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Atherosclerosis 182 (2005) 249–257

ATHEROSCLEROSIS

www.elsevier.com/locate/atherosclerosis

Genomic analysis of the response of mouse models to high-fat feeding shows a major role of nuclear receptors in the simultaneous regulation of lipid and inflammatory genes

Arja J. Kreeft[a,1], Corina J.A. Moen[a,1], Gordon Porter[b], Soemini Kasanmoentalib[c], Ronit Sverdlov[d], Patrick J. van Gorp[d], Louis M. Havekes[e], Rune R. Frants[a], Marten H. Hofker[d,*]

[a] *Department of Human and Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands*
[b] *Incyte Genomics, Palo Alto, CA, USA*
[c] *Department of Clinical Epidemiology and Biostatistics, Free University Medical Center, Amsterdam, The Netherlands*
[d] *Cardiovascular Research Institute Maastricht, Department of Molecular Genetics, Universiteit Maastricht UNS50/11, P.O. Box 616, 6200MD Maastricht, The Netherlands*
[e] *TNO-PG and Department of Cardiology and Internal Medicine, LUMC, Leiden, The Netherlands*

Received 21 May 2004; received in revised form 14 January 2005; accepted 27 January 2005
Available online 16 March 2005

Abstract

The mechanisms of diet induced hyperlipidemia and atherosclerosis have been widely studied by delineating the role of candidate genes in transgenic and gene targeted mouse models. However, diet induced hyperlipidemia represents a complex process determined by many lipid genes that is only partly understood. This study is aimed at delineating the events induced by dietary intervention in different mouse models at the level of gene expression using microarray analysis. The focus is on the liver as the organ primarily responding to diet, and crucial in determining plasma lipid levels. Firstly, the effect of the genotype was studied. Expression profiles of liver genes were compared between APOE3Leiden (E3L), APOE knockout (E−/−) and C57BL/6JIco (B6) mice using the Incyte GEM 2.03 array carrying 9552 genes. Several hundred differentially expressed genes were identified indicating that the genotype alone effects gene expression. Secondly, the response of E3L mice to high-fat feeding was investigated using a mild and severe high-fat diet (diet W and N, respectively). Diet W caused differential regulation of 200 genes, while diet N affected the expression of 788 genes in B6 and 1010 genes in E3L mice. Annotation of these genes using the Gene Ontology (GO) database showed that two major processes were strongly affected by genotype and diet, namely lipid metabolism and inflammation, the latter as determined by "immune/defense response and detoxification" processes. Many nuclear receptor target genes were differentially regulated, with the largest effects modulated by the severe high-fat diet N, leading to the suppression of genes involved in bile acid, sterol, steroid, fatty acid, and detoxification metabolism. Strikingly, a substantial part of these nuclear receptor target genes were commonly regulated during the different experimental conditions. The common regulation of many nuclear receptor target genes underlying lipid and detoxification processes as found in this study, suggest a defense mechanism involving many nuclear receptors to protect against the accumulation of toxic endogenous lipids and bile acids. These results further strengthen the close link between hyperlipidemia and inflammatory processes.

Keywords: Hyperlipidemia; Transgenic mice; Gene expression profiles; Nuclear receptor target genes

1. Introduction

Transgenic mice with an altered lipoprotein metabolism have been instrumental for the molecular and biochemical

* Corresponding author. Tel.: +31 43 388 1138; fax: +31 43 388 4574.
E-mail address: m.hofker@gen.unimaas.nl (M.H. Hofker).
[1] Both authors contributed equally to this paper.

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doi:10.1016/j.atherosclerosis.2005.01.049

analysis of hyperlipidemia and atherosclerosis. In particular, knock out and transgenic mice based on the APOE gene, such as the APOE knock out (E−/−) mouse and the APOE3Leiden (E3L) mouse have been widely used. These mice are characterized by a severe reduction of the clearance of VLDL remnants, which leads to increased plasma lipid levels and results in significant diet induced changes in the lipoprotein metabolism [1,2]. The E3L mouse proved to be particularly useful to study the effect of diet on lipoprotein levels, because their lipid profile resembles those of humans. Hence, E3L mice fed a "western type diet" will develop high levels of triglycerides and cholesterol. For atherosclerosis induction in mice, diets are used that typically contain cholesterol and bile acids [3,4]. Despite our advanced knowledge of the genes involved in hyperlipidemia and atherosclerosis, much is based on the description of a selection of ∼2000 genes that are well known. At present, however, all of the 27,000–30,000 mouse genes have been sequenced and can be accessed via databases. To begin to explore which of these genes could be linked to hyperlipidemia and/or atherosclerosis, we have studied the expression patterns of 9552 genes in liver using microarray analysis. The liver was chosen because it is the major organ determining plasma lipid levels, and can also respond to other physiological challenges by orchestrating the primary defense against toxic lipids.

To obtain a better understanding of the mechanisms underlying hyperlipidemia and atherosclerosis, two related approaches were used. Microarray analysis was used to study the effect of genetically altering the lipoprotein metabolism in mice, to see which functionally related genes would become deregulated. To this end, the gene expression in the livers of the E3L mice and the E−/− mice have been compared with the wild type C57BL/6 (B6) strain. To study if these effects would be exacerbated by different dietary interventions, microarray analysis was used to evaluate the effect of mild and severe high-fat feeding in the liver. Therefore, we fed the susceptible E3L mice a mild and severe high-fat diet and compared these mice with chow fed controls. We assume that genes that are commonly regulated during the different conditions are of particular interest for further study, because these genes may play instrumental roles under hyperlipidemic conditions.

These approaches were expected to identify a large number of differentially regulated genes. To be able to validate our hypothesis that these genes were related to the lipoprotein metabolism, we made use of recent developments in bioinformatics to annotate the output files of these microarray experiments. One of the most useful resources is the "Gene Ontology (GO) database", that is a database containing consistent descriptions of genes in terms of their associated biological processes, molecular functions and cellular compartments in a species-independent manner [5]. Interestingly, this database allows annotation of genes at different levels, depending on how much is known about a gene product. When applied to the complete content of the chip and to the output file of the chip, it should be possible to define functional classes that become particularly regulated, while other classes would not show such an over representation. Another useful resource that was used is the Kyoto Encyclopedia of Genes and Genomes (KEGG) database containing specific information on metabolic pathways [6].

The consequences of high-fat feeding on the gene expression profile in liver is likely to be complex, because numerous components have an effect on gene regulation, such as sugars and lipids, which are either present in the diet or derived during further metabolism. This study mainly focuses on the genes that are involved in the metabolism of lipids. Interestingly, the knowledge in this field has increased substantially due to the discovery of a large series of transcription factors that are directly regulated by lipids. These transcription factors include the sterol regulatory element binding proteins (SREBPs), acting as cholesterol sensors, and nuclear receptors, including the farnesoid X receptor (FXR), pregnane X receptor (PXR), constitutive androstane receptor (CAR), liver X receptor (LXR) and peroxisome proliferator activated receptors (PPARs), which are being activated by bile acids, oxysterols and fatty acids, respectively [7]. These known regulatory pathways allow further annotation of the genes detected in this study, and build towards a network of genes of which the expression is influenced by diet.

2. Methods

2.1. Animals

Transgenic E3L mice (line #2), expressing the human APOE3Leiden and human APOC1 genes and E−/− mice have been described previously [1,8]. Twenty-seven female E3L mice of the N21th generation (>99% on a B6 genetic background), 10 female E−/− (on a B6 genetic background) and 20 female B6 mice were used in experiments. Mice received standard diet (chow, Hopefarms, The Netherlands, 3883 kcal/kg), a mild HFC diet (diet W, Hopefarms, The Netherlands, 4350 kcal/kg) containing per 100 grams (g), cocoa butter 15 g, corn oil 1 g, sucrose 40.5 g, corn starch 10 g, and cholesterol 0.25 g and an 'atherogenic' high fat high cholesterol (HFC) diet (diet N, Hopefarms, 4356 kcal/kg), known to elicit fatty streak lesions in aortas of susceptible mice, containing per 100 grams, cocoa butter 15 g, corn oil 1 g, sucrose 40.5 g, corn starch 10 g, cholesterol 1 g and cholate 0.5 g [3]. Ten B6, 10 E−/− and 10 E3L mice on chow diet were sacrificed at 10 weeks of age and used for experiments. Seven E3L mice received diet W for 4 weeks and 10 B6 and 10 E3L mice received diet N for a period of 8 weeks. Mice were 10 weeks of age at the start of dietary treatment. Mice were housed under standard conditions in conventional cages with free access to food and water.

2.2. Measurement of serum lipids

After the dietary treatment period from each individual mouse, 200 μl blood was collected in a microvette tube

(Sarstedt), after a 4-h fasting period. Total serum cholesterol (TC) (kit no. 236691, Boehringer Mannheim) and triglyceride without free glycerol (TTG) (kit no. 337-B, Sigma) were measured enzymatically. The day after bleeding, mice were sacrificed by cervical dislocation and the liver was removed and immediately deep frozen in liquid nitrogen and stored at −80 °C. A small piece of the liver was fixed in 4% neutral-buffered formalin, processed, and embedded in paraffin. Three-micrometer sections were stained with hematoxylin-phloxine-saffron (HPS) and examined microscopically.

2.3. Microarray probing and data analysis

High quality total RNA from the caudate liver lobe was isolated using RNA STAT-60 (Tel-Test Inc., Friendswood, TX) according to the manufacturer's protocol. Subsequently, total RNA was pooled from individual mice for each group and poly(A)+ RNA was isolated from total RNA using the poly (A+) Tract mRNA isolation systems (Promega, Madison, WI). In this way we tried to reduce the variation due to the individual mice by averaging over 10 individuals. Poly(A)+ RNA was labeled with Cy5 and Cy3 fluorescent dyes for microarray hybridization as described [9]. Experiments were performed in duplicate using technical replication: the same RNAs were labeled, divided into two different pools and hybridized onto two different GEMs 2.03 microarrays containing 10176 spots of which 192 controls (Incyte Genomics, Palo Alto, CA). cDNA arrays were scanned for both fluorescent signals (Cy5, Cy3) and the data were analyzed using the GEMTools software version 2.5.1 (Incyte Genomics, Palo Alto, CA). For normalization, GEMTools uses a signal correction algorithm to correct for different rates of incorporation of the Cy3 and Cy5 dyes [9]. In an optimal hybridization both dyes are incorporated at similar rates. In this case, a scatterplot of the logCy3 signal versus the logCy5 signal should show a signal distribution along a line with a slope of 1. The signal correction algorithm tests whether the slope of the regression line for the logCy3 signal versus the logCy5 signal is 1. If not, GEMTools applies a regression model to rotate the regression line to a slope of 1. To determine if the signal was sufficiently high above background and was not the result of non-uniform noise on spotted DNA sites, a spot was included in the analysis if it showed a signal to background ratio of minimal 2.5, a signal intensity above 250 arbitrary units for one or both dyes, and a spot size of at least 40% of the spotted area.

2.4. Statistical analysis

Experiments were performed in duplicate and gene expression profiles (log ratios) were measured for each experiment (array). To determine significant differential gene expression the *z*-test was used on the natural log transformed expression ratios of these duplicate arrays, using all valid measurements according to the above criteria. The *z*-test is a statistical test similar to the *t*-test. In contrast with the *t*-test, for the *z*-test it is possible to achieve a sufficient sensitivity with only two replicates using a more accurate error estimation (fitted S.D.) by calculating the error from all the genes on the array. In this manner, the variance is based on a large number of observations in an experiment, instead of only two. The observed expression S.D.s based on the replicate signals are plotted against the average log signal intensities per gene. On the observed S.D.s a line is fitted to calculate a gliding mean over the errors according to the signal intensities (fitted S.D.s), because error can be large relative to low signal intensities [10,11]. A *z*-statistic with the fitted S.D. is used instead of the t-statistic using the observed S.D.(s) based on only two replicates. Based on the *z*-test, the overall minimal significant difference in expression ($p < 1 \times 10^{-3}$) was determined at fold change (FC) = 1.4. Significant differentially expressed genes were filtered using Spotfire® DecisionSite™ (Spotfire Inc., Massachusetts, USA) [12].

2.5. Functional classification of genes present on the cDNA array

Functional gene information from the Gene Ontology database (GO: http://www.geneontology.org/) (March 2003) [5] was obtained using GeneHopper [13]. To gain information on metabolic pathways, LocusLink IDs were run through the Kyoto Encyclopedia of Genes and Genomes (KEGG: www.genome.ad.jp/kegg/) (March 2003) [6]. To determine which functional classes are particularly affected, the chi-square test was performed, where the expected number of differentially expressed genes was calculated as $E = C/T \times D$, where E, expected number of genes; C, total number of genes per category on chip; T, total number of genes on chip and D, total number of differentially expressed genes.

2.6. Confirmation of differences in expression levels

Primers for genes (Sigma/Genosis) corresponding to Incyte clones were designed according to the manufacturer's protocol using Primer Express® software (Applied Biosystems). One microgram of total RNA was converted to first strand cDNA using random hexamer primers and SuperScript Reverse Transcriptase II (Revert Aid First Strand cDNA Synthesis Kit MBI/Fermentes). cDNA was diluted (2 ng/μl) and was used for the real time (RT) quantitative PCR reactions using an ABI PRISM® 7700 (Applied Biosystems). For a reaction, 5 μl cDNA (2 ng/ml), 12.5 μl 2X PCR MasterMix, 0.5 μl forward primer, 0.5 μl reverse primer, 0.75 μl SYBR green and 5.75 μl milliQ was used (Eurogentec). PCR conditions were: 2 min at 50 °C, 10 min at 95 °C, 40 cycles for 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C, 1 min at 60 °C, ramp to 95 °C (20 min), followed by 15 s at 95 °C. Data were analyzed using Sequence Detection Software version 1.7 (Applied Biosystems).

Table 1
Lipid parameters of B6, E3L and E−/− mice on standard diet and different HFC diets

Strain	Chow diet			N diet			W diet		
	TC (mmol/l)	TTG (mmol/l)	Weights (g)	TC (mmol/l)	TTG (mmol/l)	Weights (g)	TC (mmol/l)	TTG (mmol/l)	Weights (g)
B6	1.3 ± 0.3	0.3 ± 0.1	18.0 ± 1.1	$4.9 \pm 0.5^{*}$	$0.09 \pm 0.02^{*}$	19.2 ± 1.0	NA	NA	NA
E3L	$2.4 \pm 0.5^{*}$	$1.3 \pm 0.6^{*}$	19.2 ± 1.4	$54.3 \pm 9.5^{*}$	1.3 ± 0.5	21.1 ± 1.4	$14.8 \pm 3.1^{*}$	1.0 ± 0.5	19.6 ± 1.0
E−/−	$10.1 \pm 1.8^{*}$	0.5 ± 0.2	19.9 ± 1.5	NA	NA	NA	NA	NA	NA

Lipid parameters of B6, E3L and E−/− mice on different diets. TC, total cholesterol; TTG, total triglyceride. Chow, standard diet; W, moderate HFC diet; N, HFC diet. Total cholesterol and triglyceride values are the mean serum levels ±S.D. of 7–22 mice per group. Statistical analysis using the nonparametric Mann–Whitney test revealed a significant difference ($^{*}p < 0.0001$) in TC and TTG between B6 and E3L mice on chow, and in TC between B6 and E−/− mice on chow. Furthermore, a significant difference was detected ($^{*}p < 0.0001$) in TC between E3L chow and E3L W, N and in TC and TTG between B6 chow and B6 N. NA, not applicable.

3. Results

3.1. Lipid parameters and histology of the liver

E−/− mice were maintained on a standard (chow) diet and showed a significant increase in total serum cholesterol (TC) when compared with B6 (Table 1), which is in line with previous data 1. E3L mice were also maintained on a chow diet and showed significant increases in both TC and total triglyceride levels (TTG) when compared with B6 mice (Table 1). In addition, E3L mice were fed a moderate high-fat diet (W) and the severe high-fat diet (N), which resulted in a pronounced increase in TC (Table 1) when compared with E3L mice on chow, as expected from previous data [2]. In parallel, B6 mice were fed the diet N showing a significant difference in TC and TTG when compared with B6 mice on chow. Histological examination of the livers revealed fat accumulation in the livers of transgenic mice, as reported earlier, but other pathology was absent [14].

3.2. Gene expression profiles of the liver

RNA was isolated from all mice of each experimental condition and pooled to minimize the biological variation between the individual mice. For microarray experiments, Incyte cDNA arrays (GEM2.03) were hybridized simultaneously with a Cy5 labeled cDNA sample from the experimental group, and a Cy3 labeled cDNA control sample (Table 2). The use of cDNA-based arrays implied an experimental design in the form of pair-wise comparisons. Experiments were performed in duplicate, and gene expression profiles (log ratios) from duplicate experiments showed a high correlation (Pearson correlation, $r = 0.97$–0.98). To have a better understanding of the mechanisms related to hyperlipidemia and atherosclerosis, the effect of the transgenic alteration on gene expression was examined in chow fed mice. At a cutoff level of 1.4-fold expression (p-value $<1 \times 10^{-3}$, see Section 2), 150 and 187 genes were found to be regulated in E3L or E−/− mice, respectively, when compared with non-transgenic B6 mice. Upon increasing the dietary fat load using diet N 360 genes showed different expression levels in the comparison between B6 and E3L mice. To study the dietary response, E3L mice were fed diet W and compared with chow fed E3L mice. We found 200 genes to be affected by diet W. A more pronounced effect, however, was found using diet N. Some 788 genes in B6 mice and 1010 genes in E3L mice were differentially regulated. The complete dataset can be found in the supplementary materials (Table I, on the web). The majority of the genes showed a fold change of 1.4–2.0, while 184 genes displayed differences between 2- and 20-fold change.

3.3. Specific pathways involved in the dietary response

All genes on the chip were classified using the GO database, which is a hierarchical database that classifies all genes regarding their biological process, molecular function and cellular compartment. Based on GO, all 9552 genes present on the chip were categorized into 39 functional classes. Sixty percent of the genes could be categorized into these functional classes. However, the biological role of 40% of these genes remained unknown. Genes that were involved in more than one biological process were catego-

Table 2
Outline of the microarray experiments

Experimental group		Control group		Variable	Number of genes
Strain	Diet	Strain	Diet		
E3L	Chow	B6	Chow	APOE3Leiden transgene	150
E−/−	Chow	B6	Chow	ApoE knockout	187
E3L	N	B6	N	APOE3Leiden transgene	360
E3L	W	E3L	Chow	Diet	200
B6	N	B6	Chow	Diet	788
E3L	N	E3L	Chow	Diet	1010

Experimental conditions used for microarray experiments to study the effect of the APOE3Leiden transgene, ApoE deficiency and dietary gene regulation. The number of differentially regulated genes per condition are shown. Experimental group: Cy5, control group: Cy3.

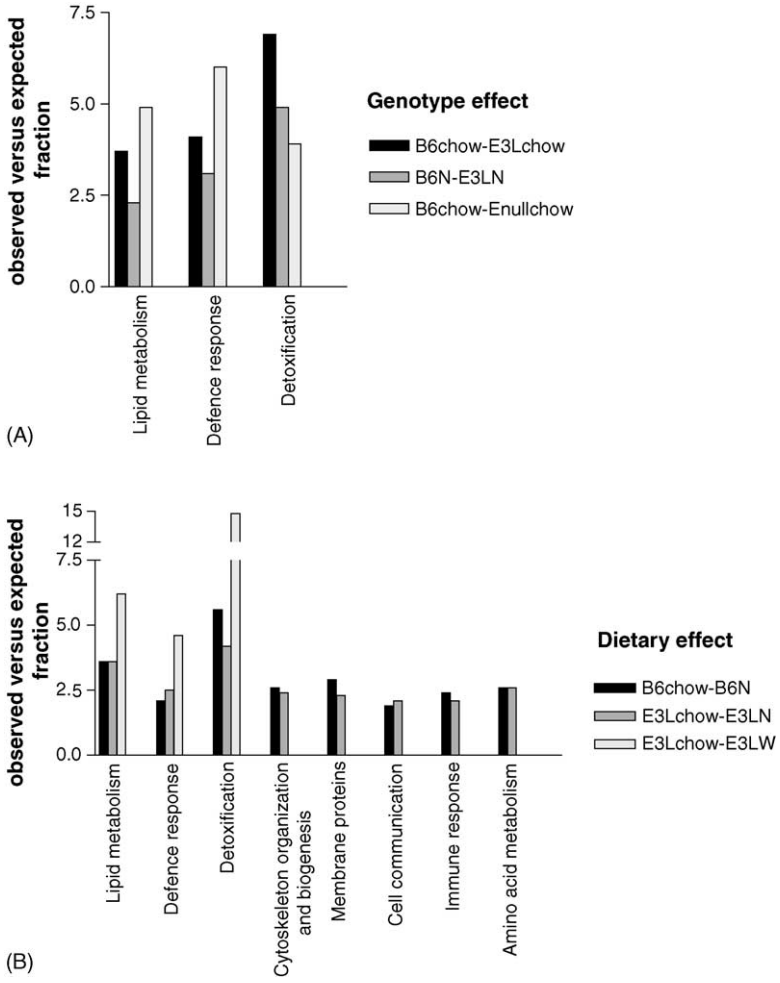

Fig. 1. Functional classes with a significantly increased number of differentially expressed genes ($p < 0.01$, chi-square test): (A) affected by genotype and (B) affected by diet.

rized in the most relevant category for our study. Notably, glutathione *S*-transferases and monooxygenases were categorized as detoxification genes, membrane proteins were categorized on the basis of the cellular compartment. Subsequently, differentially expressed genes categorized according to these 39 functional classes were identified (Table I, on the web). This allowed comparison of the functional classes over the different conditions. The major classes that were regulated over all the different conditions included lipid metabolism, transport, enzymes and inflammation, the latter represented by the immune/defense response, and detoxification. Lipid metabolism and enzymes were highly represented in the conditions studying the genotype. Strikingly, the expression profile of the response of B6 and E3L mice to diet N was highly similar. The effect of diet W in the E3L mice was less pronounced, but showed several similar functional classes as regulated by diet N, such as lipid metabolism, enzymes and detoxification processes.

To determine which processes were particularly affected upon perturbation of the lipid metabolism by genetic alterations or diet, the number of genes present on the chip that belong to a particular category was determined using the categories of the GO database (Table I, on the web). All classes with a significant increased number of differentially expressed genes are shown (Fig. 1). Lipid metabolism, the defense response and detoxification pathways were significantly affected by expression of APOE3Leiden and by ApoE deficiency. These pathways were also strongly regulated by diet W and N. Moreover, in view of the large number of genes regulated by diet N several other functional classes were identified in the experiment comparing the B6 and E3L mice. These classes were the immune response, cytoskeleton organization and biogenesis, membrane proteins, cell communication and amino acid metabolism.

3.4. Sub-classification of genes involved in lipid and detoxification metabolism revealed many nuclear receptor target genes

The two most relevant categories as indicated above, i.e. lipid and detoxification metabolism, have been further explored using the KEGG database by categorizing the genes

Table 3
Metabolic pathways involved in lipid and detoxification processes based on the KEGG database

Lipid metabolism							
Pathways	**Gene**	**B6 chow/B6N**	**E3L chow/E3LN**	**E3L chow/E3LW**	**B6 chow/E3Lchow**	**B6 N/E3LN**	**B6 chow/ Enull chow**
Fatty acid metabolism	**cytochrome P450, 1a2, aromatic compound inducible**	-2.15	-4.10	-1.70	1.15	-1.65	-1.60
	cytochrome P450, 2a4	-1.65	-4.25	-1.65	1.35	-2.00	-1.10
	cytochrome P450, 2b10, phenobarbitol inducible, type b	-3.60	-3.60	-1.40	-1.35	-1.20	-5.90
	cytochrome P450, steroid inducible 3a11	-2.60	-1.95	-2.30	-1.45	-1.20	-1.45
	fatty acid binding protein 1, liver	-2.20	-3.50	-1.55	-1.05	-1.60	-1.10
Fatty acid biosynthesis	**stearoyl-Coenzyme A desaturase 1**	3.15	2.65	1.55	-1.15	-1.10	-1.50
Bile acid biosynthesis	**cytochrome P450, 7a1 (cholesterol 7alpha-hydroxylase)**	-2.40	-2.00	2.40	-1.45	1.05	1.00
	cytochrome P450, 7b1	1.15	1.50	1.65	1.15	1.45	1.40
C21-Steroid hormone metabolism	cytochrome P450, 17	-3.45	-2.05	1.05	-2.00	-1.10	-1.80
	hydroxysteroid 11-beta dehydrogenase 1	-2.05	-2.10	-1.55	-1.50	-1.30	-1.25
Androgen and estrogen metabolism	cytochrome P450, 7b1	1.15	1.50	1.65	1.15	1.45	1.40
	hydroxysteroid 11-beta dehydrogenase 1	-2.05	-2.10	-1.55	-1.50	-1.30	-1.25
Apolipoproteins	**apolipoprotein AII**	-1.85	-2.65	-1.40	-1.15	-1.65	-1.40
	apolipoprotein E	1.85	1.40	1.25	1.40	-1.00	-20.85
Lipid transporters	solute carrier family 27 (fatty acid transporter), member 5	-2.20	-2.65	-1.10	-1.35	-1.55	-1.55
ABC transporters	**ATP-binding cassette, sub-family D (ALD), member 3**	-2.10	-2.15	-1.40	-1.50	-1.55	-1.55
	ATP-binding cassette, sub-family G (WHITE), member 5	1.75	1.60	1.55	-1.20	-1.50	-1.55
Detoxification processes							
Styrene degradation	cytochrome P450, 7b1	1.15	1.50	1.65	1.15	1.45	1.40
1,4-Dichlorobenzene degradation	cytochrome P450, 7b1	1.15	1.50	1.65	1.15	1.45	1.40
gamma-Hexachlorocyclohexane degradation	**cytochrome P450, 1a2, aromatic compound inducible**	-2.15	-4.10	-1.70	1.15	-1.65	-1.60
	cytochrome P450, 2a4	-1.65	-4.25	-1.65	1.35	-2.00	-1.10
	cytochrome P450, 2b10, phenobarbitol inducible, type b	-3.60	-3.60	-1.40	-1.35	-1.20	-5.90
	cytochrome P450, steroid inducible 3a11	-2.60	-1.95	-2.30	-1.45	-1.20	-1.45
	cytochrome P450, 4a10	-3.90	-4.85	-1.75	-1.20	-1.45	1.05
	cytochrome P450, 4a14	-5.20	-6.15	-2.35	-1.20	-1.50	1.10
	cytochrome P450, 7b1	1.15	1.50	1.65	1.15	1.45	1.40
Glutathione metabolism	**glutathione S-transferase, alpha 3**	-2.00	-3.00	-1.60	1.05	-1.60	-1.10
	glutathione S-transferase, pi 2	2.35	-1.20	-2.50	2.15	-1.30	1.50

Differentially expressed genes involved in lipid metabolism and detoxification pathways are indicated. Nuclear receptor target genes are indicated in bold. The average fold changes from the duplicate experiments are indicated. Upregulated genes are indicated in dark gray, downregulated genes are indicated in light gray.

into the following metabolic processes; (1) bile acid, (2) fatty acid, (3) sterol biosynthesis, (4) steroid metabolism and (5) detoxification pathways. Table II (on the web) shows 94 genes falling into 17 different functional classes. The greatest effects on gene expression were modulated by diet N, leading to the suppression of 70% of the genes. Twenty genes were found that were regulated in at least four conditions (Table 3). These genes included key enzymes of the bile acid biosynthesis and steroid pathway respectively; cholesterol 7-α hydroxylase (*Cyp7a1*), oxysterol 7-α hydroxylases (*Cyp7b1*), cytochrome p450 (*Cyp)*17 and hydroxysteroid (11-beta) dehydrogenase 1 (*Hsd11b*). Also several genes involved in fatty acid metabolism were regulated such as *Cyp1a2*, *Cyp2a4*, *Cyp2b10*, *Cyp3a11*, fatty acid binding protein 1 (*Fabp-1*), solute carrier family 27 gene (*Slc27*) and stearoyl-Coenzyme A desaturase 1 (*Scd1*). Two lipid transporters the ATP-binding cassette, subfamily D 3 (*Abcd3*) and *Abcg5* were also strongly regulated. *Cyp1a2*, *Cyp2a4*, *Cyp2b10* and *Cyp3a11* also play major roles in detoxification processes. Other detoxification genes that were regulated were *Cyp4a10*, *Cyp4a14* and several glutathione *S*-transferases (*GSTs*) namely *Gsta3* and *Gstp2*. Many of these genes are known to play an instrumental role during lipid metabolism, although for several genes this role remains less clear. To obtain detailed indications of the molecular mechanism underlying this gene regulation, genes have been indicated (based on literature data) that are under the regulation of the nuclear receptors FXR, PXR, CAR, LXR, hepatocyte nuclear factor 4α (HNF-4α), PPARs and SREBPs. Thirty-nine nuclear receptor target genes were identified involved in the different metabolic pathways associated with lipid metabolism and detoxification processes (Table II). An overview of these genes and the way they are regulated in this study and other studies is provided in Table 4. Intriguingly, 15 of these genes are part of the total group of

Table 4
Nuclear receptor target genes

Gene	W	N	FXR	PXR/CAR	LXR	PPARα	PPARδ	HNF-4α	SREBP 1a, 1c,2	Ref.	Tissue
Abca1	–	↑			↑					[25] *	mΦ, Intestine
Abcb1 (Mdr1)	↓	↑		↑						[26] ‡	Liver
Abcb4 (Mdr2)	↓	–				↑				[7] *	Liver
Abcc2 (Mrp2)	–	↓	↑	↑				↑		[27] *, [28] ‡	Liver
Abcd3	↓	↓				↑				[7] ‡	Liver
Abcg1	**nd**	↑			↑					[25] ‡	mΦ
Abcg5	↑	↑			↑					[25] *	Liver, intestine
Acox1	–	↓				↑	↑			[7] *, [29] ‡	Liver adipose tissue
Aldh3a2	–	↓		↑						[30] ‡	Liver
ApoA-I	–	↑	↓			↑		↑		[21] *, [7] *, [28] *	Liver
ApoA-II	↓	↓				↑		↑		[7] *, [28] *	Liver
ApoA-V	–	↓				↑				[31] *	Liver
ApoC-II	–	↑	↑		↑			↑		[20] *, [28] *	Liver
ApoC-IV	–	↓			↑					[25] *	Liver, mΦ
ApoE	–	↑	↑		↑			↑		[20] *, [25] *, [28] *	Liver, mΦ, adipocytes
Cyp1a2	↓	↓		↑						[32] ‡	Liver
Cyp2b9/10	↓	↓		↑						[30] *	Liver
Cyp2a4/2a5	↓	↓		↑						[30] ‡	Liver
Cyp3a11	↓	↓	↓	↑						[30] *, [33] †	Liver
Cyp4a10	↓	↓		↓						[30] ‡	Liver
Cyp4a14	↓	↓↓		↓	↑					[30] ‡, [25] ‡	Liver
Cyp7a1	↑	↓	↓	↓	↑			↑		[34] * [19] *, [25] * [28] *	Liver
Fas	–	↓			↑				↓	[25] *, [35] *	Liver
Ftdf1	↓	↓							↑	[36] ‡	Liver
Gst-α3	↓	↓		↑						[30] ‡	Liver
Gst-α4	↓	–		↑						[30] ‡	Liver
Gst mu1	↓	↑		↑						[30] ‡	Liver
Gst mu2	↓	↑		↑						[30] ‡	Liver
Gst pi2	↓	↑		↓						[30] ‡	Liver
Hmgcr	–	↓						↑	↑	[36] *, [28] ‡	Liver
L-fabp	↓	↓				↑		↑		[7] *, [28] ‡	Liver
Lpl	–	↑			↑	↑				[25] ‡, [7] *	Periphery
Mup2	–	↓↓		↓						[30] ‡	Liver
Pltp	–	↑	↑		↑					[37] *, [20] *	Liver
Scd-1	↑	↑			↑					[25] *	Liver
Squalene epoxidase	↓	–							↑	[38] *	Liver
Vlcad	–	↓				↑	↑			[39] *, [29] ‡	Liver

Dietary regulation (W, N) of nuclear receptor target genes is indicated and the manner of regulation found in this study and as previously reported for the several nuclear receptors. (*) Indicates a direct nuclear receptor target gene based on promoter studies, (†) nuclear receptor gene regulation based on dietary studies in mice, ‡ nuclear receptor gene regulation based on expression profiling studies in vitro and in vivo. (–) No change, (↑) upregulated, (↓) downregulated, (↑↑) or (↓↓) greater than five times FC. Genes confirmed by quantitative RT-PCR are in bold.

20 genes (i.e. 75%) that were expressed in at least four conditions (Table 3).

3.5. *Confirmation of differentially expressed genes by real time quantitative PCR*

Representative genes for the different lipid pathways and detoxification processes as well as some other genes were selected for validation using real time (RT) quantitative PCR. Differential expression of *Cyp7a1*, *Slc27*, *Scd1*, *Cyp2a4*, farnesyl diphosphate farnesyl transferase 1 (*Ftdf*), *Cyp39a1*, *Cyp17*, *Hsd11b1*, *Abca1*, the apolipoprotein *ApoH* and insulin growth factor binding protein 1 (*Igfbp1*) could be confirmed for all conditions by RT quantitative PCR using cyclophilin as reference (Table 4, Fig. I, on the web). Fold changes even as low as 1.2, could be confirmed using RT-PCR (Fig. I, on the web). Expression of the inflammatory gene, tumor necrosis factor alpha (*TNFα*), was also determined by RT-PCR and showed a strong induction (∼15-fold) by diet N in B6 and E3L mice but not in the other conditions (Fig. I, on the web).

4. Discussion

This study was aimed at delineating the events related to hyperlipidemia and atherosclerosis at the level of gene expression using microarray analysis. Therefore, two related approaches were used; the effect of genotype and environment (diet) on the gene expression profile in the mouse liver was studied using E−/− and E3L mice. The different modi-

fications of APOE in both the E3L and E−/− mice resulted in similar pathways that were affected. Interestingly, the related pathways detected in these comparisons indicated a similar compensatory mechanism responding to the changes in lipoprotein metabolism in both these genetically altered mice. In line with this similar overall response, several cholesterol responsive genes were regulated in both models. Possibly, these genes may represent the convergences in the pathways affected in the two mouse models. This study showed that the genomic analysis of transgenic and knockout models was an excellent approach to define the molecular pathways related to hyperlipidemia.

Similar to that observed in the experiments addressing the genotype effects, the lipid metabolism, detoxification and defense response genes were significantly over represented among the regulated genes when using the mild high fat diet W, which lacks cholate. A more pronounced response was observed in case the 'atherogenic' diet N, containing 0.5% cholate, had been used. The E3L and the B6 mice showed a highly similar response resulting in the differential expression of 800–1000 genes. Remarkably, although the E3L mice on diet N have a ∼9-fold higher plasma cholesterol level than the B6 mice on diet N, this much greater susceptibility to diet induced hyperlipidemia did not lead to the detection of a much higher number of response genes. Therefore, it is likely that the same pathways are activating in the same models. It is of interest to know whether this finding holds true for other models as well. The expression profiles were characterized by the over representation of the former three classes and the immune response, cytoskeleton organization and biogenesis, membrane proteins, cell communication and amino acid metabolism. These results showed that high-fat feeding had a much stronger effect than the effects observed in the previous experiments examining the effect of the genetic modifications of APOE. Moreover, a recent study using microarrays to examine the effects of a cholesterol (1.25%) and cholate (0.5%) containing diet in livers of B6 mice already indicated widespread changes in hepatic gene expression, affecting the expression of over 1200 genes. Many genes were associated with inflammatory-related processes such as immune/defense response genes and extracellular matrix proteins. Furthermore, when studying diets with only cholesterol or cholate, it was found that inflammatory gene activation was dependent on the presence of cholesterol in the diet, whereas the extracellular matrix proteins were specifically induced by cholate [16]. These results corresponded with our results; diet N had a significant effect on the cytoskeleton organization and biogenesis pathway that consisted of many extracellular matrix proteins.

Several genes were not only regulated during the dietary challenges but also during the other conditions examining the effect of the genetic modifications of APOE. These genes may be particularly of interest for further study, because they may play instrumental roles under hyperlipidemic conditions. Several of these genes were already shown to be the cause of lipid disorders. For instance, mutations in *Cyp7b1* and *Abcg5* resulted in, respectively, neonatal cholestasis [17] and sitosterolemia [18]. Intriguingly, more strongly regulated genes such as *Cyp4a10* and *Cyp4a14* were found that are not previously associated with lipid disorders.

To identify common regulatory pathways related to lipid and detoxification pathways, data regarding these pathways were integrated with published data concerning gene regulation through nuclear receptors. Nuclear receptors become activated to drive gene expression upon binding to (metabolites of) bile acids, fatty acids and cholesterol. We found many nuclear receptor target genes of which several were commonly regulated during the different conditions. HFC diets affected all nuclear receptors: FXR, PXR, CAR, LXR, PPAR-α/δ, HNF-4α and SREBPs. Except for *ApoA-I* and *Abcc2*, all FXR responsive genes were regulated by diet N in a similar manner of up/down regulation as was previously described [19–21]. Intriguingly, the regulation of *ApoA-I* by fatty acids through PPARα was dominant over the regulation by cholate through FXR. For several genes a different regulatory mechanism was seen as was described in literature. This may be due to differences in dietary composition between experiments. Moreover, the complex regulation in which lipids may not just affect one receptor may result in different effects on gene expression [7]. It is likely that the observed effects on gene regulation are partly due to the cholate present in diet N. Except for the direct regulation by bile acids of the FXR [19] and the detoxification receptors PXR/CAR [22], no other nuclear receptors are known to be directly regulated by bile acids in liver. Additionally, previous studies already indicated the effects of lipids on immune and inflammatory responses involving nuclear receptors such as the PPARs and the LXRs [23,24]. However, findings described in this study also implicated roles for other nuclear receptors in this process.

In conclusion, the common regulation of many nuclear receptor target genes underlying lipid and detoxification processes as found in this study, suggest a defense mechanism involving many nuclear receptors to protect against the accumulation of toxic endogenous lipids and bile acids. Intriguingly, most of the strongest regulated genes were also nuclear receptor target genes, which may implicate that they are key genes in regulatory pathways related to lipid metabolism. This makes these genes very good candidates for further studies. These observations further strengthen the close link between lipid metabolism and inflammatory-related processes.

Acknowledgments

This project was supported by a grant from the Dutch Organization for Scientific Research (project NWO 98010001). M.H. Hofker is Established Investigator of the Dutch Heart Association (project NHS D95022). C.J.A. Moen is research fellow of the Dutch Organization for Scientific Research (project NWO 903-39-174). We would like to thank Chris Evelo and Payman Hanifi Moghaddam for helping with data analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2005.01.049.

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