Dietary fish oil up-regulates cholesterol 7α-hydroxylase mRNA in mouse liver leading to an increase in bile acid and cholesterol excretion

Annie M. Bérard*, Marie-France Dumon, Michel Darmon

Laboratoire de Biochimie et de Biologie Moléculaire, Université Victor Ségalen Bordeaux 2, Zone Nord – Case 49-146, 146 rue Léo-Saignat, 33076 Bordeaux Cedex, France

Received 19 November 2003; revised 17 December 2003; accepted 24 December 2003

First published online 23 January 2004

Edited by Lukas Huber

Abstract To investigate the molecular events controlling reverse cholesterol transport, we compared gene expression of normal mouse liver to that of mice fed a long chain (LC) ω-3 fatty acid-enriched diet. Using cDNA microarrays, we assessed expression levels of 1176 genes, and we found that D-site binding protein (DBP) was three-fold increased in mice on a LC ω-3 fatty acid-rich diet compared to controls. DBP is known to increase transcriptional level of cholesterol 7α-hydroxylase (C7α), the rate-limiting enzyme for bile acid production and cholesterol excretion, and we found that C7α mRNA was also up-regulated by LC ω-3 fatty acids. Moreover, liver X receptor-α, another transcription factor up-regulating C7α, was three- to four-fold increased in liver of treated mice. On the other hand, we demonstrated that bile acid and cholesterol excretion were two-fold increased. These results show that LC ω-3 fatty acids control cholesterol metabolism in mice at a new endpoint.

Key words: Long chain ω-3 fatty acid; Reverse cholesterol transport; Bile acid production; D-site binding protein; Liver X receptor-α; cDNA array

1. Introduction

Dietary n-3 polyunsaturated fatty acids (PUFA) have profound effects on hepatic gene transcription leading to significant changes in lipid metabolism. The ω-3 PUFA-dependent partitioning of metabolic fuels away from storage and towards oxidation reflects the fact that these fatty acids suppress the expression of lipogenic genes, and simultaneously induce the transcription of genes encoding proteins involved in lipid oxidation and thermogenesis. It has been demonstrated that the ω-3 PUFA control of lipogenic gene transcription is due to down-regulation in liver of sterol regulatory binding protein-1 (SREBP-1) mRNA and inhibition of the release of mature SREBP-1 from the endoplasmic reticulum [1]. On the other hand, long chain (LC) ω-3 PUFA act on lipid oxidation and thermogenesis by functioning as ligands for peroxisome proliferator-activated receptor-α (PPARα) [2–4], whereas PPARα is also known to increase the transcriptional level of lipoprotein lipase, which results in a faster catabolism of triglyceride-rich lipoproteins. The consequence of LC ω-3 PUFA liganding to PPARα, associated with the fact that they decrease diacylglycerol acyltransferase mRNA, explains why LC ω-3 PUFA are used as a triglyceride-lowering drugs.

We and others have demonstrated that plasma high density lipoprotein (HDL)-cholesterol and apolipoprotein (apo) A-I and apoA-II concentrations decreased when the diet was supplemented by LC ω-3 PUFA [4,5]. The lowering of HDL-cholesterol was associated with reduced liver apoA-I mRNA levels through a PPARα-dependent pathway [4]. Furthermore we demonstrated that mice fed a LC ω-3 fatty acid-enriched diet exhibited an increased uptake of HDL-cholesterol ester in the liver, probably through an increase in scavenger receptor B-1 (SR-B1) [5]. This effect of ω-3 PUFA on HDL metabolism may contribute to their beneficial effects on reverse cholesterol transport and cardiovascular diseases.

In an attempt to identify new genes involved in the control of lipid metabolism under a LC ω-3 fatty acid-rich diet, we compared the patterns of gene expression in control and treated mouse liver using large-scale cDNA microarrays and quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) analysis. We found in treated mice an over-expression of D-site binding protein (DBP), liver X receptor-α (LXRα) and cholesterol 7α-hydroxylase (C7α) as well as an increase in bile acid and cholesterol excretion.

2. Materials and methods

2.1. Animals

Female mice, obtained from Iffa Credo (L'Arbresle, France), were on the C57Black background. They were housed in a full-barrier animal facility on a 12 h light/dark cycle, with free access to food and water. Since ω-3 fatty acids can be considered a possible supplementation or treatment in human diet for their beneficial roles, we decided in this study to add these fatty acids to the standard mouse diet. Eight week old female mice were thus placed either on a regular diet, or on a regular diet enriched in LC ω-3 fatty acids for 16 weeks as previously described [5]. The ω-3-enriched diet contained 19% eicosapentaenoic acid (20:5n-3) and 13% docosahexaenoic acid (22:6n-3) from total fatty acids. Mice were fed the diet for 16 weeks. They did not lose weight and had normal hepatic tests (ALAT, ASAT, LDH) throughout the experiment. As the study described here was part of a larger study ($n = 20$ mice for each study group), we used five mice on a standard diet and four mice on a LC ω-3 fatty acid-rich diet. The use of the research protocol was in accordance with the French Ministry of Agriculture, section of Health and Animal Protection (approval 04476).

2.2. RNA isolation

Mouse livers were obtained from 6 month old female mice on a standard diet ($n = 5$) or a LC ω-3 fatty acid-rich diet ($n = 4$). Samples

*Corresponding author.
E-mail address: annie.berard@u-bordeaux2.fr (A.M. Bérard).

Abbreviations: DBP, D-site binding protein; LXRα, liver X receptor-α; C7α, cholesterol 7α-hydroxylase; S15αOH, steroid 15α-hydroxylase; PUFA, polyunsaturated fatty acids

0014-5793/04/$30.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(04)00049-3

were immediately put into a Trizol® solution (Gibco BRL, InVitrogen, Cergy, France) and stored at −80°C pending RNA isolation. Total RNAs were isolated according to Chomczynski and Sacchi [6] from each individual liver. The amount of RNA was determined by measuring absorption at 260 nm. Quality of the isolated RNAs was controlled by the 260/280 nm ratio (1.8–2.0) and by denaturing gel electrophoresis.

2.3. cDNA array procedures and analyses

Aliquots of RNAs from mice on a standard diet were pooled (untreated pool) and aliquots of RNAs from mice on a LC ω-3 fatty acid-rich diet were pooled (treated pool).

Samples of total RNA pools (5 μg) were used to synthesize [α-^{32}P]dATP cDNA probes for hybridization according to the manufacturer's procedure (Clontech, Palo Alto, CA, USA). The ^{32}P-labelled cDNA probes were further purified by column chromatography (NucTrap® Probe purification column, Stratagene, Amsterdam, The Netherlands). The Atlas Mouse 1.2 Array, containing 1176 mouse cDNAs, was purchased from Ozyme (Clontech). The probes (3×10^6 cpm/ml) were hybridized overnight (68°C) on separate filter arrays, and washed according to the manufacturer's protocol. After exposure to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA, USA) for 2–4 days, the images were analyzed using the ImageQuaNT software (Molecular Dynamics). Two different hybridizations were performed with the untreated pool probes and with the treated pool probes.

After subtraction of the background, signal values were normalized by considering the mean filter hybridization signal. No signals were detected in the spots of plasmid or bacteriophage DNA, confirming the specificity of the hybridization. For each gene, the treated/untreated ratio was calculated by dividing the average value obtained for the two independent hybridizations performed with the treated pool probes, by the average value of two independent hybridizations performed with the untreated pool probes.

2.4. qPCR analysis

For performing qPCR, RNAs from each individual liver from treated and untreated mice were reverse transcribed to cDNA using random primers and GeneAmp® RNA PCR kit (Applied Biosystems, Courtaboeuf, France). The specific primers and TaqMan® probes were designed using Primer Express (Applied Biosystems) or Primer3 software, and synthesized by Eurogentec (Seraing, Belgium). Probes were double labelled with the fluorescent reporter dye 6-carboxyfluorescein (FAM) covalently linked to the 5′ end of the probe and the quencher dye 6-carboxytetramethylrhodamine (TAMRA) attached to the 3′ end. qPCR was performed in 96 well reaction plates with optical caps. Fluorescence was continuously followed for each reaction. Gene expression was normalized for ribosomal 18S RNA amount. The qPCR data are given as the mean of treated/untreated ratios of gene expression (and S.E.M.) calculated from amplifications of cDNAs prepared from individual treated ($n=4$) and untreated ($n=5$) mice. Moreover, amplifications from each individual cDNA were performed in duplicate and the duplicate data matched closely, indicating that there was no technical problem with the qPCR; the mean of the duplicates was used for the subsequent calculations (mean treated/untreated ratios and S.E.M.).

2.5. Bile acid and cholesterol excretion

For measurement of the bile acid and cholesterol excretion, feces samples from 10 treated or untreated mice were pooled and prepared as described [7]. Briefly, feces samples were lyophilized and ground to a fine powder which was extracted with 90% ethanol. The extract was subsequently dried down completely and resuspended in 50% ethanol. The bile acid and cholesterol contents of all samples were measured colorimetrically using analysis kits (Sigma Diagnostics, L'Isle d'Abeau-Chesnes, France; bioMérieux, Marcy l'Etoile, France).

3. Results and discussion

3.1. cDNA arrays

Liver RNA samples obtained from four treated mice were pooled as well as the samples from five untreated mice. They were used to prepare ^{32}P-labelled cDNA probes which were then hybridized to individual Atlas 1.2 Arrays (Clontech) containing cDNA for 1176 mouse genes. Two independent cDNA array experiments were performed. 100% of the 1176 genes gave a signal above the background value (data in pixels) in both types of mice (http://cbi.labri.fr/outils/data/). Only 20 genes were significantly expressed differentially in hepatic samples from treated or untreated mice. Four genes gave a ratio larger than two-fold and 16 genes had a ratio smaller than 50% in treated mice (Table 1). Among the four genes up-regulated in the livers from treated mice according to cDNA array results, the three first genes gave reproducible hybridization values whereas the last one (platelet endothelial cell

Table 1
Genes differentially expressed (transcriptional arrays) in livers from mice on an ω-3-enriched diet for 16 weeks (treated) compared to livers from control mice (untreated)

GenBank accession number	Gene/protein name	Treated/untreated ratio[a]
U29762	D-binding protein (albumin D box-binding protein) (DBP)	3.100
U28423	58 kDa inhibitor of RNA-activated protein kinase (P58^{IPK})	2.199
D78645	78 kDa glucose-regulated protein (GRP78)	2.139
L06039	CD31; platelet endothelial cell adhesion molecule 1 (PECAM-1)	2.056
X97052	MAPKK6; MAP kinase kinase 6 (MKK6)	0.498
D28492	caspase-2 precursor (CASP2)	0.497
U34960	transducin β2 subunit	0.497
AF069542	NF-κB essential modulator; IκB kinase γ subunit (IKK-γ) essential regulatory subunit	0.493
X76292	desert hedgehog homolog precursor (DHH); HHG3	0.491
X70472	myb-related protein B; B-myb; mybL2	0.480
U03279	PI3-K p110; phosphatidylinositol 3-kinase catalytic subunit	0.478
U10551	Gem-induced immediate early protein; Ras family member	0.441
AF033585	frizzled homolog 9 (FZD9)	0.439
U02887	DNA repair protein XRCC1	0.429
D31788	BST-1; lymphocyte differentiation antigen CD38	0.422
Y14019	Rab-3b ras-related protein	0.393
U12273	DNA-(apurinic/apyrimidinic) lyase; AP endonuclease 1 (APEX nuclease; APEN; APEX)	0.378
U92456	WBP6; pSK-SRPK1; WW domain binding protein 6 serine kinase for SR splicing factors	0.375
U44088	T-cell death-associated protein (TDAG51)	0.350
U12273	DNA excision repair protein ERCC1	0.288

[a]Ratios were calculated by dividing the average value (from two independent hybridizations) obtained for the control sample pool ($n=5$) by the average value of the treated sample pool ($n=4$).

Table 2
qPCR data concerning genes either found overexpressed in cDNA array experiments or involved in cholesterol metabolism

Gene/protein name	qPCR results
DBP	12.9 ± 9.40
$P58^{IPK}$	3.20 ± 2.18
GRP78	2.96 ± 1.64
PECAM-1	0.75 ± 0.32
Cholesterol 7α-OH	2.27 ± 0.53
Steroid 15α-OH	2.37 ± 1.47
LXRα	3.41 ± 0.60
PXRα	6.88 ± 2.14
RXRα	8.72 ± 2.78

qPCR analysis shows the determination of the relative quantity of mRNA from individual livers of four mice on a LC ω-3 fatty acid-enriched diet compared to mRNA from individual livers of five control mice. Target and endogenous control (18S) amplifications were done in duplicate and results were normalized to 18S. Data are expressed as the mean (±S.E.M.) of the treated/untreated ratio of gene expression.

adhesion molecule 1, PECAM-1) did not give close values in the two experiments.

3.2. qPCR

qPCR analysis was used first to check array results and, second, to explore further the metabolic pathways related to lipid metabolism.

Table 2 shows that only the three first genes were found to be at least two-fold up-regulated in the liver of treated mice. These were the D-binding protein (also called albumin D box-binding protein, DBP), the 58 kDa inhibitor of RNA-activated protein kinase ($P58^{IPK}$) and the 78 kDa glucose-regulated protein (GRP78). $P58^{IPK}$ is an inhibitor of RNA-activated protein kinase known to interfere in the regulation of normal cellular processes such as signal transduction, cellular differentiation, growth and proliferation as well as gene expression at the transcriptional level [8]. GRP78 is an endoplasmic reticulum chaperone protein whose expression is induced during oxidative stress [9]. Its induction is required to maintain endoplasmic reticulum function, to facilitate protein folding and thus to protect cells from the toxic consequences of oxidative stress. It has been suggested that GRP78 may protect the host cells against cell death by suppressing oxyradical accumulation and stabilizing mitochondrial functions [10,11]. Omega-3 PUFA are believed to be molecular targets for lipid peroxide formation due to their high degree of unsaturation. Some authors have reported a protective role in vivo for ω-3 PUFA in combating free radicals [12,13]. Thus the up-regulation of the GRP78 gene could explain, at least in part, how ω-3 PUFA would protect tissues from the effects of oxyradical accumulation. Moreover, GRP78 can form a complex with caspase-7 and caspase-12 preventing activation and release of caspase-12. It is noteworthy that the array results showed that an ω-3 diet decreased levels of mRNA encoding different proteins involved in apoptosis, as caspase-2 precursor (×0.497), caspase-7 (×0.531), and apoptotic protease activating factor 1 (×0.614). The DBP, a member of the proline- and acid-rich domain subfamily of basic/leucine zipper proteins, accumulates according to a robust circadian rhythm in liver and several other tissues [14]. It has been demonstrated that DBP seems to be more a component of the circadian output pathway rather than a master gene of the clock [15].

It has been shown that DBP is involved in the transcriptional regulation of several hepatic genes [16]. In particular, DBP is a positive regulator of the circadian expression of the gene encoding cholesterol 7α-hydroxylase (C7α) [14,17], the rate-limiting enzyme in the conversion of cholesterol to bile acids [18], and steroid 15α-hydroxylase (S15αOH) [19], an enzyme involved in liver steroid catabolism. That suggests that DBP may play an important role in cholesterol homeostasis through transcriptional regulation of C7α and S15αOH. Other transcription factors have been shown to up-regulate C7α and S15αOH genes, namely two nuclear receptors: the liver X receptor α (LXRα) [20] and the pregnane X receptor α (PXRα) [21] respectively. By qPCR, we showed that mRNAs of LXRα, PXRα and RXRα (a receptor which forms active heterodimers with LXRα and PXRα) are dramatically up-regulated in the livers of treated mice as shown in Table 2. Furthermore, we demonstrated by qPCR that C7α and S15αOH mRNA levels were both two- to three-fold increased in livers of treated mice (Table 2). In contrast, Pawar et al. [22] concluded that LXRα was not a target for ω-3 PUFA regulation in rat livers because LXR-regulated transcripts such as C7α were not up-regulated. However, their study design was different from ours: the rats were fed a high carbohydrate diet supplemented with fish oil at 10% w/w and livers were removed ∼2 h after completing the meal. On the other hand, α-linolenic acid, which is an ω-3 fatty acid precursor of LC ω-3 PUFA, has been described to be able to induce LXRα expression in vitro by two-fold [23]. The fact that LC ω-3 PUFA were shown, in this last study and ours, to increase the transcriptional level of LXRα is particularly important because LXR is known to regulate transcripts encoding proteins involved in HDL metabolism and reverse cholesterol transport [24], such as scavenger receptor-B1 whose mRNA was found two- to three-fold increased in liver from mice fed a LC ω-3 fatty acid-rich diet [5] and C7α. Besides that, several authors have reported that C7α protein level and

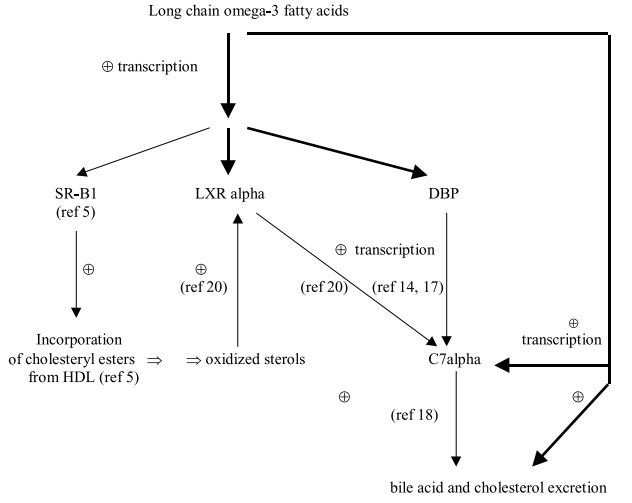

Fig. 1. Putative pathways of stimulation of bile acid and cholesterol excretion by LC ω-3 fatty acids. Thick arrows represent pathways identified in this work. Thin arrows represent pathways identified in the literature with corresponding references. It is presently unknown whether stimulation of C7α as well as bile acid and cholesterol excretion by LC ω-3 fatty acids represent a new additional pathway or follow the presently identified pathways through LXRα and DBP.

activity are regulated primarily at the level of gene transcription [17,25].

3.3. Bile acid and cholesterol excretion

We evaluated bile acid and cholesterol excretion in feces samples from treated mice compared to untreated mice. Mice on a LC ω-3 fatty acid-rich diet excreted bile acid and cholesterol two times more than untreated mice (0.101 versus 0.051 μmol/g of feces and 0.80 versus 0.28 μmol/g of feces, respectively). For the first time, we demonstrated that mice on a LC ω-3 fatty acid-rich diet were able to up-regulate bile acid and cholesterol excretion. Another study demonstrated that incubation of cells with remnants derived from corn oil rich in n-6 PUFA led to increased bile acid production, while addition of palm oil remnants to the medium did not affect bile acid synthesis [26]. In our study, the most likely explanation for the effect on bile acid and cholesterol excretion is an increase in C7α by LC ω-3 PUFA through an induction of DBP and LXR (Fig. 1). Since it has been established that the control of lipogenic gene transcription by ω-3 is mainly due to a reduction of SREBP-1 in liver [1], and that PPARα has probably a minor role in the lowering of triglyceride and HDL-cholesterol levels by fish oil in mice [4], our results suggest the existence of a new pathway through which fish oil could exert its lipid-lowering action. In our previous study [5], we reported that LC ω-3 PUFA induce an increase in SR-B1 mRNA, suggesting an increase in SR-B1 amount at the surface of liver cells. This effect on the last step of reverse cholesterol transport – an important mechanism by which HDL are thought to reduce the development of atherosclerosis – could account, at least in part, for the beneficial effect of diets rich in LC ω-3 PUFA reported in epidemiological studies. In this study, we demonstrated for the first time an increase in bile acid and cholesterol excretion from the body of treated mice, probably through an increase in C7α secondary to DBP and LXRα induction.

Acknowledgements: This work was supported by Pierre Fabre Santé, France. We thank Antonio Palos-Pinto for preparation of feces extracts and the Centre de Bioinformatique de Bordeaux (CbiB), Plateforme Génomique Fonctionnelle, Université Victor Segalen Bordeaux 2.

References

[1] Kim, H.J., Takahashi, M. and Ezaki, O. (1999) J. Biol. Chem. 274, 25892–25898.
[2] Ren, B., Thelen, A.P., Peters, J.M., Gonzalez, F.J. and Jump, D.B. (1997) J. Biol. Chem. 272, 26827–26832.
[3] Ren, B., Thelen, A. and Jump, D.B. (1996) J. Biol. Chem. 271, 17167–17173.
[4] Dallongeville, J., Bauge, E., Tailleux, A., Peters, J.M., Gonzalez, F.J., Fruchart, J.C. and Staels, B. (2001) J. Biol. Chem. 276, 4634–4639.
[5] Le Morvan, V., Dumon, M.-F., Palos-Pinto, A. and Bérard, A.M. (2002) Lipids 37, 767–772.
[6] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[7] Sinal, C.J., Tohkin, M., Miyata, M., Ward, J.M., Lambert, G. and Gonzalez, F.J. (2000) Cell 102, 731–744.
[8] Tang, N.M., Ho, C.Y. and Katze, M.G. (1996) J. Biol. Chem. 271, 28660–28666.
[9] Kaufman, R.J. (1999) Genes Dev. 13, 1211–1233.
[10] Liu, H., Miller, E., van de Water, B. and Stevens, J.L. (1998) J. Biol. Chem. 273, 12858–12862.
[11] Rao, R.V., Peel, A., Logvinova, A., del Rio, G., Hermel, E., Yokota, T., Goldsmith, P.C., Ellerby, L.M., Ellerby, H.M. and Bredesen, D.E. (2002) FEBS Lett. 514, 122–128.
[12] Yavin, E., Brand, A. and Green, P. (2002) Nutr. Neurosci. 5, 149–157.
[13] Barbosa, D.S., Cecchini, R., El Kadri, M.Z., Rodriguez, M.A., Burini, R.C. and Dichi, I. (2003) Nutrition 19, 837–842.
[14] Fonjallaz, P., Ossipow, V., Wanner, G. and Schibler, U. (1996) EMBO J. 15, 351–362.
[15] Lopez-Molina, L., Conquet, F., Dubois-Dauphin, M. and Schibler, U. (1997) EMBO J. 16, 6762–6771.
[16] Mueller, C.R., Maire, P. and Schibler, U. (1990) Cell 61, 279–291.
[17] Lavery, D.J. and Schibler, U. (1993) Genes Dev. 7, 1871–1884.
[18] Shefer, S., Hauser, S., Bekersky, I. and Mosbach, E.H. (1970) J. Lipid Res. 11, 404–411.
[19] Lavery, D.J., Lopez-Molina, L., Margueron, R., Fleury-Olela, F., Conquet, F., Schibler, U. and Bonfils, C. (1999) Mol. Cell. Biol. 19, 6488–6499.
[20] Peet, D.J., Turley, S.D., Ma, W., Janowski, B.A., Lobaccaro, J.M., Hammer, R.E. and Mangelsdorf, D.J. (1998) Cell 93, 693–704.
[21] Akiyama, T.E. and Gonzalez, F.J. (2003) Biochim. Biophys. Acta 1619, 223–234.
[22] Pawar, A., Botolin, D., Mangelsdorf, D.J. and Jump, D.B. (2003) J. Biol. Chem. 278, 40736–40743.
[23] Tobin, K.A., Steineger, H.H., Alberti, S., Spydevold, O., Auwerx, J., Gustafsson, J.A. and Nebb, H.I. (2000) Mol. Endocrinol. 14, 741–752.
[24] Lund, E.G., Menke, J.G. and Sparow, C.P. (2003) Arterioscler. Thromb. Vasc. Biol. 23, 1169–1177.
[25] Stravitz, R.T., Hylemon, P.B., Heuman, D.M., Hagey, L.R., Schteingart, C.D., Ton-Nu, H.T., Hofmann, A.F. and Vlahcevic, Z.R. (1993) J. Biol. Chem. 268, 13987–13993.
[26] Bravo, E., Cantafora, A., Marinelli, T., Avella, M., Mayes, P.A. and Botham, K.M. (1996) Life Sci. 59, 331–337.