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Marked variability in hepatic expression of cytochromes CYP7A1 and CYP27A1 as compared to cerebral CYP46A1. Lessons from a dietary study with omega 3 fatty acids in hamsters

Natalia Mast^{*}, Marjan Shafaati[‡], Wahiduz Zaman^{**}, Wenchao Zheng^{*}, Deborah Prusak[#], Thomas Wood[#], G. A. S. Ansari[#], Anita Lövgren-Sandblom[‡], Maria Olin[‡], Ingemar Björkhem[‡], and Irina Pikuleva^{*,¶}

^{*}Department of Ophthalmology and Visual Sciences, Case Western Reserve University, Cleveland, OH, USA

^{**}Department of Pharmacology and Toxicology, University of Texas, Medical Branch, Galveston, TX, USA

[#]Department of Biochemistry and Molecular Biology, University of Texas, Medical Branch, Galveston, TX, USA

[‡]Division of Clinical Chemistry, Department of Laboratory Medicine, Karolinska Institute, Huddinge, Sweden

Abstract

Two diets simulating the recommendations of the American Heart Association to increase the intake of n-3 polyunsaturated fatty acids (n-3 PUFAs) were tested on Golden Syrian hamsters and compared to the diet simulating the current estimated consumption of fat in the United States. N-3 PUFAs were evaluated for their effects on serum and brain lipids and on the three cytochrome P450 enzymes (CYPs 7A1, 27A1, and 46A1) that play key roles in cholesterol elimination from different organs. Hamsters on the highest concentration of n-3 PUFAs had a statistically significant decrease in LDL and HDL cholesterol and no change in serum total cholesterol and triglycerides levels. CYP27A1 and CYP46A1 mRNA levels were increased in the liver and brain, respectively, whereas possible effects on CYP7A1 were obscured by a marked intergroup variability at mRNA, protein and sterol product levels. Increased levels of CYP46A1 mRNA in the brain did not lead to significant changes in the levels of lathosterol, 24S-hydroxycholesterol or cholesterol in this organ. The data obtained are discussed in relation to inconsistent effects of n-3 PUFAs on serum lipids in human trials and reported positive effects of fish oil on cognitive function.

Keywords

fish oil; n-3 polyunsaturated fatty acids; American Heart Association; dietary guidelines; cholesterol turnover; brain; CYP7A1; CYP27A1; CYP46A1

[¶]To whom correspondence should be addressed. Telephone (216)368-3823; fax (216) 368-3482; irina.pikuleva@case.edu.

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1. Introduction

There have been significant nutritional changes during human evolution. Some of them, like increased consumption of meat and saturated fat along with a 10-fold increase in the ratio of n-6 to n-3 polyunsaturated fatty acids (PUFAs), are associated with the increased mortality from cardiovascular disease (CVD) [1-3]. A general consensus is that saturated fat should be reduced in our diet, whereas consumption of n-3 PUFAs should be increased. Yet, specific quantitative recommendations vary widely among different countries as well as among nutritionists, and we still do not know the intake of n-3 PUFAs and the ratio of n-6 to n-3 PUFAs that would achieve optimum health benefits. N-3 PUFAs are essential fatty acids that exert diverse effects on human health and act via multiple mechanisms which include production of eicosanoids, cell signaling and regulation of gene expression [1,4,5]. In addition, n-3 PUFAs, as well as all fatty acids, alter physico-chemical properties of cellular membranes because upon ingestion they are distributed to virtually every cell in the body and become incorporated into membrane lipids. The membrane lipid content is changed, and this change may affect the microenvironment and function of membrane proteins [4-7].

The goal of the present study was to investigate how dietary recommendations of the American Heart Association (AHA) regarding fat consumption affect cholesterol-metabolizing cytochromes P450, CYPs 7A1, 27A1 and 46A1, the enzymes that play important roles in maintenance of cholesterol homeostasis [8]. CYP7A1 is expressed in the liver, where it catalyzes the conversion of cholesterol to 7 α -hydroxycholesterol, the first step in the quantitatively most important (under normal physiological conditions) pathway of cholesterol removal via bile acids [9]. Complete deficiency of CYP7A1 activity results in significant elevation of total and LDL cholesterol (T-C and LDL-C, respectively) and substantial accumulation of cholesterol in the liver [10]. CYP27A1 is expressed ubiquitously and hydroxylates cholesterol and some other sterols on the side chain producing 27-hydroxysterol. This enzyme is important for cholesterol elimination from extrahepatic tissues, bile acid biosynthesis in the liver and vitamin D₃ hydroxylation in the kidney [11-13]. Partial or complete deficiency of CYP27A1 leads to abnormal cholesterol and cholestanol (reduced form of cholesterol) accumulation in multiple tissues and is often manifested by premature atherosclerosis [14]. Finally, CYP46A1 is mainly expressed in the brain where it converts cholesterol to 24S-hydroxycholesterol, thus controlling cholesterol turnover in this organ [15-18]. Medical significance of CYP46A1 is still under investigation but an increased activity is suggested to be beneficial on neurodegeneration [19-21]. CYPs 7A1, 27A1, and 46A1 are all membrane-bound proteins residing either in the endoplasmic reticulum (CYPs 7A1 and 46A1) or mitochondria (CYP27A1).

To conduct our nutritional studies, we selected hamsters. Hamsters are believed to be one of the best animal models for human cholesterol and bile acid metabolism [22]. They are also a recognized model for atherosclerotic research and represent an appropriate and acceptable model to address effects of dietary fatty acids and cholesterol [22-25]. Like humans, hamsters respond readily to changes in dietary fat composition and have been used previously by others to examine the effect of PUFAs [26-29]. We tested three isocaloric diets (Table 1), each supplemented with modest, non-atherogenic (for hamsters) amounts of cholesterol (0.025%). The first diet simulated the current estimated intake in the US of total fat (34% of total dietary calories or 34% en), saturated fat (12-15% en) and polyunsaturated fat (7% en) and the mean ratio (9.9:1) between n-6 and n-3 PUFAs [2,30]. The energy intake from n-3 PUFAs in this diet corresponded to a human equivalent of 1.4 g/day @ 2000 kcal of α -linolenic acid (ALA) and 0.1-0.2 g/day of a sum of eicosapentaenoic (EPA) and docosahexaenoic acids (DHA). The second diet simulated the recommendations of the AHA to people without documented CVD to increase intake of DHA and EPA to 0.55 g/day and have the n-6/n-3 PUFAs ratio equal to 6:1 [31]. The third diet simulated the suggestions of the AHA to people with documented CVD

to consume ~ 1g/day of EPA plus DHA [31]. The n-6/n-3 PUFAs ratio in this diet was 5.0:1. Duration of feeding was 6 weeks because it takes 4-5 weeks in hamsters for new steady states to be established [22]. We demonstrate here that n-3 PUFAs affect the expression of CYPs 27A1 and 46A1 mRNA and possibly the cholesterol turnover in the brain. The data obtained may help to explain the conflicting results of human trials on the serum lipid profiles and provide new information on cholesterol homeostasis in the brain.

2. Methods

2.1. Animals and Diets

Forty two 8-week-old male Golden Syrian hamsters (Harlan Sprague Dawley, Inc., Indianapolis, IN), each weighing ~100 g, were housed 2 animals per cage in a temperature-controlled environment with a 12 h light/dark cycle. After a 1-week adaptation period on a standard rodent diet AIN-93M (the diet of the American Institute of Nutrition for maintenance of mature rodents), 6 animals were sacrificed after the overnight fasting (~16 hrs), and the other 36 animals were randomly assigned to three groups (n=12) and placed on experimental diets for 6 weeks. The experimental diets were custom prepared by Research Diets Inc. (New Brunswick, NJ) and represent the modified formulas of the diet AIN-93M. The modifications included an increased fat content at the expense of the carbohydrate content (on a cal/cal basis), and a different fat composition (Supplementary Table 1). All diets also contained cholesterol in the amount (0.025%) which is non-atherogenic for hamsters and equivalent to human intake of <300 mg/day [22]. Palm oil, olive oil, safflower oil, and pharmaceutical grade fish oil ROPUFA 75 N-3 EE (DSM Nutritional Products, Parsippany, NJ) were the major sources of SFAs, MUFAs, n-6 and n-3 PUFAs, respectively. ROPUFA 75 N-3 EE contained 75% of n-3 PUFAs in the form of ethyl esters with the EPA to DHA ratio equal to 2.1:1. A weekly supply of each diet was nitrogen purged and vacuum packed in foil-lined bags. Unopened and opened bags were stored at -80°C. Food and fresh water were provided *ad libitum*, body weight was monitored weekly, and food intake daily at the end of the dark period. While all dietary groups had unrestricted access to food and always had some food left before a new portion of food was given to them, the total food consumption was reduced by 8% in group 3 fed the highest fish oil diet. This reduced food consumption led to a decreased by ~20% gain in the body weight in this group, however, did not seem to affect animal behavior or cause visible signs of malnutrition. After six weeks on custom diets, hamsters were deprived from food, and ~16 hrs later sacrificed in a chamber containing carbon dioxide. Blood was withdrawn immediately via cardiac puncture. Internal organs were excised, rinsed in cold 0.9% NaCl, blotted, cut into pieces, weighed, and flash-frozen in liquid nitrogen. All tissues were stored at -80°C. The research was conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and approved by the University of Texas Medical Branch at Galveston Institutional Animal Care and Use Committee (protocol # 0707030).

2.2. Serum lipid analysis

Serum was obtained by placing the blood samples at 25°C for 30-60 min to allow the clot formation followed by centrifugation at 1,000 g for 15 min to separate the serum. T-C, HDL cholesterol (HDL-C), LDL-C and triglycerides (TG) in the serum were measured in triplicate using commercial enzymatic kits (Stanbio Laboratory, Boerne, TX).

2.3. Cloning of the hamster cDNAs for CYPs 7A1, 46A1, and 27A1

Total RNA was isolated using the RNAqueous Kit (Ambion, Austin, TX) from the stored frozen samples of the hamster brain cortex, liver and eyeballs that were placed in the RNAlater solution (Ambion, Austin, TX) immediately after the organ excision. The quality of the RNA samples was assessed by the analysis on an RNA Nano chip using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Synthesis of cDNA was performed using 1 µg of

total RNA and the RetroScript Kit (Ambion, Austin, TX) under the conditions recommended by the manufacturer. To clone CYP7A1, the forward primer was 5'-AGATCTATGATGACCATCTCTTTGATTTGGGG-3' and reverse primer was 5'-AAGCTTCACAGATGTTTCAGTTTATATTTAACTC-3'. These primers were designed based on the available hamster CYP7A1 sequence (the GenBank accession number L04690). PCR was performed using 2 µl of hamster liver cDNA and the Fail Safe PCR System (Epicentre Technologies, Madison, WI) Buffer E. The target DNA was amplified using 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 6 minutes. The 1514 bp product was cloned into a transcription plasmid (T7PA) at the BglII and HindII sites and confirmed by DNA sequence analysis. To clone CYP46A1, the forward primer was 5'-ATGAGCCCCGGGCTGCTGCTG-3' and reverse primer was 5'-TCAGCAGGGTGGGGGTGGGG-3'. These primers were designed based on the mouse CYP46A1 sequence (the GenBank accession number NM_010010). PCR was performed using 2 µl of hamster cortex cDNA and Fail Safe Buffer D. The conditions of the amplification were 40 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 6 minutes. The 1503 bp product was cloned using the TA Topo Kit (Invitrogen) and confirmed by sequence analysis. To clone CYP27A1, the forward primer was 5'-ATGGCTGCGTGGAGCCGCACG-3' and reverse primer was 5'-ATGATCCGGGAGTTTGTGGG-3'. These primers were designed based on the mouse CYP27A1 sequence (the GenBank accession number NM_024264). PCR was performed using 2 µl of hamster liver cDNA and Fail Safe Buffer D. The conditions of the amplification were 40 cycles of 94°C for 30 seconds, 48°C for 30 seconds, and 72°C for 5 minutes. The 1230 bp product was cloned using the TA Topo Kit (Invitrogen) and confirmed by sequence analysis. The partial sequences for hamster CYP46A1 and 27A1 have been submitted to the GenBank (accession numbers FJ915127 and FJ915128, respectively).

2.4. Quantitative Real Time PCRs (RT-QPCR)

Synthesis of cDNA was performed in a 20 µl reaction using 1 µg of total RNA and the reagents in the Taqman Reverse Transcription Reagents Kit (Applied Biosystems Inc., Foster City, CA). The reaction conditions were as follows: 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. QPCR amplifications (performed in triplicate) were carried out using 2 µl of cDNA, SYBR green primers (see below) and the SYBR Green PCR Master Mix (Applied Biosystems Inc.) as specified by the manufacturer. The reaction volume was 25 µl and the final concentration of the primers was 900 nM. Relative RT-QPCR assays were performed with 18S RNA as a normalizer, whereas the absolute RT-QPCR was based on the known amounts of a synthetic transcript of the CYP7A1 gene. All PCR assays were run in the ABI Prism 7000 Sequence Detection System. The conditions of the amplification were 50°C for 2 min, 95°C for 10 min and then 40 cycles of 95°C for 15 sec, and 60°C for 1 min. The SYBR primers for hamster CYP7A1 were 5'-TACTAGATAGCATCATCAAGGAGGCTC-3' (forward) and 5'-CCATCCTCAAGGTGCAGAGTG-3' (reverse). The SYBR primers for hamster CYP46A1 were 5'-GGTCACGAGACTTCTGCCAAC (forward) and 5'-AACCAACAACCTCATCCACCTC (reverse). The SYBR primers for hamster CYP27A1 were 5'-GGAGCAGTGAAGGATCACC (forward) and 5'-TGTTCAAAGCCTGGCGC (reverse). Relative mRNA level changes were calculated by the $2^{-\Delta\Delta C_t}$ method [32].

2.5. Preparation of the liver microsomes and mitochondria

Frozen liver samples (~5 g) were thawed, sliced and homogenized in 20 ml of homogenization buffer by Dounce homogenizer and Teflon pestle. The homogenization buffer contained 50 mM potassium phosphate buffer, pH 7.4, 20% glycerol, 1 mM EDTA, 0.2 mM dithiothreitol, complete EDTA-free Protease inhibitor Cocktail (2 tablets per 100 ml of buffer, Hoffman-La Roche Inc, Nutley, NJ), 1 mM sodium fluoride and 1 mM activated sodium orthovanadate). The crude homogenate was subjected to centrifugation at 600 g for 10 min at 4°C. The supernatant was collected and kept in a separate tube on ice. The pellet was resuspended in 10

ml of homogenization buffer and spun down again at 600 g for 10 min. The two 600 g-supernatants were combined and subjected to centrifugation at 9,000 g for 20 min. The 9,000 g-supernatant was transferred to a separate tube, whereas the pellet was resuspended in 20 ml of homogenization buffer and spun down again. Pellets and supernatants after two 9,000 g-centrifugations were combined. The pellet represented the mitochondrial fraction and supernatant used for isolation of the microsomal fraction by a 100,000 g centrifugation for 1 hr.

2.6. Quantification of CYP7A1 protein in the liver

Proteins from the microsomal fraction were separated by SDS-PAGE and transferred to a PVDF membrane. Western blot analysis was carried out using goat polyclonal antibodies against human CYP7A1 (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:100 dilution and the fluorescently-labeled donkey anti-goat IRDye 680 secondary antibodies (LI-COR Biosciences, Lincoln, NE) at a 1:5,000 dilution. Quantification of fluorescence was carried out using the ODYSSEY Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Protein expression of CYP7A1 was estimated based on the fluorescence intensity of the known amounts of purified recombinant human CYP7A1. The amino acid sequence identity between human and hamster CYP7A1 is 82% [33].

2.7. Quantification of CYP46A1 protein in the brain

Total brain protein (20 µg) was separated by SDS-PAGE and transferred to a nitrocellulose membrane followed by the incubation with the rabbit polyclonal antibodies against human CYP46A1 (a generous gift from Dr. D Russell, UT Southwestern) and sheep anti-rabbit immunoglobulins conjugated with horseradish peroxidase. CYP46A1 and beta actin (used as a loading control) were detected by using the SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL). Chemiluminescent signals from both proteins were recorded and quantified by the Bio-Rad Universal hood II (Bio-Rad Laboratories, Hercules, CA), and the CYP46A1/actin ratio calculated.

2.8. Quantification of steroids

7 α -Hydroxy-4-cholesten-3-one and lathosterol in the serum were measured by liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS), respectively, as described [34,35]. 24-Hydroxycholesterol, 27-hydroxycholesterol, lathosterol, cholesterol and 7-ketocholesterol in the brain were measured by GC-MS [36,37].

3. Results

3.1. Serum lipids

The baseline levels of serum lipids were determined prior to testing the effect of diets. Measurements were carried out after a 1-week adaptation period during which the animals were fed regular rodent diet AIN-93M (Table 2). Mean T-C (103 mg/dL), LDL-C (61mg/dL), HDL-C (48 mg/dL), and TG (175 mg/dL) were at or around the levels suggested as desirable in humans by the AHA (<200 mg/dL T-C, <100 mg/dL LDL-C, >40 mg/dL HDL-C, and <150 mg/dL TG) (<http://www.americanheart.org/presenter.jhtml?identifier=183>). Switching to the “human”-like diets 1-3 led to an increase in T-C, HDL-C and TG and a change in the LDL-C to HDL-C ratio. This result is consistent with the previous findings by others showing that unlike rats and mice, which are highly resistant to the effects of dietary cholesterol, hamsters are moderately responsive [27], and thus represent a better model for nutritional studies. The AIN-93M diet was cholesterol-free and only 9% of calories were from fat, whereas diets 1-3 contained 0.025% cholesterol and fat provided 34% of calories (Supplementary Table 1). Changes in the levels of serum lipids were also observed between the diets. Increased intake

of the fish oil led to a statistically significant decrease in the levels of LDL-C and HDL-C in diet 3.

3.2. Expression of genes for CYPs 7A1, 27A1, and 46A1 (*cyp7a1*, *cyp27a1*, and *cyp46a1*)

Expression of *cyp7A1* was first investigated by the relative RT-QPCR and then to confirm the data by the absolute RT-QPCR. *Cyp27a1* and *cyp46A1* were analyzed by the relative RT-QPCR only. To design good primers and obtain reliable results, we cloned all three hamster genes (*cyp7a1*, *cyp27a1* and *cyp46A1*). As assessed by mRNA levels, *cyp7A1* showed a significant, from ~ 17- to 44-fold, intergroup variability, *cyp27a1* a moderate, 4-6-fold, intergroup variability, and *cyp46a1* a low, only 2-3-fold, intergroup variability (Table 3). Because of this high variability, a decrease in *cyp7a1* in groups 2 and 3 was not statistically significant. *Cyps 27a1* and *46a1*, however, did show a statistically significant increase in group 3 in the liver and brain, respectively.

Since CYPs 27A1 and 46A1 are also expressed in the retina [38-40], and dietary supplementation with n-3 PUFAs is currently under investigation in the Age-Related Eye Disease Study 2 (<http://www.clinicaltrials.gov/ct2/results?term=NCT00345176>), we measured *cyp27a1* and *cyp46a1* levels in the eyeball as well. In these experiments we combined three eyeballs, each from a different animal, to obtain one mRNA sample, and, thus, had four mRNA samples per group. No differences in abundance of *cyp27a1* and *cyp46a1* in the eyeball were found between the three dietary groups.

3.3. Protein expression of CYP7A1

To assess how mRNA levels correlate with protein expression, we tested three animals in each group (Table 4). Overall, in each group there was a correlation between the CYP7A1 mRNA and protein levels but not in all cases this correlation was quantitative.

3.4. Levels of sterol markers for cholesterol synthesis and degradation in the liver

Steady state levels of cholesterol in the liver as well as in any organ reflect a balance between the pathways of cholesterol accumulation and elimination. Since the liver is the major organ where cholesterol degradation takes place, and CYP7A1 is the key enzyme in this process, we assessed how diets affected hepatic cholesterol accumulation and elimination. Serum levels of cholesterol precursor lathosterol, an established marker for cholesterol biosynthesis in the liver [35], and of cholesterol degradation product 7 α -hydroxy-4-cholesten-3-one, a suitable plasma marker for the activity of CYP7A1 and bile acid synthesis [34,41-44] were determined (Table 5). We did not measure the CYP7A1 product 7 α -hydroxycholesterol because this sterol may be formed non-enzymatically from cholesterol, and this non-enzymatic formation increases with the storage time of plasma [45,46]. Both pooled and several individual serum samples were used. Of the three diets tested, a pooled serum sample from animals on diet 2 had the lowest levels of lathosterol, and the pooled sample from hamsters on diet 3 had the highest serum lathosterol. Likewise, the ratio between serum lathosterol and cholesterol, used as an indicator of cholesterol biosynthesis as well [47], was the lowest in the pooled sample in group 2, and the highest in group 3. Variations of lathosterol in the individual serum samples were similar in the three groups and did not exceed a 2.2-fold range (not shown). Levels of 7 α -hydroxy-4-cholesten-3-one were similar in diets 1 and 2 and lower in diet 3. This sterol showed a significant variability within each group, up to ~11-fold, yet this variability was not as high as the variations in the CYP7A1 mRNA. CYP7A1 mRNA is known to have a rapid turnover and short half-life of 2-3 hrs [48]. It is possible that not all of the mRNA is translated into protein and 7 α -hydroxy-4-cholesten-3-one.

3.5. Levels of sterol markers for cholesterol synthesis and degradation in the brain

Most of cholesterol in the brain is derived from local synthesis and eliminated primarily via 24S-cholesterol hydroxylation catalyzed by CYP46A1 [15,18,49]. Since diet 3 resulted a statistically significant increase in the cerebral CYP46A1 mRNA levels, we compared this diet to diet 1 for its effect on the brain markers for cholesterol synthesis and degradation as well as on CYP46A1 protein expression and serum 24S-hydroxycholesterol, which is also used as marker for CYP46A1 activity in the brain. Of the three sterols measured in the brain, lathosterol, 24S-hydroxycholesterol, and cholesterol, the former showed the highest intergroup variability (up to 6.7-fold), 24S-hydroxycholesterol varied up to 2.1-fold and cholesterol only up to 1.5-fold (not shown). Increased fish oil content in diet 3 seemed to slightly increase cholesterol biosynthesis in the brain as reflected in the higher mean level of cholesterol precursor lathosterol (Table 6). This difference was not statistically significant, nevertheless, there appeared to be a compensatory increase in cholesterol elimination from the brain as indicated by the slightly elevated levels of 24S-hydroxycholesterol (Table 6). This difference was not statistically significant, and this was the case also with the slightly increased ratio between 24S-hydroxycholesterol and lathosterol in group 3 (4.65 vs 4.48). A tendency to increased cholesterol biosynthesis and elimination remained when sterol levels were normalized to cholesterol content (Fig. 1). CYP46A1 protein expression in the brain was assessed by comparing the signals from CYP46A1 and actin on Western blots. The CYP46A1/actin ratio was slightly increased in diet 3 but this increase was not statistically significant and was not translated in increased serum 24S-hydroxycholesterol levels, which were the same in the diets (Table 6). Thus, diet 3 had an effect on the CYP46A1 mRNA levels in the brain and a trend to the increased cerebral cholesterol turnover, yet changes in the levels of either CYP46A1 protein or sterol markers were not significant.

Ubiquitous CYP27A1 is also expressed in the brain, and its product, 27-hydroxycholesterol, is a stable compound. We determined brain levels of 27-hydroxycholesterol (Table 6). In both dietary groups they were more than 170-times lower than those of 24S-hydroxycholesterol. Thus, similar to humans and mice [16,18], 24S-hydroxylation is the major route for enzymatic cholesterol elimination from the brain in hamsters. The mean levels and the 27-hydroxycholesterol to cholesterol ratio were slightly higher in group 3 than in group 1 (Table 6, Fig. 1). The intergroup variability in 27-hydroxycholesterol in the brain was up to 2-fold (not shown).

Increased n-3 PUFAs consumption was positively associated with increased oxidative stress in several studies (reviewed in [50]). Therefore, we measured 7-ketocholesterol, a toxic oxysterol, formed non-enzymatically from cholesterol. There was only a slight increase in this oxysterol level in group 3.

4. Discussion

Fish oil has been shown to have a beneficial effect on many cardiovascular risk factors and is also known to decrease TG levels in humans [51,52]. Changes in other serum lipids are less consistent [53], although many studies show a small non-significant net increase in TC, LDL-C, and HDL-C (<http://www.ahrq.gov/clinic/tp/o3cardrisktp.htm>). Much of the data variability on serum lipids in human studies is attributed to confounding contributions of dietary cholesterol and fat, fatty acid composition of the diet and genetic background [54-57]. To minimize the effect of these confounders in our study, we carried out experiments on animals and carefully controlled for a number of parameters. Animals were healthy and of about the same age, sex and weight; they lived in a very controlled environment and were fed exactly the same diets respective of their dietary group. Further, we used hamsters that represent a better animal model for the evaluation of the effect of dietary cholesterol and n-3 PUFAs than

mice and rats. Also, unlike previous animal studies, in which n-3 PUFAs were added to the standard rodent chow [26-29], we used “human-like” diets.

Hamsters on diet 3 had a statistically significant decrease in LDL-C and HDL-C. This effect was the opposite to that seen in many, but not all, human trials; both lipoproteins were significantly lower in hamsters on diet 3 containing the highest amount of n-3 PUFAs but the LDL-C/HDL-C ratio did not seem to change in the three test groups. We also observed a very marked intra- and intergroup variability of CYP7A1, and this variability was present at all levels; mRNA, protein and enzyme activity. This result is consistent with the previous findings in humans demonstrating that CYP7A1 activity vary significantly, over a 5-10- fold range in healthy individuals [45,58-60]. It is likely effects on *cyp7a1* transcription that determine different mRNA levels and consequently enzyme activity. In hamsters and humans, the *cyp7a1* gene transcription is not sensitive to cholesterol but is suppressed by bile acids [61-64]. Transcription factors that negatively regulate *cyp7a1* (the farnesoid X receptor, fibroblast growth factors 4 and 15 and a protein β Klotho [61]) are responsive to different stimuli, and the multitude of such stimuli may explain interindividual variability in the human gene expression as well as our data.

Despite the carefully controlled conditions in our study, hamsters still had a marked intergroup variability suggesting that factors other than age, sex, health status and diet affect CYP7A1 transcription. One such factor could be starvation or individual differences in the time of the last food intake. Starvation was reported to markedly decrease mRNA, protein and enzyme activity levels of CYP7A1 in rats [48,65], and affect animal's response to the estradiol treatment [65]. Another factor could be genetic differences because humans are known to carry several polymorphisms in *CYP7A1* [8,66,67]. We did not investigate whether hamster *cyp7a1* is polymorphic as well. However, even in the absence of this information, it becomes clear that characterization of the effects of n-3 PUFAs in humans should be carried out on the individuals that have the same CYP7A1 genotype and similar basal levels of CYP7A1 activity with the latter serving as an indicator of the lifestyle and dietary preference. The combination of these two factors will likely determine the individual's response to n-3 PUFAs.

The levels of CYP27A1 mRNA in the liver varied considerably less than those of CYP7A1 and showed a statistically significant increase in hamsters fed the diet with the highest amounts of n 3-PUFA. This increase was, however, reflected in only slightly higher levels of 27-hydroxycholesterol in the brain. A higher activity of CYP27A1 would be expected to lead to a higher rate of cholesterol elimination via the alternative pathway of bile acid formation. Upregulation of CYP27A1 also leads to the increased metabolism of 24S-hydroxycholesterol with decreasing levels of this oxysterol in the circulation and liver [68].

The marked variability in expression of hepatic enzymes CYP7A1 and CYP27A1 is in contrast to the stable expression of cerebral CYP46A1. The promoter region of *CYP46A1* bears the classical hallmarks of a gene with putative housekeeping function. Only oxidative stress was initially shown to cause a significant increase in *CYP46A1* transcription. [69]. Subsequent work implicating the SP family of transcription factors in the regulation of CYP46A1 expression has provided a possible mechanistic explanation for this observation [70], as assembly of functional SP oligomers is known to be regulated by oxidative stress. Very recently evidence was obtained that epigenetic mechanisms may also be important for the expression of CYP46A1 [71]. In our study we detected a clear and statistically significant effect of n-3 PUFAs on the P450 mRNA levels in the brain of hamsters in group 3. This effect is of interest in relation to a previous failure to demonstrate enzyme regulation at the transcriptional level by a number of hormonal and dietary factors [69]. The protein and 24S-hydroxycholesterol levels in group 3, however, were increased non-significantly, and serum 24S-hydroxycholesterol levels were unchanged. It is possible that there is a time lag between the mRNA and protein

syntheses and consequently the production of 24S-hydroxycholesterol. Brain cholesterol biosynthesis was also increased non-significantly in group 3, and cholesterol levels were slightly lower.

Studies in mice indicate that in the brain cholesterol biosynthesis and elimination are tightly coupled. Mice lacking CYP46A1 (CYP46A1 knockout mice) have a decreased cholesterol elimination from the brain and a compensatory decrease in cholesterol biosynthesis [18]. The steady state cerebral cholesterol levels in CYP46A1 knockout mice remain the same as in wild type animals but cholesterol turnover is decreased leading to deficiencies in memory and learning [18]. The consequences of the increased cholesterol elimination from the brain were investigated in a recent gene therapy study [20]. The *cyp46A1*-treated animals, which represent mouse models of Alzheimer's disease, had increased brain content of 24S-hydroxycholesterol but unchanged cholesterol levels suggesting increased cerebral cholesterol biosynthesis and turnover. These animals showed improved cognitive performance and also had a reduced amyloid plaque formation [20]. Thus, although we could not demonstrate a statistical increase in the cerebral cholesterol turnover in hamsters on diet 3, the observed changes are in the direction expected for the increased CYP46A1 mRNA level and consistent with the results of studies that have found association between higher intakes of oily fish or n-3 PUFAs and reduced risk of dementia [72-77]. It should be noted that our data does not exclude a possible difference at the $\pm$ 10-20% level.

In summary, our nutritional studies in hamsters demonstrate marked interindividual variations in *cyp7a1* gene transcription, CYP7A1 protein levels and enzyme activity. We suggest that such high variations may underlie the differential effect of n-3 PUFAs on serum cholesterol and inconsistent results of human trials. Much less variations were seen in *cyp46a1* expression and we show that increased dietary intake of n-3 PUFAs may lead to a stimulation of cholesterol turnover in the brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Effect of the highest fish oil diet (diet 3) on the ratios of lathosterol to cholesterol (A), 24S-hydroxycholesterol to cholesterol (B), and 27-hydroxycholesterol to cholesterol (C) in the hamster brain.

Table 1
Fatty acid composition of the standard rodent diet AIM-93 and diets tested in the present study

Diet	Total fat, % en	SFAs, % en	MUFAs, % en	PUFAs, % en		n-3 PUFAs composition, % en		Human equivalent of (EPA+DHA), (g/day)	
				n-6	n-3	n-6/n-3	ALA		
AIM-93	9.3	1.3	2.3	5.0	0.7	7.1	0.7	0	0
1	34	13.4	13.6	6.2	0.63	9.8	0.57	0.06	≤ 0.1
2	34	13.4	13.6	5.9	1.0	5.9	0.70	0.26	0.55
3	34	13.2	13.6	5.8	1.2	4.8	0.65	0.53	1.1

Table 2

Effect of diets on hamster serum lipids^a

Diet	T-C (mg/dl)	LDL-C (mg/dl)	HDL-C (mg/dl)	LDL-C/HDL-C	TG (mg/dl)
AIN-93M ^b	103 ± 8	61 ± 11	48 ± 7	1.3	175 ± 17
1	184 ± 24	78 ± 24	122 ± 15	0.6	260 ± 58
2	165 ± 21	59 ± 16	112 ± 10	0.5	254 ± 78
3	171 ± 35	55 ± 25 ^c	106 ± 19 ^c	0.5	231 ± 64

^aData for 35 hamsters excluding hamster 9 in diet group 3. This hamster was excluded because it had the LDL-C value of 170, which was >3 SD away from the group mean, based on all 12 animals (mean = 65, SD = 45). Values in the Table are means ± SD for n=12 (diets 1 and 2) or n=11 (diet 3).

^bAnimals after a 1-week adaptation period on the AIN-93M diet.

^cSignificantly different from diet 1 at $p \leq 0.05$. Statistical analysis was performed using 1-factor ANOVA with pairwise comparisons using Tukey's HSD test.

Table 3
Effect of diets on mean, minimum and maximum mRNA levels of hamster CYPs 7A1, 27A1 and 46A1

Diet	Absolute CYP7A1 mRNA levels in the liver, # of copies, $\times 10^3$				Relative CYP27A1 mRNA levels in the liver				Relative CYP46A1 mRNA levels in the brain				Relative mRNA levels in the eyeball	
	Mean $\pm$ SD	Min.	Max		Mean $\pm$ SD	Min.	Max.		Mean $\pm$ SD	Min.	Max.		CYP27A1, mean $\pm$ SD	CYP46A1, mean $\pm$ SD
1 (n=10)	94 $\pm$ 97	16	282		1.0 $\pm$ 0.5	0.3	1.9		1.0 $\pm$ 0.2	0.8	1.6		1.0 $\pm$ 0.7	1.0 $\pm$ 0.2
2 (n=10)	27 $\pm$ 13	3	46		1.2 $\pm$ 0.5	0.6	2.3		1.1 $\pm$ 0.4 ^a	0.7	2.1		0.8 $\pm$ 0.2	1.2 $\pm$ 0.2
3 (n=11)	61 $\pm$ 49	4	179		1.6 $\pm$ 0.6 ^b	0.8	3.0		1.5 $\pm$ 0.4 ^{b,c}	1.0	2.9		0.8 $\pm$ 0.3	1.1 $\pm$ 0.3

^aSignificantly different from diet 3 at $p \leq 0.05$.

^bSignificantly different from diet 1 at $p \leq 0.05$.

^cSignificantly different from diet 2 at $p \leq 0.05$.

Table 4

A comparison of CYP7A1 mRNA levels in the hamster liver to the protein expression in the liver microsomes

Diet/Hamster	CYP7A1 mRNA ¹ , # of copies, $\times 10^3$	CYP7A1, pmol/mg of total microsomal protein ²
1/4	58	1.4
1/5	282	3.3
1/9	22	Undetectable
2/2	30	1.9
2/6	36	2.3
2/10	16	1.3
3/2	63	16.6
3/3	4	Undetectable
3/12	72	2.3

^{1, 2}The mRNA and protein levels were determined as described under Methods.

Table 5

Effect of diets on serum markers for cholesterol synthesis and degradation in the hamster liver

Diet	Serum lathosterol, $\mu\text{g/mL}$	Serum T-C, mg/dL	Lathosterol/T-C, $\mu\text{g/mg}$, $\times 10^{-4}$	Serum 7 α -hydroxy-4-cholestene-3-one, ng/mL
1	0.18 ^{a,b}	184 $\pm$ 24 ^c (n=12)	9.8	85 ^{a,b}
2	0.14 ^{a,b}	165 $\pm$ 21 ^c (n=12)	8.5	90 ^{a,b}
3	0.19 ^{a,b}	171 $\pm$ 35 ^c (n=12)	11.1	63 ^{a,b}

^a Average of duplicate determinations.^b Sterol content in a pooled sample.^c Mean $\pm$ SD of individual samples.

Table 6

Effect of the highest fish oil diet (diet 3) on sterol markers of cholesterol synthesis and degradation in the hamster brain, CYP46A1 protein expression and serum 24-hydroxycholesterol^a

Diet	Lathosterol, ng/mg tissue	24S-Hydroxy-cholesterol, ng/mg tissue	Cholesterol, µg/mg tissue	Serum 24S-hydroxy-cholesterol, ng/ml	CYP46A1/actin ratio	27-Hydroxy-cholesterol, ng/mg tissue	7-Keto-cholesterol, ng/mg tissue
1	10.5 ± 1.8	47.0 ± 2.8	15.0 ± 0.6	24.0 ± 2.2	0.18 ± 0.05	0.13 ± 0.06	0.71 ± 0.06
3	11.0 ± 1.8	51.2 ± 0.6	14.2 ± 0.3	23.9 ± 2.7	0.23 ± 0.07	0.14 ± 0.09	0.74 ± 0.04

^aThe results are means ± SEM of individual samples (n=12 for each diet).