Microarray analysis indicates an important role for FABP5 and putative novel FABPs on a Western-type diet[S]

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Abstract **Liver parenchymal cells play a dominant role in hepatic metabolism and thereby total body cholesterol homeostasis. To gain insight into the specific pathways and genes involved in the response of liver parenchymal cells to increased dietary lipid levels under atherogenic conditions, 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 LDL receptor-deficient mice, an established atherosclerotic animal model. Using ABI Mouse Genome Survey Arrays, we were able to detect 7,507 genes (28% of the total number on an array) that were expressed in parenchymal cells isolated from livers of LDL receptor-deficient mice at every time point investigated. Time-dependent gene expression profiling identified fatty acid binding protein 5 (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, because 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 the cellular toxicity of cholesterol, free fatty acids, and/or lipid oxidants. Furthermore, as a secondary response to the Western-type diet, liver parenchymal cells of LDL receptor-deficient mice stimulated glycolysis and lipogenesis pathways, resulting in a steady, more atherogenic serum lipoprotein profile (increased VLDL/LDL).—** Hoekstra, M., M. Stitzinger, E. J. A. van Wanrooij, I. N. Michon, J. K. Kruijt, J. Kamphorst, M. Van Eck, E. Vreugdenhil, T. J. C. Van Berkel, and J. Kuiper. **Microarray analysis indicates an important role for FABP5 and putative novel FABPs on a Western-type diet.** ***J. Lipid Res.*** **2006.** 47: **2198–2207.**

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High levels of circulating cholesterol attributable to the 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 causes of death in the Western world. Several mutations in the LDL receptor are associated with familial hypercholesterolemia, a dominantly inherited error of metabolism characterized by increased plasma LDL levels, xanthomas of skin and tendons, and premature heart disease caused by atherosclerosis of the coronary arteries (2).

The liver is an essential organ in the regulation of serum cholesterol levels because 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 VLDL and HDL, respectively (5, 6). Because of 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 with these types of microarray studies is the heterogeneity of the liver, which contains several different cell types, each of which has its 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 liver cells are 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.

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Abbreviations: ACLY, ATP-citrate lyase; cRNA, complementary RNA; Ct, threshold cycle number; FABP, fatty acid binding protein; HPRT, hypoxanthine guanine phosphoribosyl transferase; PKLR, liver pyruvate kinase; SREBP, sterol-regulatory element binding protein; 36B4, acidic ribosomal phosphoprotein P0.

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[S] The online version of this article (available at http://www.jlr.org) contains additional two tables and one figure.

Importantly, in earlier studies, we showed that not only the function of parenchymal cells is different from that of other hepatic cells but also the expression and regulation of genes involved in lipid metabolism are 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 as well as 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 from microarray studies that are based on total liver mRNA. Therefore, in this study, using microarray technology, we focused on the specific response of liver parenchymal cells to atherogenic diet feeding in LDL receptor-deficient mice, an established atherosclerosis mouse model.

MATERIALS AND METHODS

Animals

Homozygous LDL receptor-deficient mice (15, 16) were obtained from the Jackson Laboratory as mating pairs and bred at the Gorlaeus Laboratories. Female mice were maintained on sterilized regular chow containing 4.3% (w/w) fat and no cholesterol (RM3; Special Diet Services, Witham, UK) or were fed a semisynthetic Western-type diet containing 15% (w/w) cacao butter and 0.25% (w/w) cholesterol (Diet W; Special Diet Services) for 2, 4, or 6 weeks. Subsequently, parenchymal liver cells were isolated essentially according to the method of Nagelkerke, Barto, and Van Berkel (17) as modified for mice by Van Berkel et al. (18). The purity and viability of the cells were analyzed 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 of >99% parenchymal cells with a viability of >95% under both standard and Western-type diet feeding conditions.

Serum lipid analyses

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 analyzed by fractionation of 30 μl of serum from 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 reaction was used to synthesize UTP-digoxigenin-labeled complementary RNA (cRNA). Equal amounts of cRNA from two pooled RNA samples of two mice (total of four mice) per time point were 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 control spots were scanned for 5 and 25 s using an AB1700 Chemiluminescence Analyzer (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 ratio for every spot was obtained, which needed to be at least 1 at each time point (>90% spot confidence) 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 of 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 with 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 five clusters, whereas similarity between gene expression profiles was determined using a cosine correlation (Spotfire software).

Confirmation of gene expression changes by real-time quantitative PCR

Quantitative gene expression analysis of isolated liver parenchymal cells was performed as described (10). In short, total RNA

TABLE 1. Primers used for real-time quantitative PCR

Gene	GenBank Accession Number	Forward Primer	Reverse Primer	Amplicon Size
Acidic ribosomal phosphoprotein P0	NM007475	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG	85
Acetyl-CoA carboxylase	XM109883	AGAATCTCCTGGTGACAATGCTTATT	GCTCTGTGAGGATATTTAGCAGCTC	87
ATP-citrate lyase	NM134037	AGGTACCCTGGGTCCACATTC	CTACGATCATCTTGACTCCTGGAGT	73
Fatty acid binding protein 5	BC002008	GGAAGGAGAGCACGATAACAAGA	GGTGGCATTGTTCATGACACA	73
Glyceraldehyde-3-phosphate dehydrogenase	NM008084	TCCATGACAACTTTGGCATTG	TCACGCCACAGCTTTCCA	103
Hypoxanthine guanine phosphoribosyl transferase	J00423	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG	91
Malic enzyme	NM008615	TTAAGGATCCACTGTACATCGGG	GGCGTCATACTCAGGGCCT	62
Liver pyruvate kinase	NM013631	AAGACAGTGTGGGTGGACTACCA	CGTCAATGTAGATGCGGCC	70
Sterol-regulatory element binding protein-1	AB017337	GACCTGGTGGTGGGCACTGA	AAGCGGATGTAGTCGATGGC	74

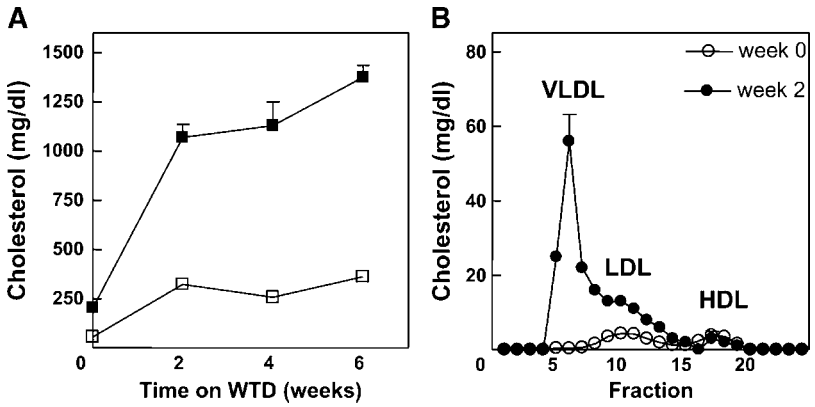


Fig. 1. A: Effect of a Western-type diet (WTD) on serum-free (open squares) and total (closed squares) cholesterol levels in LDL receptor-deficient mice. B: Effect of a Western-type diet on serum cholesterol distribution in LDL receptor-deficient mice. Blood samples were drawn on a chow diet (open circles) and 2 weeks on a Western-type diet (closed circles). Sera from individual mice were loaded onto a Superose 6 column, and fractions were collected. Fractions 3–7 represent VLDL, fractions 8–15 represent LDL, and fractions 15–19 represent HDL. Error bars represent means ± SEM (n = 4) per group.

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 listed in **Table 1**. Hypoxanthine guanine phosphoribosyl transferase (HPRT), 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 HPRT, GAPDH, and 36B4 ($Ct_{housekeeping}$) and raising 2 to the power of this difference. The average Ct of three housekeeping genes was used to exclude the possibility that changes in relative expression were caused by variations in the expression of separate housekeeping genes.

Immunoblotting

Pelleted liver parenchymal cells were suspended in 500 μl of 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

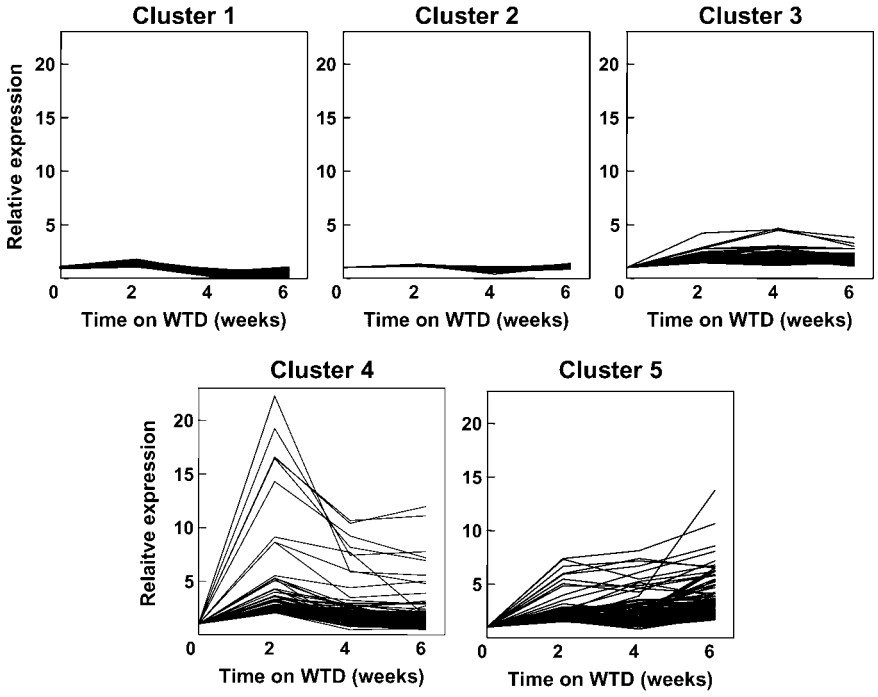


Fig. 2. Gene clusters detected in liver parenchymal cells of LDL receptor-deficient mice. K-means clustering of genes was performed based upon similarity in their regulation profiles upon Western-type diet (WTD) feeding.

TABLE 2. List of genes involved in the primary response of liver parenchymal cells and whose expression was >5-fold changed after 2 weeks of Western-type diet feeding

Celera Gene Identifier	GenBank Accession Number	Fold Change on Western-Type Diet Compared with Chow Diet			Gene
		2 Weeks	4 Weeks	6 Weeks	
mCG1638	NM010634	22	5.9	5.5	Fatty acid binding protein 5, epidermal (FABP5)
mCG22278	AC134443	19	7.4	7.7	Unassigned FABP, chromosome 8
mCG5289	AC130218	17	10	12	Unassigned FABP, chromosome 18
mCG22653	AC147992	16	11	11	Unassigned FABP, chromosome 18
mCG7050	NM009381	16	8.1	6.9	Thyroid hormone-responsive SPOT14 homolog (THRSP)
mCG9729	AL954662	14	9.2	7.2	Unassigned FABP, chromosome 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 β
mCG141310	—[a]	5.5	4.4	5.0	Unassigned acetyl-CoA carboxylase, chromosome 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, chromosome 6

[a] Unassigned Celera transcript is 99% homologous to AY451393 (acetyl-CoA carboxylase 1).

pelleted by a 10 min centrifugation (13,000 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 13,000 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 membranes (Schleicher and Schuell). Immunolabeling was performed using either goat polyclonal fatty acid binding protein 5 (FABP5; R&D Systems) or rabbit polyclonal sterol-regulatory element binding protein-1 (SREBP-1) (H160; Santa Cruz Biotechnology) as primary antibody and donkey anti-goat IgG or goat anti-rabbit IgG (Jackson ImmunoResearch), respectively, as secondary antibody. Finally, immunolabeling was detected by enhanced chemiluminescence (Amersham Biosciences). Changes in protein expression levels were quantified using ImageQuant software.

RESULTS

Serum lipid levels

Feeding LDL receptor-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 increase in free and total serum cholesterol levels compared with 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 LDL receptor-deficient mice, because the circulating serum cholesterol levels of both LDL and VLDL were markedly induced upon feeding the Western-type diet compared with the chow diet (Fig. 1B). The dramatic increase 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, whereas the clearance is greatly inhibited by the absence of the LDL receptor.

Gene expression profiles of liver parenchymal cells

To gain insight into the primary and possible secondary response of liver parenchymal cells to an increase 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 LDL receptor-deficient mice on the regular chow or Western-type diet (2, 4, or 6 weeks). Two RNA samples containing

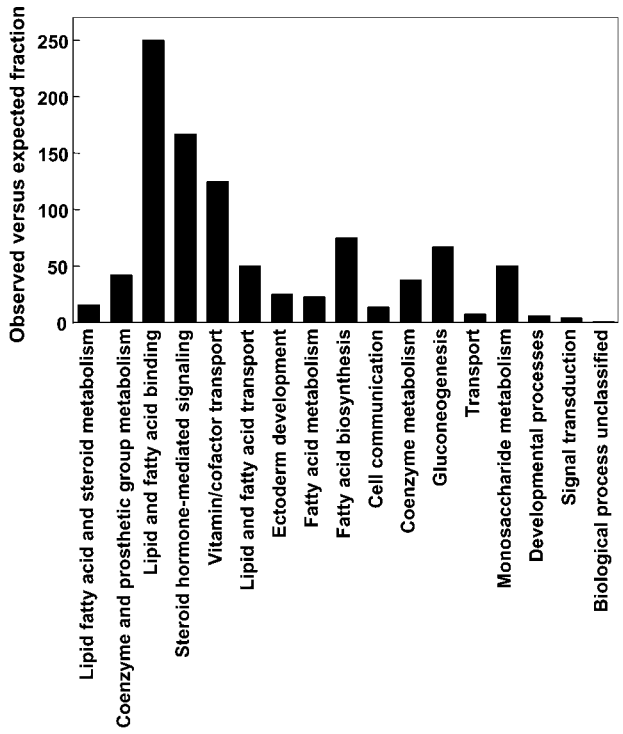
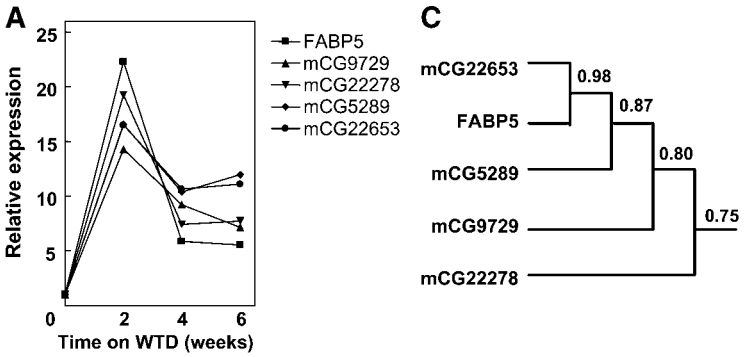


Fig. 3. Biological process classes with a significantly ($P < 0.05$; binomial test; PANTHER software) enhanced number of highly regulated genes associated with the primary response of liver parenchymal cells to increased dietary lipid levels.

pooled RNA from two separate mice per group (total group size, four 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 AB1700 software. Using a cutoff minimal signal-to-noise ratio of 1 (>90% spot confidence) for every time point, we were able to detect 7,507 genes (28% of the total number on an array) that were expressed in parenchymal cells isolated from livers of LDL receptor-deficient mice at every time point investigated. Subsequently, using K-means clustering, five 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 = 1,305), 2 (n = 2,110), and 3 (n = 2,408) contain ~78% of the total number of genes detected in liver parenchymal cells. Gene cluster 1 contains some genes whose expression was >5-fold downregulated after 6 weeks of Western-type diet feeding (for a more detailed view of cluster 1, see the supplementary Figure I and Table I). 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 2 weeks of diet feeding, after which the gene expression rapidly declines even sometimes to the basal level, indicating that these genes play an important role in the primary response of liver parenchymal cells to an increase 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, because their expression gradually but steadily increases over time upon Western-type diet feeding for 6 weeks.

Identification of genes and biological processes involved in the primary response of liver parenchymal cells to increased dietary lipid levels

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 increase in dietary lipid levels, because 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 2 weeks of Western-type diet feeding. The known/assigned genes in this list include FABP5, thyroid hormone-responsive SPOT14 homolog (THRSP), DNA damage-inducible transcript 4 (DDIT4), fatty acid elon-



B

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FABP5      MASLKDLEGKWRLMESHGFEEYMKELGVGLALRKMAAMAKPDCIITCDGNNITVKTESTV
mCG22653   MASLKDLEGKWCLMESHGFEEYMKELRVGLALRKMAAMAKPDCIITCDGNNITVKTESTV
mCG5289    MASLKDLEGKWRL LESHSFEEYMKELGVGLALRKMAAMAKSDCIII ICDGNNITFKTESTV
mCG22278   MASLKDLEGKWRLMESHGFEEYAKELGVGLALRKMAAMAKPDSIITCDGNNIT I KTESTV
mCG9729    MANLKDLKGKWCLMESHGFEEYTKELGVGLALRKMAAMAKPDCIITCDGNNITVKTVSTV

FABP5      KTTVFSCNLGEKFDETTADGRKTETVCTFQDGALVQHQQWDGKEST I TRKLKDGKMIVEC
mCG22653   KTTVFSCNLGETFDETTADGRKTETVCTFQDGALVQHQQWDGKEST I TRKLKDGKMI VEC
mCG5289    KTTVFSCTLGEKFDETTADGRKTETVCTFQDGAL IQHQQWEGKES TVTGNLKDAKMTVER
mCG22278   KTTVFSCTLGEKFDETMADGRKTEMVCTFQDGALVQHQQWEGEHDNRKTEGWEVDCGACH
mCG9729    KTTLFSCTLGEKFDETTADGRKTETVCTFKDGAWSS TSNGKGRRT

FABP5      VMNNATCTRVYEKVQ
mCG22653   VMNNATCTRVYEKVQ
mCG5289    VTNNATC TRLYEKVQ
mCG22278   EQCHLHSGLREGAMRASSSSWTGVSCQSEYAQFSERT
mCG9729
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Fig. 4. A: Time-dependent effect of a Western-type diet (WTD) on parenchymal liver cell gene expression of fatty acid binding protein 5 (FABP5) and four putative novel FABPs located on chromosomes 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 compared with 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.

gase 6 (ELOVL6), acetyl-CoA carboxylase, fatty acid synthase (Fasn), acetoacetyl-CoA synthetase, 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 with the total list of genes detected in liver parenchymal cells using PANTHER.

Figure 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 LDL receptor-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) overrepresented compared with expected. Strikingly, the five 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, Fazio, and Linton (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 an increase in dietary lipid levels.

Identification of genes and biological processes involved in the secondary response of liver parenchymal cells to increased dietary lipid levels

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 LDL receptor-deficient mice the diet enriched in cholesterol. Therefore, it is 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, among others, the expression of glucosidase β2 (GBA2), stearoyl-CoA desaturase 1, 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 compared with 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**). 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

TABLE 3. List of genes involved in the secondary response of liver parenchymal cells and whose expression was >5-fold changed after 6 weeks of Western-type diet feeding

Celera Gene Identifier	GenBank Accession Number	Fold Change on Western-Type Diet Compared with Chow Diet			Gene
		2 Weeks	4 Weeks	6 Weeks	
mCG19285	NM172692	0.9	0.4	14	Glucosidase β2 (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.[a]	1.2	0.7	9.8	Unassigned high-mobility group protein, chromosome 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, chromosome 19
mCG11880	XM001004685	7.3	5.5	6.8	Unassigned malic enzyme, chromosome 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), γ-transducing activity polypeptide 2 (GNGT2)
mCG129478	AL831708	1.1	1.8	5.5	Unassigned, chromosome 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, β 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)

[a] N.F., no homologous GenBank accession number found for the given unassigned Celera transcript sequence.

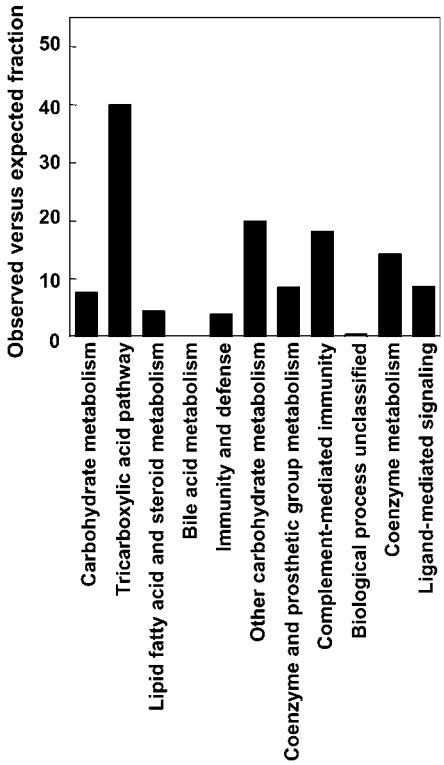

Fig. 5. Biological process classes with a significantly ($P < 0.05$; binomial test; PANTHER software) enhanced number of highly regulated genes associated with the secondary response of liver parenchymal cells to increased dietary lipid levels.

6 weeks include ATP-citrate lyase (ACLY; 6.5-fold), liver pyruvate kinase (PKLR; 5.8-fold), glucose-6-phosphate dehydrogenase X-linked (G6PDX; 5.9-fold), acyl-CoA synthetase short-chain family member 2 (8.6-fold), and an unassigned transcript coding for malic enzyme (6.8-fold). ACLY, PKLR, G6PDX, and malic enzyme are key enzymes involved in endogenous cholesterol, fatty acid, triacylglycerol, and phospholipid synthesis (lipogenesis) in the liver (24). Interestingly, 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 of changes in gene expression by real-time quantitative PCR

Representative genes with the different responses were selected for validation using real-time quantitative PCR. Time-dependent regulation of the expression of genes involved in the primary response (i.e., FABP5 and acetyl-CoA carboxylase) and secondary response (i.e., pyruvate kinase, ACLY, malic enzyme, 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**; for Ct values and absolute mRNA expression levels, see supplementary Table II). FABP5 mRNA expression is readily detectable under normal feeding conditions [Ct = 23.07; the no-RT control Ct value is 38 (data not shown)], and it is markedly enhanced upon Western-type diet feeding (Ct values of 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; therefore, it was determined whether Western-type diet-induced changes in gene expression levels of FABP5 were translated into similar changes at 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 levels of 9.3 (FABP5), 50.3 (mCG22278), 13.5 (mCG5289), 19.0 (mCG22653), and 9.6 (mCG9729), respectively] compared with, for instance, the expression of FABP1/L-FABP (relative expression, 7,058), an established liver-expressed FABP (22).

Because SREBP-1 is an important transcription factor that can affect the expression of the highly upregulated genes involved in lipogenesis (i.e., stearoyl-CoA dismutase-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 kDa), which has to be cleaved into a smaller active mature SREBP-1 protein (66 kDa) (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 2 weeks of atherogenic diet feeding in both the nuclear and cytoplasmic fractions (**Fig. 7**). Quantification using ImageQuant software revealed that nuclear mature SREBP-1 protein expression was decreased by 40% (n = 2) on the Western-type diet, whereas cytoplasmic protein expression was decreased by 12% (n = 2). The marked decrease in nuclear SREBP-1 protein expression indicates that SREBP-1 activity was decreased in liver parenchymal cells from LDL receptor-deficient mice upon Western-type diet feeding.

DISCUSSION

The purpose of this study was to gain insight into the response of the liver parenchymal cell, which is mainly responsible for the liver's metabolic function, to an increase in dietary lipid levels in an atherosclerosis-prone mouse model. More specifically, liver parenchymal cell gene expression profiles in LDL receptor-deficient mice on a Western-type (atherogenic) diet containing 0.25% cholesterol and 15% fat for 2, 4, and 6 weeks were compared with those of mice 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 an increase in dietary lipid levels, because their expression increases highly (>10-fold) within the first 2 weeks of diet feeding, with a subsequent decline

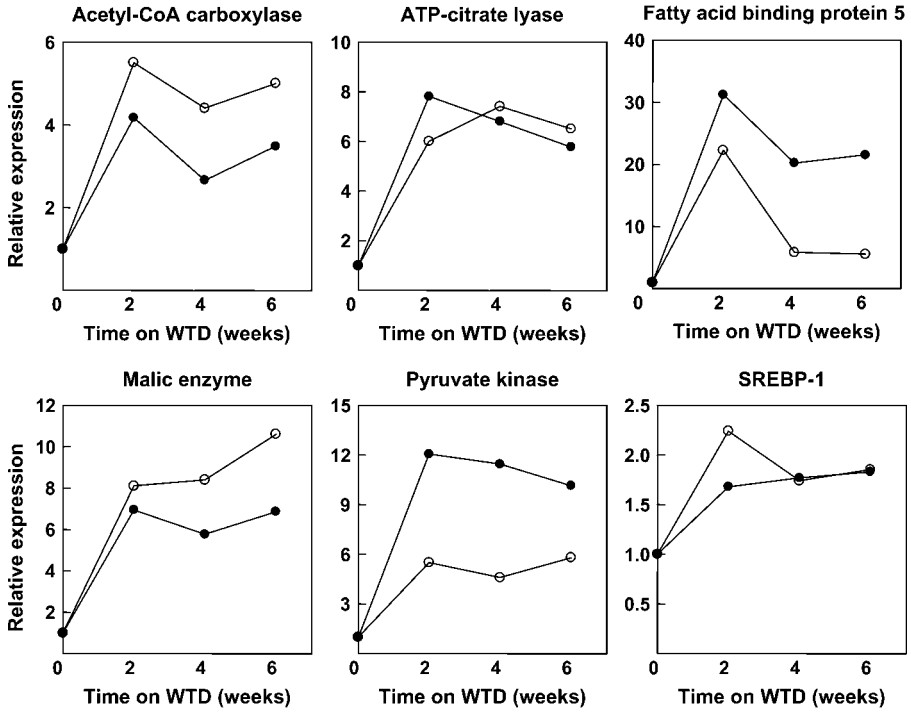

Fig. 6. Real-time quantitative PCR validation (closed circles) of the Western-type diet (WTD)-induced changes in the expression of genes involved in the response of liver parenchymal cells to increased dietary lipid levels in LDL receptor-deficient mice, as observed with microarray analysis (open circles). SREBP-1, sterol-regulatory element binding protein-1.

in the subsequent 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 the peroxisomal β-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 (37) and leukotriene A_4 (38). In addition, FABP5 also plays a role in basal and hormone-stimulated lipolysis in adipose tissue (39).

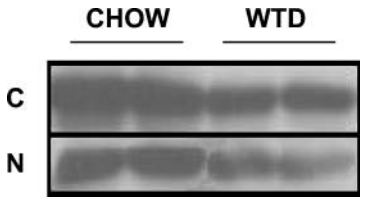

Fig. 7. Effect of Western-type diet (WTD) feeding for 2 weeks on the protein expression of mature SREBP-1 in liver parenchymal cell nuclear (N) and cytoplasmic (C) fractions.

It thus seems that the 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 FABP5-like transcripts is highly induced upon Western-type diet feeding suggests that, in liver parenchymal cells, these proteins may play an important role in protection against the cellular toxicity of reactive lipids such as 4-hydroxynonenal and leukotriene A_4, through transporting them to intracellular compartments for subsequent metabolism. Because the four putative FABPs share homology with respect to their sequences, expression, and regulation with FABP5, it will be 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, because its cholesterol diet-induced regulation profile in total liver correlated with that of 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 attributable to extensive lipid loading, as Wang et al. (26) have shown that cholesterol or oxysterol loading of cells results in a rapid decay of mature SREBP-1 protein attributable to an impaired cleavage of the precursor protein. Although data from Maxwell et al. (26) 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 2 weeks on the Western-type diet in this study was not caused by 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, the retinoic acid receptor, and peroxisome proliferator-activated receptors (40–44). Therefore, it will 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 malic enzyme (24)] 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 de Fourmestraux et al. (9) that indicate 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 LDL receptor-deficient mice on the Western-type diet, because Grefhorst et al. (47) have also shown that stimulation of lipogenesis through pharmacological activation of the nuclear receptor liver X receptor leads to the production of large triglyceride-rich VLDL particles. In accordance, LDL receptor-deficient mice that have an increased hepatic lipogenesis rate attributable to crossing with SREBP-1 transgenic mice accumulate large lipid-rich lipoproteins (VLDLs) as a result of increased synthesis and secretion and blocked degradation via the LDL receptor (48).

In conclusion, using a microarray-based approach, we have identified FABP5 and four putative novel FABP5-like FABPs as important genes involved in the primary response of liver parenchymal cells from LDL receptor-deficient mice to Western-type diet feeding, because 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 the glycolysis and lipogenesis pathways, resulting in a subsequent increase in serum levels of the atherogenic lipoproteins VLDL and LDL.

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