. *Biochem J*. **320 (Pt 3)**: 729-733.
36. Woodford JK, Behnke WD and Schroeder F (1995) Liver fatty acid binding protein enhances sterol transfer by membrane interaction. *Mol Cell Biochem*. **152**: 51-62.
37. Bennaars-Eiden A, Higgins L, Hertzel AV, Kapphahn RJ, Ferrington DA and Bernlohr DA (2002) Covalent modification of epithelial fatty acid-binding protein by 4-hydroxynonenal in vitro and in vivo. Evidence for a role in antioxidant biology. *J Biol Chem*. **277**: 50693-50702.
38. Dickinson Zimmer JS, Voelker DR, Bernlohr DA and Murphy RC (2004) Stabilization of leukotriene A4 by epithelial fatty acid-binding protein in the rat basophilic leukemia cell. *J Biol Chem*. **279**: 7420-7426.
39. Hertzel AV, Bennaars-Eiden A and Bernlohr DA (2002) Increased lipolysis in transgenic animals overexpressing the epithelial fatty acid binding protein in adipose cells. *J Lipid Res*. **43**: 2105-2111.
40. Janowski BA, Willy PJ, Devi TR, Falck JR and Mangelsdorf DJ (1996) An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature*. **383**: 728-731.
41. Peet DJ, Janowski BA and Mangelsdorf DJ (1998) The LXRs: a new class of oxysterol receptors. *Curr Opin Genet Dev*. **8**: 571-575.
42. Dreyer C, Keller H, Mahfoudi A, Laudet V, Krey G and Wahli W (1993) Positive regulation of the peroxisomal beta-oxidation pathway by fatty acids through activation of peroxisome proliferator-activated receptors (PPAR). *Biol Cell*. **77**: 67-76.
43. Keller H, Dreyer C, Medin J, Mahfoudi A, Ozato K and Wahli W (1993) Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proc Natl Acad Sci U S A*. **90**: 2160-2164.
44. Bonilla S, Redonnet A, Noel-Suberville C, Pallet V, Garcin H and Higueret P (2000) High-fat diets affect the expression of nuclear retinoic acid receptor in rat liver. *Br J Nutr*. **83**: 665-671.
45. Amemiya-Kudo M, Shimano H, Hasty AH, Yahagi N, Yoshikawa T, Matsuzaka T, Okazaki H, Tamura Y, Iizuka Y, Ohashi K, Osuga J, Harada K, Gotoda T, Sato R, Kimura S, Ishibashi S and Yamada N (2002) Transcriptional activities of nuclear SREBP-1a, -1c, and -2 to different target promoters of lipogenic and cholesterogenic genes. *J Lipid Res*. **43**: 1220-1235.
46. Sato R, Okamoto A, Inoue J, Miyamoto W, Sakai Y, Emoto N, Shimano H and Maeda M (2000) Transcriptional regulation of the ATP citrate-lyase gene by sterol regulatory element-binding proteins. *J Biol Chem*. **275**: 12497-12502.
47. Grefhorst A, Elzinga BM, Voshol PJ, Plosch T, Kok T, Bloks VW, van der Sluijs FH, Havekes LM, Romijn JA, Verkade HJ and Kuipers F (2002) Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. *J Biol Chem*. **277**: 34182-34190.

48. Horton JD, Shimano H, Hamilton RL, Brown MS and Goldstein JL (1999) Disruption of LDL receptor gene in transgenic SREBP-1a mice unmasks hyperlipidemia resulting from production of lipid-rich VLDL. *J Clin Invest*. **103**: 1067-1076.