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ORIGINAL CONTRIBUTION

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Effects of dietary fat and oxidized cholesterol on gene expression in rat liver as assessed by cDNA expression array analysis

Summary *Background* Specific oxysterols acting as ligands for nuclear transcription factors were shown to affect expression of genes involved in lipid metabolism. However, the various biological effects of oxysterols such as cytotoxicity, atherogenicity or mutagenicity suggest that other genes may be also affected by oxysterols than lipid metabolism. *Aim of the study* The present study was conducted to investigate the effects of dietary oxidized cholesterol containing significant amounts of oxysterols and its interactions with different dietary fats on gene expression profiles as assessed by DNA array technology in rats. *Methods* 54 male Sprague-Dawley rats were assigned to six groups and were fed six semisynthetic diets, which varied in the type of dietary fat (coconut oil vs. salmon oil) and dietary cholesterol (none cholesterol vs. 5 g unoxidized cholesterol/kg vs. 5 g oxidized cholesterol/kg). *Results* Changes in gene expression as observed in response to dietary oxidized cholesterol were strongly dependent on the type of fat. In the rats fed coconut oil, the expression of 7 genes (5 up- and 2 down-regulated) was altered by dietary oxidized cholesterol, while in the rats fed salmon oil, the expression of 50 genes (16 up- and 34 down-regulated) was altered. 29 genes (22 up- and 7 down-regulated) were identified as possible targets for an altered gene expression by dietary salmon oil as compared to dietary coconut oil. *Conclusion* The present study showed that dietary oxidized cholesterol transcriptionally affects many genes involved in xenobiotic metabolism and stress response – an effect that was amplified by the administration of fish oil as dietary fat.

Key words oxysterols – salmon oil – hepatic gene expression – cDNA expression array – rats

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Introduction

It is well known that various lipid components of human and animal diets such as fatty acids, cholesterol or oxidized derivatives of cholesterol (oxysterols) influence serum lipid concentrations, lipid profiles and lipid metabolism by affecting transcription of genes involved in the absorption, extracellular transport, cellular uptake, intracellular trafficking, metabolism and elimination of lipids in the organism [1–4].

For example, dietary fats rich in polyunsaturated fatty acids (PUFA), such as fish oil, lower plasma lipid concentrations by stimulating fatty acid oxidation mediated by enhanced transcription of genes involved in mitochondrial, peroxisomal and microsomal β-oxidation, which are controlled by peroxisome proliferator activated receptors (PPAR) α and γ, and by simultaneously suppressing lipogenesis due to decreased expression of genes controlled by sterol regulatory element binding protein (SREBP)–1c [1, 5, 6]. It is also known that oxidized derivatives of cholesterol (oxysterols) such as 27-hydroxycholesterol, 24S-hydroxycholesterol, 22R-hydroxycholesterol, and 24S,25-epoxycholesterol, affect transcription of genes involved in maintaining cholesterol homeostasis [3, 7]. Transcriptional regulation of cholesterol homeostasis is mediated by SREBPs controlling genes involved in the biosynthesis and uptake of

cholesterol (LDL receptor, β-HMG-CoA reductase) and fatty acids (lipogenic enzymes) [8, 9]. In addition, cholesterol metabolism is also regulated by the oxysterol-binding nuclear hormone receptors liver X receptor α and β (LXRα and LXRβ) stimulating transcription of genes involved in reverse cholesterol transport, e. g. cholesterol ester transfer protein, apolipoprotein E, and lipoprotein lipase [10]. This shows that administration of dietary lipids to the mammalian organism may affect a wide spectrum of genes at a transcriptional level.

Until now most studies dealing with the effects of oxysterols on differential gene expression are considering genes involved in lipid metabolism. However, the various biological effects of oxysterols such as cytotoxicity, atherogenicity or mutagenicity suggest that other genes may be also affected by oxysterols than lipid metabolism. In addition, *in vivo* studies investigating the effects of dietary oxysterols on gene transcription are lacking so far. Employing DNA arrays, which are powerful screening tools to analyze differential gene expression, in nutrition research offers the chance to gain insight into the gene activation or suppression of more than 1,000 different genes in response to various feeding regimes [11–14]. Therefore, the present study was conducted to investigate the effects of dietary oxidized cholesterol on gene expression profiles as assessed by DNA array technology in rats. Considering the lack of information about the interactions of dietary fat and dietary oxidized cholesterol with regard to gene transcription, we planned to study possible interactions between oxidized cholesterol and the type of fat. Therefore, we used diets with different fats, salmon oil and coconut oil, mainly differing in their concentrations of n-3 PUFAs. To investigate the effect of the dietary fat on hepatic gene expression in isolation, we further analyzed expression profiles of rats fed cholesterol-free diets, containing either fish oil or coconut oil as dietary fat. The liver was used as the object of investigation for gene expression analysis because dietary lipids such as oxysterols are at first transported to the liver, where they exert various biological effects, after being ingested and absorbed from the gut [15, 16]. Therefore, we determined the oxysterol 7β-hydroxycholesterol in liver and plasma in view of controlling the different dietary intervention regimens used. It was repeatedly demonstrated that dietary fats rich in PUFA such as fish oil but also oxysterols influence the antioxidant status [17, 18]. Therefore, we further intended to determine concentrations of antioxidants (tocopherol, vitamin C, glutathione) in liver and plasma as parameters of the antioxidant status.

Materials and methods

Animals

A total of 54 male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) with an initial body weight of 72 g (± 7 g, SD) were assigned to six groups of nine rats each and kept as described previously in detail [19]. All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt.

Diets

The animals were fed six semisynthetic diets, which varied in the type of dietary fat (coconut oil vs. salmon oil) and dietary cholesterol (no cholesterol vs. 5 g unoxidized cholesterol/kg vs. 5 g oxidized cholesterol/kg) (Table 1). Oxidized cholesterol was prepared by heating cholesterol (Sigma-Aldrich, Steinheim, Germany) placed as a thin film on a glass Petri dish at 115 °C for 48 hours in an electric oven. Oxysterols (Sigma-Aldrich, Steinheim, Germany) in the oxidized cholesterol preparation were determined using a quantitative GC-MS method with selective ion monitoring [20]. The composition of the oxidized cholesterol preparation was as previously reported [19]. The peroxide values of the dietary fats, which were extracted from the diets with a mixture of hexane and isopropanol (3:2, as described in [21]) and measured according to official methods [22], were 3.9 and < 0.1 mEq O_2 per kg salmon oil and coconut oil, respectively.

To equalize the vitamin E concentrations of the diets irrespective of the dietary fats used, the native tocopherol concentrations of the two fats were analyzed using an established HPLC method [23]. Based on these native concentrations, diets were supplemented individually with all-*rac*-α-tocopheryl acetate (Merck Eurolab, Darmstadt, Germany), allowing for a biopotency of 67 % compared to α-tocopherol. After supplementation the vitamin E concentrations of both types of diets were 40 mg α-tocopherol equivalents/kg. The fatty acid composition of coconut oil (Palmin, Hamburg, Germany) and salmon oil (Caelo, Hilden, Germany) was as reported previously [19].

The diets were prepared weekly by solubilizing the all-*rac*-α-tocopheryl acetate and unoxidized cholesterol or the oxidized cholesterol preparation in the fat and mixing with the dry components and water. The diets were then freeze dried and stored at –20 °C to prevent autoxidation of lipids, e. g. polyunsaturated fatty acids and cholesterol. The water content after freeze drying was below 5 g per 100 g of diet.

To standardize the feed intake, the diets were fed daily

Table 1 Composition of the experimental diets (g/kg diet)

Diet	1	2	3	4	5	6
Dietary fat	Coconut oil	Salmon oil	Coconut oil	Salmon oil	Coconut oil	Salmon oil
Dietary cholesterol	none	none	unoxidized	unoxidized	oxidized	oxidized
Corn starch	398	398	398	398	398	398
Casein	200	200	200	200	200	200
Saccharose	200	200	200	200	200	200
Coconut oil	100	–	100	–	100	–
Salmon oil	–	100	–	100	–	100
Cellulose	40	40	35	35	35	35
Mineral mixture[1]	40	40	40	40	40	40
Vitamin mixture[2]	20	20	20	20	20	20
Cholesterol	–	–	5	5	–	–
Oxidized cholesterol	–	–	–	–	5	5
DL-methionine	2	2	2	2	2	2

[1] Minerals supplemented (per kg diet): calcium carbonate, 7.56 g; dicalcium phosphate, 8.67 g; potassium chloride, 6.87 g; sodium bicarbonate, 3.77 g; magnesium oxide, 1.01 g; ferrous sulfate hydrate, 116 mg; zinc oxide, 38 mg; manganese oxide, 16 mg; copper sulfate pentahydrate, 24 mg; calcium iodate, 3.2 mg; sodium selenite pentahydrate, 3.3 mg

[2] Vitamins supplemented (per kg diet): all-trans-retinol, 1.34 mg; cholecalciferol, 25 µg; menadion sodium bisulfite, 7.5 mg; thiamine hydrochloride, 5 mg; riboflavine, 6 mg; pyridoxine hydrochloride, 6 mg; biotin, 0.2 mg; calcium pantothenate, 15 mg; nicotinic acid, 30 mg; vitamin B_{12}, 25 µg; folic acid, 2 mg; choline chloride, 1 g

in restricted amounts at 0800 h. The amount of food administered in the present study was 15 % less than the amounts of diets with identical nutrient composition consumed ad libitum by growing rats in preliminary studies. The diet intake was recorded daily and increased from 7.0 g/d to 15.0 g/d during the experiment resulting in an average diet intake of 14.4 g per rat and day. In this feeding regimen, the food offered was completely consumed by all the rats. Thus all the rats consumed identical amounts of food. The experimental diets were fed for 35 days. Water was provided ad libitum from nipple drinkers.

Sample collection

After 35 days of feeding the experimental diets the rats were starved overnight to ensure that all rats had nearly the same interval of starvation until killing. Rats were anesthetized with diethyl ether and killed by decapitation. The liver was excised immediately and frozen with liquid nitrogen and stored at –80 °C pending analysis. Hepatic tocopherol concentrations were determined according to [23]. The fatty acid composition of liver phosphatidyl choline was determined by gas chromatography (GC) of fatty acid methyl esters (FAME) as described previously in detail [24]. 7ß-hydroxycholesterol in liver and plasma were analyzed using the same GC-MS method as used for determination of dietary oxysterols [20]. Determination of ascorbic acid in plasma was performed by high-performance liquid chromatography according to Ross [25]. The ratio of oxidized to reduced glutathione (GSSG/GSH ratio) was calculated from the corresponding concentrations. GSSG and GSH concentrations in the liver were determined as reported previously [24].

RNA isolation, cDNA probe synthesis and hybridization to Atlas cDNA array rat 1.2

Total RNA was isolated from rat liver using Trizol™ reagent (Invitrogen, Karlsruhe, Germany). Afterwards three RNA samples within the same dietary treatment group were pooled and subjected to DNase treatment according to the Atlas pure total RNA labeling system user manual (Clontech, Palo Alto, CA). The cDNA expression array analysis was performed as described in the Atlas pure total RNA labeling system user manual (Clontech). In brief, cDNA probes of three DNase-treated RNA pools (containing 40 µg total RNA) per dietary treatment group were prepared by simultaneous reverse transcription using a mix of primers complementary to the genes represented on the array and radiolabeling with [α^{33}P]-dATP (Perkin Elmer, Boston, MA). In order to purify the labelled cDNA from unincorporated ^{33}P-labelled nucleotides and small cDNA fragments the cDNA probes were purified using NucleoSpin extraction spin columns (Clontech). After hybridization for 18 h at 68 °C with continuous agitation using a hybridization oven the nylon array membrane was washed performing the following wash program to remove unbound and nonspecifically bound probes: wash 1 (2x SSC, 1 % SDS) for 30 min at 68 °C, wash 2 (0.1x SSC, 0.5 %

SDS) 30 min, 68 °C and a final wash (2x SSC) for 5 min at 25 °C. Images of specifically bound probes were obtained by phosphorimaging using an Imaging plate (Raytest, Straubenhardt, Germany) for 24 h to reveal the expression profiles. After scanning the images with a Bio-Imaging-Analyzer (BAS-1500, Raytest) results were evaluated using AtlasImage software version 2.0 (Clontech). The applied Atlas cDNA array contained only one dot per gene on the nylon membrane. The signal intensities of each gene spot were corrected for background and normalized using an average of all of the gene signals on the array. We did not normalize using an average of housekeeping gene signals as we observed a different gene expression of hypoxanthine-guanine phosphoribosyltransferase in response to dietary oxidized cholesterol (Table 2). Only genes showing adjusted signal intensities of 5 and more were considered for the analysis in order to avoid overinterpretation of the regulation of genes with low signal intensities caused by slight differences between adjusted signal intensities. A difference in gene expression between the dietary treatment groups was considered to be meaningful at a ratio threshold of 1.6 or more – a threshold range that is often used in the literature because independent analysis such as Northern blotting or real-time RT-PCR revealed similar or even more changes in transcript levels than detected by array analysis in most cases [14, 26].

Real-time polymerase chain reaction

In order to confirm mRNA expression data obtained from cDNA array experiments relative gene expressions of apolipoprotein A-I precursor (apoAI) (Accession # M00001), carbonic anhydrase III (CAIII) (M22413), glutathione S-transferase subunit 13 (GST13) (S83436), alcohol dehydrogenase A subunit (ADH) (M15327) and cytochrome P450 4A1 (CYP4A1) (X07259) compared to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined by means of quantitative real-time RT-PCR using Rotorgene real-time PCR cycler (LTF Labortechnik, Wasserburg, Germany). In contrast to the cDNA array experiments RNA of all nine rats per group was used for real-time RT-PCR (n = 9). cDNA synthesis and quantitative real-time RT-PCR were performed as previously described [27]. Gene specific primers coding for apoAI, CAIII, GST13, ADH, CYP4A1 and GAPDH were obtained from Roth (Karlsruhe, Germany). Identification of product length of the amplified product was confirmed using 2 % agarose gel electrophoresis. Relative quantification was performed using the "delta-delta CP-method" as applied in a recent paper [28]. The crossing points, which are essential for calculation of relative expression ratios, were obtained using Rotorgene Software 5.0. Relative expression ratios were calculated according to Eq. [4] as shown in [28] presuming an optimal and identical real-time amplification efficiency of target genes and reference gene (GAPDH) = 2. Relative expression ratios are expressed as fold changes for the target genes normalized to GAPDH. The primer sequences used for real-time RT-PCR and lengths of the amplified products were as follows: 5'-CAAGATGAGCCCCAGTCCCAATG-3' (forward) and 5'-TCCTCGGCCACAACCTTTAGATGC-3' (reverse) for rat apoAI (428 bp); 5'-TGCCAAAGGGGACAACCAGT-3' (forward) and 5'-CTCGCCTTTCTCCCGTCCTATCTT-3' (reverse) for rat CAIII (400 bp); 5'-ATCACGGAGTCCCAGAACATTTTG-3' (forward) and 5'-CCCCAGGGCCACAGAAGACA-3' (reverse) for rat GST13 (352 bp); 5'-TGCGAAGGCCAAAGAGTTAGGTG-3' (forward) and 5'-GCTGGCGCTTGATTCGGTAGG-3' (reverse) for rat ADH (497 bp); 5'-CAGAATGGAGAATGGGGACAGC-3' (forward) and 5'-TGAGAAGGGCAGGAATGAGTGG-3' (reverse) for rat CYP4A1 (459 bp); 5'-GCATGGCCTTCCGTGTTCC-3' (forward) and 5'-GGGTGGTCCAGGGTTTCTTACTC-3' (reverse) for rat GAPDH (337 bp).

Table 2 Comparison of cDNA array and real-time RT-PCR data. Shown is the Δ-fold change of the expression of various genes in rats fed oxidized cholesterol compared to rats fed unoxidized cholesterol with either coconut oil or salmon oil[1, 2]

	oxidized Cholesterol vs. unoxidized Cholesterol: Δ-fold change			
	Coconut oil		Salmon oil	
Encoded protein	cDNA array	RT-PCR	cDNA array	RT-PCR
CAIII[3]	±	1.0	↓2.1	↓1.5
ApoAI	±	↑1.4	↑1.8	↑1.4
GST13	±	↑1.3	↑1.9	↑1.8
ADH	±	↑1.2	↑2.4	↑1.7
CYP4A1	±	↑1.5	↓1.8	1.0

[1] Results are means of n = 3 (cDNA array) and n = 9 (RT-PCR) rats per group. (↑) up-regulation, (↓) down-regulation. (±) no change in gene expression (< 1.6-fold change)
[2] Results from real-time RT-PCR are relative expression ratios presented as fold-changes for the target genes normalized to reference gene (GAPDH)
[3] *CAIII* carbonic anhydrase; *apoAI* apolipoprotein A-I precursor; *GST13* glutathione S-transferase subunit 13; *ADH* alcohol dehydrogenase A subunit; *CYP4A1* cytochrome P450 4A1

Statistics

Data were subjected to ANOVA using the Minitab Statistical Software (Minitab, State College, PA, USA). Classification factors were dietary fat, cholesterol and the interaction of both factors. For statistically significant *F*-values, individual means of the treatment groups were compared by Fisher's multiple range test. Means were considered significantly different for $P < 0.05$.

Results

Final body weights, plasma lipid concentrations and concentrations of 7β-hydroxycholesterol and antioxidants in liver and plasma

Final body weights of the rats were influenced by the type of dietary fat (Table 3). Rats fed the salmon oil diets had significantly higher final body weights than rats fed the coconut oil diets. There was no difference in final body weights between rats fed diets without cholesterol or with unoxidized or oxidized cholesterol.

Plasma triacylglycerols and cholesterol were influenced by dietary fat and dietary cholesterol; rats fed the salmon oil diets had significantly lower concentrations of triacylglycerols and cholesterol than rats fed the coconut oil diets. Rats fed the coconut oil diets containing unoxidized or oxidized cholesterol had 3-fold higher plasma cholesterol concentrations than those fed a cholesterol-free diet. On the other hand, in the rats fed salmon oil dietary cholesterol had no effect on plasma cholesterol concentrations. Plasma triacylglycerol concentrations were higher in the rats fed a cholesterol-free diet compared with those fed diets containing unoxidized or oxidized cholesterol, irrespective of the dietary fat.

Concentrations of α-tocopherol in liver and plasma were significantly effected by the dietary fat; rats fed the coconut oil diets had higher concentrations of α-tocopherol in liver and plasma than those fed the salmon oil diets. Feeding oxidized cholesterol compared with unoxidized cholesterol had no effect on tocopherol concentrations in liver and plasma. Plasma concentrations of vitamin C were unaffected by dietary fat and dietary cholesterol. The GSSG/GSH ratio was significantly higher in rats fed salmon oil than in rats fed coconut oil. This ratio tended to be higher in rats fed oxidized cholesterol than in rats fed unoxidized cholesterol (oxidized cholesterol, 0.45 ± 0.11; unoxidized cholesterol, 0.39 ± 0.10; $P < 0.15$, $n = 18$).

Fatty acid composition of hepatic phosphatidyl choline

The fatty acid composition of hepatic phosphatidyl choline was strongly influenced by the dietary factors fat, cholesterol and the interaction between those factors (Table 4). Rats fed salmon oil as dietary fat had significantly higher amounts of total PUFA and long-chain n-3 PUFAs such as 20:5 n-3, 22:5 n-3 and 22:6 n-3, but significantly lower amounts of total MUFA and n-6 PUFA. Rats fed cholesterol-supplemented diets had significantly lower amounts of total PUFA and total SFA, but higher amounts of total MUFA than rats fed cholesterol-free diets. The effects of dietary oxidized cholesterol as compared to unoxidized cholesterol on the fatty acid composition of the hepatic phospholipid fraction were mainly dependent on the type of fat; the rats fed salmon oil diets showed lower amounts of total MUFA and 20:5

Table 3 Final body weights, plasma lipid concentrations and concentrations of 7β-hydroxycholesterol and antioxidants in liver and plasma of rats fed a cholesterol-free, a cholesterol-supplemented and an oxidized cholesterol-supplemented diet with either coconut oil or salmon oil as dietary fat

Diet	1	2	3	4	5	6
Dietary fat	Coconut oil	Salmon oil	Coconut oil	Salmon oil	Coconut oil	Salmon oil
Dietary cholesterol	none	none	unoxidized	unoxidized	oxidized	oxidized
Final body weight[1] (g)	264 ± 12^{b}	279 ± 10^{a}	261 ± 13^{b}	284 ± 10^{a}	265 ± 10^{b}	283 ± 11^{a}
Plasma lipid concentrations, mmol/L						
Triacylglycerols[1, 2, 3]	1.20 ± 0.33^{a}	0.67 ± 0.18^{b}	0.79 ± 0.25^{b}	0.54 ± 0.13^{c}	0.59 ± 0.12^{c}	0.44 ± 0.19^{c}
Cholesterol[1, 2, 3]	1.49 ± 0.20^{b}	1.73 ± 0.23^{b}	4.80 ± 0.47^{a}	1.94 ± 0.25^{b}	4.45 ± 0.63^{a}	1.68 ± 0.17^{b}
7β-hydroxycholesterol						
Plasma[1, 2, 3], nmol/mmol Chol	65 ± 17^{e}	133 ± 24^{d}	86 ± 37^{e}	278 ± 73^{b}	224 ± 55^{c}	402 ± 65^{a}
Liver[1, 2, 3], nmol/g	1.4 ± 0.8^{e}	$4.4 \pm 3.0^{d, e}$	6.5 ± 1.9^{d}	25.7 ± 3.7^{b}	13.6 ± 1.9^{c}	40.2 ± 13.0^{a}
Liver antioxidants						
α-Tocopherol[1, 2, 3], nmol/g	$114 \pm 22.4^{a, b}$	54 ± 15^{c}	$125 \pm 40^{a, b}$	96 ± 21^{b}	163 ± 37^{a}	83 ± 16^{b}
Glutathione ratio (GSSG/GSH)[1, 2]	0.52 ± 0.09^{b}	0.72 ± 0.13^{a}	0.37 ± 0.07^{c}	0.42 ± 0.12^{c}	0.41 ± 0.12^{c}	$0.49 \pm 0.08^{b, c}$
Plasma antioxidants						
α-Tocopherol[1, 2, 3], μmol/mmol lipids*	7.08 ± 1.49^{a}	6.16 ± 0.39^{b}	4.86 ± 0.47^{c}	4.46 ± 0.46^{d}	4.93 ± 0.47^{c}	4.22 ± 0.90^{d}
Ascorbic acid, μmol/L	81 ± 18	77 ± 14	77 ± 32	77 ± 11	100 ± 23	83 ± 7

Results are means ± SD, n = 9

Results of analysis of variance: Means within a row not sharing the same superscript letters differ significantly by Fisher's multiple range test ($P < 0.05$). Significance of factors ($P < 0.05$): [1] fat, [2] cholesterol, [3] fat x cholesterol

SFA saturated fatty acids; *MUFA* monounsaturated fatty acids; *PUFA* polyunsaturated fatty acids; *Chol* cholesterol

* mmol lipids = sum of mmols triacylglycerols and mmols cholesterol

Table 4 Fatty acid composition of hepatic phosphatidyl choline of rats fed a cholesterol-free, a cholesterol-supplemented and an oxidized cholesterol-supplemented diet with either coconut oil or salmon oil as dietary fat

Diet	1	2	3	4	5	6
Dietary fat	Coconut oil	Salmon oil	Coconut oil	Salmon oil	Coconut oil	Salmon oil
Dietary cholesterol	none	none	unoxidized	unoxidized	oxidized	oxidized
Fatty acid composition of hepatic phosphatidyl choline, g/100 g fatty acids						
Total SFA[1, 2]	40.3 ± 0.7[a]	40.1 ± 0.7[a]	36.8 ± 0.9[c]	38.0 ± 1.2[b, c]	37.2 ± 1.1[c]	38.3 ± 0.8[b]
Total MUFA[1, 2, 3]	13.8 ± 0.5[d]	12.8 ± 0.7[d]	20.0 ± 1.2[a]	17.4 ± 0.8[b]	19.3 ± 1.2[a]	16.0 ± 1.2[c]
Total PUFA[1, 2]	36.5 ± 1.0c	40.9 ± 1.1[a]	33.1 ± 0.7[d]	37.2 ± 0.8[b, c]	33.8 ± 1.0[d]	37.5 ± 0.8[b]
Total n-6-PUFA[1, 2, 3]	33.1 ± 1.1[a]	15.7 ± 0.9[d]	29.7 ± 0.7[b]	15.3 ± 0.8[d]	30.3 ± 1.1[b]	17.0 ± 0.3[c]
18:2 n-6[1, 2, 3]	7.18 ± 0.83[b]	3.22 ± 0.42[d]	9.43 ± 0.65[a]	3.98 ± 0.41[c]	9.53 ± 0.71[a]	4.32 ± 0.29[c]
20:4 n-6[1, 2, 3]	17.1 ± 1.4[a]	11.1 ± 0.6[c]	11.5 ± 0.5[b, c]	9.83 ± 0.50[d]	12.0 ± 1.1[b]	11.1 ± 0.3[c]
Total n-3-PUFA[1, 2, 3]	3.27 ± 0.18[d]	25.2 ± 1.7[a]	3.16 ± 0.35[d]	21.8 ± 1.1[b]	3.07 ± 0.18[d]	20.5 ± 1.15[c]
20:5 n-3[1, 2, 3]	0.06 ± 0.02[c]	11.4 ± 0.5[a]	0.31 ± 0.10[c]	11.3 ± 0.6[a]	0.34 ± 0.05[c]	10.3 ± 0.7[b]
22:5 n-3[1, 2, 3]	0.13 ± 0.02[c]	2.73 ± 0.46[a]	0.11 ± 0.02[c]	2.06 ± 0.19[b]	0.13 ± 0.02[c]	1.90 ± 0.26[b]
22:6 n-3[1, 2, 3]	2.96 ± 0.15[c]	10.4 ± 1.7[a]	2.44 ± 0.31[c]	7.80 ± 0.49[b]	2.34 ± 0.09[c]	7.61 ± 0.51[b]

Results are means ± SD, n = 9
Results of analysis of variance: Means within a row not sharing the same superscript letters differ significantly by Fisher's multiple range test ($P < 0.05$). Significance of factors ($P < 0.05$): [1] fat, [2] cholesterol, [3] fat x cholesterol
SFA saturated fatty acids; *MUFA* monounsaturated fatty acids; *PUFA* polyunsaturated fatty acids

n-3, but higher amounts of total n-6 PUFA and 20:4 n-6 in response to dietary oxidized cholesterol as compared to unoxidized cholesterol.

Validation of cDNA array data by quantitative real-time RT-PCR

Relative expression ratios, presented as fold-changes between dietary groups, as obtained from cDNA array experiments and real-time RT-PCR for 5 selected genes are shown in Table 4. In the rats fed the coconut oil diets RT-PCR results were similar to those obtained from cDNA array experiments; none of the genes were regulated above the threshold level of 1.6. Except for CYP4A1 RT-PCR and cDNA array data were also similar in the rats fed the salmon oil diets

Identification of genes affected by the dietary fat (fish oil compared to coconut oil) by means of cDNA array technique

Of the genes represented on the cDNA expression array, 154 (= 13 %) showed signal intensities above the arbitrary level of 5; 29 of those genes were identified as possible targets for an altered gene expression by dietary salmon oil as compared to coconut oil considering the threshold level of 1.6 (Table 5). 22 genes were shown to be up-regulated and 7 to be down-regulated in response to dietary salmon oil as compared to coconut oil. The genes affected by dietary fat were classified according to the functions *lipid metabolism* (n = 9), from which 6 were up- and 3 were down-regulated, and *xenobiotic metabolism/stress response* (n = 12), from which 9 were up- and 3 were down-regulated. The remaining genes regulated in response to dietary fat, which are involved in transcription, DNA repair, protease inhibition, etc. were not classified according to similar function (*others,* n = 8; 7 up- and 1 down-regulated).

Identification of genes affected by dietary oxidized cholesterol as compared to unoxidized cholesterol using either coconut oil or salmon oil as dietary fat by means of cDNA array technique

Changes in gene expression observed in response to dietary oxidized cholesterol were dependent on the type of fat. In the rats fed coconut oil, the expression of 7 genes (5 up- and 2 down-regulated) was altered by dietary oxidized cholesterol, while in the rats fed salmon oil, the expression of 50 genes (16 up- and 34 down-regulated) was altered (Table 6). Within the rats fed coconut oil, genes affected by dietary oxidized cholesterol could be classified according to the functions *lipid metabolism/energy metabolism* (n = 1) and *xenobiotic metabolism/stress response* (n = 1), whereas 5 genes, from which 4 were up- and 1 was downregulated, could not be assigned to one of those classifications (*others*). In the rats fed salmon oil, genes differently expressed in response to dietary oxidized cholesterol could be functionally classified to *lipid metabolism/energy metabolism* (n = 13; 8 up- and 5 down-regulated) and to *xeno-*

Table 5 Genes encoding proteins that were up(↑)- or down(↓)-regulated at least 1.6-fold in the liver of rats fed salmon oil (diet 2) compared with rats fed coconut oil (diet 1)[1, 2]

Gene[3]	Encoded protein	Salmon oil vs. Coconut oil:	
		Δ-fold change ± SD	Function of the encoded protein
Lipid metabolism/Energy metabolism			
M00001	apolipoprotein A-I precursor	↓2.4±0.1	Apolipoprotein
M00002	apolipoprotein A-IV precursor	↓1.7±0	Apolipoprotein
L46791	liver carboxylesterase 10 precursor	↑2.5±1.1	Triglyceride degradation
M10149	fructose-bisphosphate aldolase B	↓1.6±0.1	Carbohydrate metabolism
D90109	long chain acyl-CoA synthetase 2	↑1.9±1.3	Fatty acid synthesis
J02791	medium chain acyl-CoA dehydrogenase precursor	↑1.7±0.7	Fatty acid degradation
M33648	mitochondrial hydroxymethylglutaryl-CoA synthase precursor	↑1.6±0.8	Ketone body synthesis, cholesterol synthesis
M32801	3-ketoacyl-CoA thiolase A + 3-ketoacyl-CoA thiolase B	↑1.6±0.4	Fatty acid degradation
U62803	lecithin:cholesterol acyltransferase	↑2.0±1.5	Extracellular cholesterol esterification
Xenobiotic metabolism/Stress response			
K01931	glutathione S-transferase Ya subunit	↑1.8±0.4	Stress response
K02422	cytochrome P450 IA2 (CYPIA2)	↓1.9±0.3	Biotransformation, phase I
J02657	cytochrome P450 2C11 (CYP2C11)	↑1.8±0.3	Biotransformation, phase I
M10161	cytochrome P450 3A1 (CYP3A1)	↑3.1±1.5	Biotransformation, phase I
M18335	cytochrome P450 2C7 (CYP2C7)	↓1.7±0.2	Biotransformation, phase I
M33936	cytochrome P450 4A3 (CYP4A3); lauric acid omega-hydroxylase	↑4.4±2.3	Biotransformation, phase I
M37828	cytochrome P450 4A8 (CYP4A8)	↑4.3±3.2	Biotransformation, phase I
U39943	cytochrome P450 2J3 (CYP2J3)	↓1.7±0.2	Biotransformation, phase I
X07259	cytochrome P450 4A1 (CYP4A1)	↑5.0±2.1	Biotransformation, phase I
X55446	cytochrome P450 2C23; arachidonic acid epoxygenase	↑2.1±1.3	Biotransformation, phase I
M64723	Clusterin, antimyocarditis apolipoprotein J	↑2.2±0.3	Stress response, Apoptosis inhibition
M22413	carbonic anhydrase III; carbonate dehydratase III	↑3.7±1.5	Antioxidant, anti-apoptotic
Others			
X63594	I-kB (I-kappa B) alpha chain; RL/IF-1 gene product	↑2.0±1.2	Transcription
D10863	Id-2; DNA-binding protein inhibitor; HLH protein	↓2.0±0.3	Transcription
M89791	insulin-like growth factor binding protein 1 precursor	↑1.8±1.3	GH-IGF axis
L27843	nuclear tyrosine phosphatase	↑1.8±1.1	Intracellular protein phosphatase
U41744	PDGF-associated protein	↑1.9±1.4	Enhances mitogenic effect of PDGF
J02897	proteasome component C3	↑1.6±0.6	Proteosomal protein
D44495	apurinic/apyrimidinic endonuclease	↑1.6±0.5	DNA repair
V01217	cytoplasmic beta-actin	↑2.0±0.4	Housekeeping genes

[1] Results are means ± SD of three independent experiments
[2] *GH-IGF* growth hormone/insulin-like growth factor, *PDGF* plateled derived growth factor
[3] GenBank accession number

biotic metabolism/stress response (n = 11; 4 up- and 7 down-regulated), whereas the main part of the differently expressed genes (n = 26; 4 up- and 22 down-regulated) could not be classified (*others*).

Discussion

The present study was performed to investigate the effects of dietary fat and oxidized cholesterol on differential gene expression in rat liver. For gene expression profiling we applied a commercially available cDNA macroarray containing 1176 unique cDNAs spotted on a nylon membrane. For validation of the results of the cDNA array gene expression of five genes was also determined by independent quantitative real-time RT-PCR. Except for one gene DNA array data could be confirmed by real-time RT-PCR. This suggests that employing DNA arrays is a suitable means to analyze differential gene expression in response to various feeding regimes.

Growing rats were used for the present study as we expected that the effects of the dietary treatments would be greater than in adult, non-growing rats. Although the experimental rats were fed a restrictive diet in order to exclude secondary effects which might result from different feed intakes, the rats fed the salmon oil diets had higher final body weights than the rats fed the coconut oil diets. Coconut oil contains low levels of essential fatty acids. However, signs of essential fatty acid deficiency such as an increased triene:tetraene ratio in tissues regarding the fatty acid analysis of liver phosphatidyl choline (PC) were not observed. A previous study from Yaqoob et al. [29] demonstrated that feeding similar

Table 6 Genes encoding proteins that were up(↑)- or down(↓)-regulated at least 1.6-fold in the liver of rats fed oxidized cholesterol (diet 5 and 6) compared with rats fed unoxidized cholesterol (diet 3 and 4) with two different dietary fats (coconut oil and salmon oil)[1]

Gene[2]	Encoded protein	Oxidized Cholesterol/Unoxidized Cholesterol: Δ-fold change ± SD		
		Coconut oil	Salmon oil	Function of the encoded protein
Lipid metabolism/Energy metabolism				
M00002	apolipoprotein A-IV precursor	±	↑2.2±1.9	Apolipoprotein
D90109	long chain acyl-CoA synthetase 2	±	↓1.6±0.4	Fatty acid synthesis
M32801	3-ketoacyl-CoA thiolase A + 3-ketoacyl-CoA thiolase B	±	↑2.4±2.1	Fatty acid degradation
M38566	sterol 26-hydroxylase (CYP27) mitochondrial precursor	±	↑2.2±0.8	Bile acid synthesis
U72497	fatty acid amide hydrolase	±	↓1.9±0.3	Lipid metabolism
M67465	3-beta hydroxy-5-ene steroid dehydrogenase type III	±	↓1.7±0.1	Lipid metabolism
Y08172	2-arylpropionyl-CoA epimerase	±	↑2.1±0.9	Lipid metabolism
M10149	fructose-bisphosphate aldolase B	±	↑2.0±0.8	Carbohydrate metabolism
M86240	fructose-16-bisphosphatase, liver	±	↑2.8±1.4	Carbohydrate metabolism
D10854	NADP + alcohol dehydrogenase; aldehyde reductase	±	↓2.0±0.2	Ethanol metabolism
X54080	cytochrome c oxidase, subunit VIIa	↓1.7±0.1	↓3.7±0.1	Energy metabolism
Xenobiotic metabolism/Stress response				
X54793	heat shock 60-kDa protein; 60-kDa chaperonin	±	↓1.7±0.3	Stress response, chaperon
X62660	glutathione transferase, subunit 8	±	↓1.7±0.1	Stress response
J02627	cytochrome P450 2E1 (CYP2E1)	±	↑2.0±1.3	Biotransformation, phase I
J02657	cytochrome P450 2C11 (CYP2C11)	±	↓2.4±0.1	Biotransformation, phase I
M10161	cytochrome P450 3A1 (CYP3A1)	↑1.6±0.1	±	Biotransformation, phase I
M58041	cytochrome P450 2C22 (CYP2C22)	±	↓1.8±0.2	Biotransformation, phase I
M94548	cytochrome P450 4F1 (CYP4F1); hepatic tumour	±	↑1.7±0.7	Biotransformation, phase I
U39207	cytochrome P450 4F5 (CYP4F5)	±	↑1.8±1.2	Biotransformation, phase I
X55446	cytochrome P450 2C23; arachidonic acid epoxygenase	±	↓2.2±0.1	Biotransformation, phase I
Others				
J02998	ras-related protein rab1A	±	↓2.4±0.2	Mitogenic effect
X74401	rab GDI, beta species, ras related GTPase	±	↓1.6±0.1	Mitogenic effect
L12138	thymidylate synthase	±	↓2.2±0.3	Nucleotide metabolism
X13817	calmodulin	±	↓1.6±0.3	Calcium-binding protein
X63594	I-kB (I-kappa B) alpha chain; RL/IF-1 gene product	±	↓2.0±0.2	Transcription
M20035	prothymosin-alpha	±	↑1.7±0.9	Cell proliferation
X78327	ribosomal protein L13	±	↓1.9±0.2	Ribosomal protein
L14684	mitochondrial elongation factor G precursor	±	↓1.8±0.1	Translation
J02646	eukaryotic translation initiation factor 2 alpha subunit	±	↓2.8±0.3	Translation
D17711	dC-stretch binding protein	±	↓1.6±0.3	RNA processing, turnover & transport protein
M64986	high mobility group protein 1; amphoterin	±	↓1.7±0.1	DNA-binding protein
M29014	insulin receptor precursor	↓1.6±0.3	±	Hormone receptor
M15480	insulin like growth factor I	±	↑2.2±1.3	GH-IGF axis
U62326	macrophage migration inhibitory factor	±	↓1.5±0.2	Cytokines & chemokines
D14592	dual-specificity mitogen-activated protein kinase kinase 2	±	↑2.1±0.9	Intracellular kinase network members
M17526	guanine nucleotide-binding protein G(O) alpha subunit	±	↓1.7±0.4	Signal transduction
U41744	PDGF-associated protein	±	↓1.6±0.4	Enhances mitogen effect of PDG
M81397	thrombin	±	↓1.6±0.2	Serine protease
D90404	dipeptidyl-peptidase I precursor; cathepsin C	↑1.7±0.8	↓2.8±0.2	Cysteine protease
M36320	cathepsin H	↑2.1±0.2	±	Cysteine protease
M29859	proteasome component C2	±	↓2.5±0.1	Proteosomal protein
J02897	proteasome component C3	±	↓2.0±0.1	Proteosomal protein
M58593	proteasome component C8	±	↓1.7±0.3	Proteosomal protein
D10755	proteasome iota subunit; macropain iota subunit	±	↓2.7±0.3	Proteosomal protein
D21800	proteasome subunit RC10-II	±	↓2.0±0.3	Proteosomal protein
D44495	apurinic/apyrimidinic endonuclease	↑2.2±0.7	↓1.9±0.5	DNA repair
X61381	interferon induced protein	↑1.7±1.3	↓1.9±0.4	interferon induced protein
M63983	hypoxanthine-guanine phosphoribosyltransferase	±	↑1.6±1.0	Housekeeping gene

[1] Results are means ± SD of three independent experiments. (±) no change in gene expression (< 1.6-fold change)
[2] GenBank accession number

amounts of a coconut oil- or a menhaden oil-containing diet resulted in higher weight gains of the fish oil-fed rats. Although the actual cause for this effect remained unexplained the authors observed significantly increased tissue weights and altered serum lipid levels and fatty acid compositions of serum and tissues in response to dietary fish oil. Similar to our results Yaqoob et al. [29] and other investigators [30, 31] reported lower serum triacylglycerol and cholesterol concentrations in fish oil-fed rats compared with those fed coconut oil. Regardless of the reason for different body weight gains the different final body weights are unlikely to jeopardize the results of the present study with regard to the effects of dietary oxidized cholesterol.

As expected, feeding the salmon oil diets led to a significant incorporation of oxidation susceptible n-3 PUFAs into liver lipids which also led to reduced vitamin E concentrations [32]. Gene expression analysis in the present study demonstrated that feeding fish oil as compared to coconut oil resulted in an enhanced transcription of PPARα-responsive genes such as mitochondrial β-HMG-CoA synthase, 3-ketoacyl-CoA thiolase A and B, medium chain acyl-CoA dehydrogenase and the cytochrome P450 species CYP4A1 and CYP4A3 with the strongest change in gene expression being observed within the latter two enzymes. This is in accordance with results from other investigators reporting that n-3 PUFA stimulate expression of PPARα target genes [1, 5]. The induction of genes of the cytochrome P450 subfamilies CYP2C (CYP2C11, CYP2C23) and CYP4A (CYP4A1, CYP4A3, CYP4A8) observed in response to dietary fish oil is possibly related to the beneficial effects of the consumption of marine oils, because these P450 species metabolize arachidonic acid to biologically active epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs). Abundant functional studies indicate that EETs and HETEs are powerful regulators of vascular tonus and systemic blood pressure [33].

The induction of oxidative stress as observed after feeding fish oil to different experimental animals was shown to be accompanied by a coordinated induction of gene expression of stress proteins, endogenous antioxidants such as glutathione and antioxidant and detoxifying enzymes [34]. In this regard enhanced transcription rates of carbonic anhydrase III, clusterin/apolipoprotein J and glutathione-S-transferase (GST) subunit Ya indicate an adaptive response to fish oil-induced oxidative stress. Carbonic anhydrase was reported to protect proteins from oxidation catalyzed by iron-containing degradation products of haemoglobin and myoglobin, whereas clusterin and GST subunit Ya were shown to be up-regulated in the course of ROS formation. This is in accordance with a recent study demonstrating an altered hepatic gene expression in response to dietary fish oil to defend against PPARα activation and ROS production using high-density oligonucleotide arrays [34].

Changes in the transcription of genes involved in lipid and energy metabolism were only poorly observed in the present study by dietary fish oil, although it is well known that fish oil reduces transcription of several lipogenesis-related genes caused by the reduction of SREBP-1c mRNA [1, 5, 6]. In the present study we could not observe any suppression of SREBP-1c regulated genes; however cDNAs of important SREBP-1c target genes were not spotted on the macroarray applied in the present study. The observed lowering effect of fish oil-treatment on apolipoprotein AI and AIV mRNA in the present study is in accordance with results from other researchers [35, 36].

Feeding dietary oxidized cholesterol compared with unoxidized cholesterol resulted in markedly increased levels of oxysterols in liver and plasma as also shown from others [15, 16]. The effects of dietary oxidized cholesterol were strongly dependent on the type of dietary fat. Significantly more genes involved in xenobiotic metabolism and stress response were transcriptionally affected by oxidized cholesterol when salmon oil was used as dietary fat as compared to coconut oil. This suggests that the effects of oxysterols on gene transcription were amplified by oxidative stress induced by dietary n-3 PUFA. Prooxidative effects of oxysterols and oxidized cholesterol were demonstrated in vitro as well as in vivo [18, 37]. However, in the present study the administration of dietary oxidized cholesterol did not reduce vitamin E and vitamin C concentrations and caused only a slight increase in the GSSG/GSH ratio. This suggests that dietary oxidized cholesterol caused only moderate oxidative stress in the current study. As a consequence of oxidative stress induction of genes involved in stress response and biotransformation is often reported [33]. Especially the induction of different cytochrome P450 species such as CYP2E1, involved in the metabolism of a variety of endo- and xenobiotica, is responsible for detoxification and protection from cellular damage [38, 39]. In contrast, the reduced gene expression of the CYP subfamilies 2C (CYP2C11, CYP2C22, CYP2C23), which catalyze the oxidative metabolism of arachidonic acid, could be responsible for the raised proportions of arachidonic acid in the hepatic PC, although others demonstrated an increased activity of Δ6 desaturase by dietary administration of oxidized cholesterol as compared to unoxidized cholesterol [40].

A suppression of cellular defense mechanisms was also demonstrated as a consequence of oxidative stress [41]. In this regard, the reduced transcription of proteosomal and lysosomal enzymes (cathepsin C), which was observed in response to dietary oxidized cholesterol within the rats fed salmon oil, possibly indicates a decreased capacity to protect the cell from toxic effects caused by oxidized proteins. The proteasomal pathway

is responsible for processes essential for cell viability, including the selective degradation of oxidized proteins. A loss in proteasome function contributes to the accumulation of oxidized proteins and increases irreversible damage caused by oxidative stress [42].

It is well known that lipid metabolism is affected by specific oxysterols via activation of the oxysterol-binding receptors LXRα and β [10]. However, it is still unclear whether oxysterols of dietary origin contribute to an activation of LXR as well. The present study revealed that significantly more genes belonging to lipid and energy metabolism were altered by dietary oxidized cholesterol in the rats fed fish oil than in the rats fed coconut oil. A possible explanation could be that feeding fish oil was shown to increase hepatic concentrations of oxysterols as compared to lard or coconut oil [19, 24]. The induction of reverse cholesterol transport mechanisms (apoAI and apoAIV) and cholesterol elimination pathways (sterol-27 hydroxylase) by dietary oxidized cholesterol must be considered beneficial in view of the protective effects from coronary heart disease. A stimulation of reverse cholesterol transport, an increase of plasma HDL cholesterol, and an inhibition of cholesterol absorption was also observed as a consequence of pharmacological activation of LXRs [43]. However, as mentioned above it remains to be elucidated whether these favourable effects are achieved by high concentrations of exogenous oxysterols as well.

In conclusion, expression profiling using cDNA arrays revealed that dietary oxidized cholesterol affects transcription of many genes, mainly involved in xenobiotic metabolism and stress response. In addition, it was shown that the effects of oxidized cholesterol on gene expression are presumably amplified by the administration of dietary fish oil. The study suggests that at least some of the observed effects on gene transcription are possibly responsible for the pathophysiological effects of oxysterols such as atherogenicity and mutagenicity.

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