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## Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce $\beta$ -oxidation in white fat

Received: 10 March 2005 / Accepted: 13 June 2005 / Published online: 5 October 2005  
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**Abstract** *Aims/hypothesis:* Intake of *n*-3 polyunsaturated fatty acids reduces adipose tissue mass, preferentially in the abdomen. The more pronounced effect of marine-derived eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids on adiposity, compared with their precursor  $\alpha$ -linolenic acid, may be mediated by changes in gene expression and metabolism in white fat. *Methods:* The effects of EPA/DHA concentrate (6% EPA, 51% DHA) admixed to form two types of high-fat diet were studied in C57BL/6J mice. Oligonucleotide microarrays, cDNA PCR subtraction and quantitative real-time RT-PCR were used to characterise gene expression. Mitochondrial proteins were quantified using immunoblots. Fatty acid oxidation and synthesis were measured in adipose tissue fragments. *Results:* Expression screens revealed upregulation of genes for mitochondrial proteins, predominantly in epididymal fat when EPA/DHA

concentrate was admixed to a semisynthetic high-fat diet rich in  $\alpha$ -linolenic acid. This was associated with a three-fold stimulation of the expression of genes encoding regulatory factors for mitochondrial biogenesis and oxidative metabolism (peroxisome proliferator-activated receptor gamma coactivator 1 alpha [*Pparg1a*, also known as *Pgc1 $\alpha$* ] and nuclear respiratory factor-1 [*Nrf1*] respectively). Expression of genes for carnitine palmitoyltransferase 1A and fatty acid oxidation was increased in epididymal but not subcutaneous fat. In the former depot, lipogenesis was depressed. Similar changes in adipose gene expression were detected after replacement of as little as 15% of lipids in the composite high-fat diet with EPA/DHA concentrate, while the development of obesity was reduced. The expression of *Pparg1a* and *Nrf1* was also stimulated by *n*-3 polyunsaturated fatty acids in 3T3-L1 cells. *Conclusions/interpretation:* The anti-adipogenic effect of EPA/DHA may involve a metabolic switch in adipocytes that includes enhancement of  $\beta$ -oxidation and upregulation of mitochondrial biogenesis.

**Electronic Supplementary Material** Supplementary material is available in the online version of this article at <http://dx.doi.org/10.1007/s00125-005-1944-7>

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**Keywords** Adipose tissue · Carnitine palmitoyltransferase 1 · Fish oil · Flax-seed oil · Metabolism · Mitochondria · *n*-3 Polyunsaturated fatty acids · PGC-1 · PPAR $\gamma$ 1A

**Abbreviations** ACOX1: acyl-Coenzyme A oxidase 1 · ALA:  $\alpha$ -linolenic acid · ATP5A1: subunit  $\alpha$  of F<sub>1</sub>-ATPase · AU: arbitrary units · cHF diet: composite high-fat diet based on chow · cHF-F1 and sHFf-F2: high-fat diets with partial replacement of lipids by fish oil concentrate · COX6: cytochrome c oxidase subunit VI · CPT1A: carnitine palmitoyltransferase 1A · DHA: docosahexaenoic acid · EPA: eicosapentaenoic acid · EPA/DHA concentrate: concentrate of *n*-3 polyunsaturated fatty acids from fish oil (6% EPA and 51% DHA) · ESM: Electronic Supplementary Material · MT-ATP6 and MT-ATP8: subunits 6 and 8 of mitochondrial ATPase · MT-CO1 and MT-CO3: mitochondrial cytochrome *c* oxidase subunits I and III · MT-ND1: subunit 1 of mitochondrial NADH dehydrogenase (complex I) · MT-ND4: subunit 4 of mitochondrial NADH dehydrogenase (complex I) · NRF1: nuclear respiratory factor-1 · PPAR: peroxisome

proliferative activated receptor · PPAR GC1A: PPAR $\gamma$  coactivator-1 $\alpha$  · PTP4A1: protein tyrosine phosphatase 4a1 · PUFA: polyunsaturated fatty acids · qRT-PCR: quantitative real-time RT-PCR · SCD1: stearyl-CoA desaturase 1 · SDHA: 70 kDa protein subunit of mitochondrial complex II · sHff diet: semisynthetic high-fat diet · UCP: uncoupling protein

## Introduction

Type 2 diabetes exhibits a much stronger dependence on obesity than other health consequences of excessive accumulation of body fat. One of the major causes of obesity is high intake of lipids [1]. Studies in rodents [2–7] have demonstrated that saturated fats facilitate adipose tissue deposition more effectively than polyunsaturated fatty acids (PUFA). The long-chain *n*-3 PUFA eicosapentaenoic acid (EPA; 20:5 *n*-3) and docosahexaenoic acid (DHA, 22:6 *n*-3), which are abundant in marine fish oil, are more potent in reducing adiposity than *n*-3 PUFA from plants, such as  $\alpha$ -linolenic acid (ALA, 18:3 *n*-3 [4, 8]). The intake of DHA appears to be particularly important [5, 8]. In mammals, ALA is a precursor of EPA and DHA, but is rapidly oxidised in the organism and its conversion to EPA and DHA is quite inefficient [6, 9, 10]. Hence, dietary fish oil supplement is a more effective source of tissue DHA than ALA [6, 10]. Also in humans, dietary fish oil may decrease body fat content [11]. Furthermore, EPA and DHA act as hypolipidaemics, exert prophylactic effects on cardiovascular disease and may improve insulin sensitivity (references, see [4, 9]). Therefore, current dietary recommendations favour a substantial decrease in the *n*-6: *n*-3 PUFA ratio [6, 9].

The effect of *n*-3 PUFA on fat deposition does not result from a reduction in food intake [2–6, 8, 11], but rather reflects metabolic changes in several tissues [6, 12], such as stimulation of lipid oxidation and inhibition of lipogenesis in liver (references, see [6, 12]), stimulation of fatty acid oxidation in muscle [13], as well as adaptive thermogenesis mediated by mitochondrial uncoupling protein 1 (UCP1) in brown fat [2, 14]. In humans, whole-body lipid oxidation was increased by dietary fish oil [11]. The anti-adipogenic effect of *n*-3 PUFA may be also mediated by changes occurring in white adipose tissue itself [5, 15]. Studies in rodents indicate a complex modulation of gene expression in white adipose tissue by *n*-3 PUFA [6, 12], especially in fat depots in the abdominal region, which are preferentially reduced [3, 5, 6, 8, 16]. The changes in gene expression suggest a decrease in lipogenesis and fatty acid release from adipocytes [5] and an enhancement of mitochondrial respiration [3] and glucose uptake [8] in these cells.

The main goals of this study were to investigate the effects of EPA and DHA, compared with ALA, on gene expression in white adipose tissue, and to test the hypothesis that these effects limit the development of obesity. Experiments were conducted in mice fed two types of high-fat diet, either a semisynthetic diet (sHff) or a composite diet based on chow (CHF; see [Materials and methods](#) and

[8]). The sHff diet is well defined and made it possible to study the separate effects of EPA and DHA, and ALA, respectively. Similar purified diets have been used in previous studies in rodents. While these diets do not promote obesity in mice [2–5, 8, 13, 14], the CHF diet does [8, 17]. We show here that replacement of some of the dietary lipids with EPA and DHA induced expression of mitochondrial genes in white adipose tissue, as well as the gene encoding their upstream regulatory factor, PPAR $\gamma$  coactivator-1 $\alpha$  (PPARGC1A, also known as PGC1 $\alpha$ ). This factor links nuclear receptors to the transcriptional program of mitochondrial biogenesis and oxidative metabolism in adipocytes and muscle cells [18–20] and to gluconeogenesis in liver [21]. We also demonstrate that the expression of the gene for carnitine palmitoyltransferase 1A (*Cpt1a*) and oxidation of fatty acids was stimulated in epididymal but not subcutaneous white adipose tissue. Our data suggest that upregulation of mitochondrial biogenesis and induction of  $\beta$ -oxidation, as well as suppression of lipogenesis in adipocytes, are involved in the preferential reduction of lipid accumulation by EPA and DHA in abdominal fat.

## Materials and methods

### Animals and diet

Male C57BL/6J mice were imported from the Jackson Laboratory (Bar Harbor, ME, USA) and bred at the Institute of Physiology for up to 16 generations. Male mice were used for the experiments. Animals were housed in a controlled environment (20°C; 12-h light–12-h dark cycle; light from 06.00 h) with free access to water and standard chow [17]. At 4 months of age, singly caged animals were randomly assigned for 4 weeks to one of the high-fat diets described previously (see Tables 1, 2, 3 of Ref. [8] and the Electronic Supplementary Material [ESM] Tables 1 and 2); the sHff diet, which contained 20% (wt/wt) flax-seed oil (rich in ALA) as the only lipid; or the sHff-F2 diet, which had the same composition as the preceding diet except that 44% of lipids were replaced by *n*-3 PUFA concentrate containing 6% EPA and 51% DHA (EPAX 1050TG; Pronova Biocare, Lysaker, Norway; EPA/DHA concentrate). Some 4-month-old animals were also habituated for 2 weeks to the CHF diet, derived from standard chow and containing 35% (wt/wt) lipids of very low *n*-3 PUFA content. These were then assigned for 5 weeks to the CHF diet or to the CHF-F1 diet, which had the same composition as the CHF diet except that 15% (wt/wt) of lipids were replaced with EPAX 1050TG [8]. Individual food intake was determined as before [8]. Mice were killed by cervical dislocation and subcutaneous dorsolumbar and epididymal white adipose tissue depots [8] were dissected. Tissues were either used immediately for measurement of fatty acid metabolism, or stored in liquid nitrogen for RNA analysis and immunoblotting. The experiments were conducted under the guidelines for the use and care of laboratory animals of the Institute of Physiology, Academy of Sciences of the Czech Republic.

**Table 1** Effects of EPA/DHA concentrate on body weight, fat depots and food consumption

	Diet			
	sHFf	sHFf-F2	cHF	cHF-F1
Body weight (g)				
Initial <sup>a</sup>	26.4±0.5	26.4±0.4	27.5±0.6	27.8±0.7
Final <sup>a</sup>	26.9±0.7	23.4±0.6 <sup>b</sup>	35.4±1.1	33.4±0.7
Change <sup>a</sup>	0.4±0.5	-2.9±0.8 <sup>b</sup>	7.75±0.7	6.25±0.6 <sup>c</sup>
Epididymal fat weight (mg)	481±38	326±24 <sup>b</sup>	2093±97	1741±122 <sup>c</sup>
Dorsolumbar fat weight (mg)	215±12	197±16	679±45	640±40
Food consumption <sup>a</sup> (kJ/day per animal)	71.2±6.2	70.8±5.1	65.5±3.1	66.3±4.2
<i>n</i>	12	12	11	12

Data are means±SEM

Mice were fed semisynthetic diets (sHFf or sHFf-F2) and used for the analysis of gene expression in adipose tissue (Table 2, Fig. 2), or fed composite diets (cHF or cHF-F2) and used for the analysis of gene expression in collagenase-liberated adipocytes (Table 4)

<sup>a</sup>With respect to the whole period of dietary treatment

<sup>b</sup>Statistically significant difference compared with sHFf diet

<sup>c</sup>Statistically significant difference compared with cHF diet

### Differentiation of 3T3-L1 adipocytes

Eleven days after induction of cell differentiation [22] and 24 h before RNA isolation, a complete change of the medium was performed using serum-free Dulbecco's modified Eagle medium containing 0.5% fatty acid-free BSA, 830 nmol/l insulin and 0.5 mmol/l oleic acid in the form of BSA/fatty acid sodium salt. Medium was further supplemented with ethanolic solution of fatty acid: (1) 0.2 mmol/l oleic acid; (2) 0.2 mmol/l ALA; or (3) 0.2 mmol/l DHA, and incubated for 1 h before use.

### RNA isolation

Total RNA was isolated and purified using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol of the supplier with additional phenol/chloroform extraction.

### DNA and protein content

DNA was measured fluorometrically in tissue samples digested with proteinase K, and protein concentration was assessed using the bicinchoninic acid procedure and BSA as standard [23].

### cDNA PCR subtraction

RNA was isolated from epididymal fat pads pooled from six mice fed the sHFf or the sHFf-F2 diet. cDNA was generated using the SMART PCR cDNA Synthesis Kit (BD Biosciences, San Jose, CA, USA). To detect the genes up-regulated by the sHFf-F2 diet, PCR-based cDNA subtraction was carried out with the PCR-Select cDNA Subtraction

Kit (BD Biosciences). Subtracted cDNA libraries were subcloned (T/A cloning) into bacteria and analysed by colony array hybridisation using <sup>32</sup>P-labelled cDNA probes (DECaprime II DNA Labeling Kit; Ambion, Austin, TX, USA). Selected cDNAs were sequenced (DNA sequencer CEQ2000XL; Beckman Coulter, Harbor Boulevard, CA, USA) and DNA sequences were analysed using BLAST (GenBank, NIH).

### Oligonucleotide microarray

Each RNA sample isolated from dorsolumbar and epididymal fat of sHFf and sHFf-F2 mice (nine animals per group) was indirectly labelled [24] and processed separately. In the reverse transcription step, aminoallyl dUTP was incorporated and then chemically coupled to fluorophores. RNA samples (30 µg) were separately labelled with Cy5 dye. A common reference sample (a pool representing all the investigated samples) was also labelled with Cy3. High-density microarrays, based on commercial oligonucleotide library (Mouse 10k A set; MWG Biotech, Ebersberg bei München, Germany) were printed on epoxy slides (Quantifoil, Jena, Germany) using a MicroGrid II (Genomic Solutions, Ann Arbor, MI, USA), pretreated, hybridised overnight at 42°C in a humid hybridisation chamber, and read using a laser scanner (ScanArray Express; Perkin-Elmer, Boston, MA, USA). To correct for fluctuations in RNA sample size and the amount of DNA spotted, signals were normalised [25]. All Cy3 and Cy5 signals lower than 2.5-fold background value were excluded from the analysis (approximately 60% of spots). Identification of differentially expressed genes was done using discriminant analysis, which is a supervised form of principal components analysis (Genemath software; Applied Maths, Sint-Martens-Lathem, Belgium). Samples were first assigned to their diet intervention group, then separated along the dimension of maximum

**Table 2** Differential gene expression in mice fed sHFF and sHFF-F2 diets

Transcript	Accession number	Function	Epididymal fat		Dorsolumbar fat			
			cDNA subtraction <sup>a</sup>	Microarray <sup>b</sup> (fold change)	Microarray <sup>b</sup> (fold change)	qRT-PCR <sup>c</sup> (AU)	qRT-PCR <sup>c</sup> (AU)	
				sHFF diet	sHFF-F2 diet	sHFF diet	sHFF-F2 diet	
<b>Induction by sHFF-F2 diet</b>								
<i>mt-Col1</i>	J01420_3	OXPPOS		1.6 <sup>d</sup>	0.41±0.01	1.21±0.02 <sup>d</sup>	1.2	0.78±0.02
<i>mt-Co3</i>	AB042809_7	OXPPOS	Yes	1.8 <sup>d</sup>	0.79±0.02	1.44±0.02 <sup>d</sup>	1.7 <sup>d</sup>	0.63±0.02
<i>mt-Nd1</i>	AB049357_1	OXPPOS		1.8 <sup>d</sup>			1.7 <sup>d</sup>	
<i>mt-Nd4</i>	AB042809_10	OXPPOS		1.9 <sup>d</sup>			1.8 <sup>d</sup>	
<i>mt-Atp6</i>	AB042432_6	OXPPOS	Yes	1.7 <sup>d</sup>			1.8 <sup>d</sup>	
<i>mt-Atp8</i>	AB042432_5	OXPPOS		1.7 <sup>d</sup>			1.7 <sup>d</sup>	
<i>Adipsin</i>	NM_013459_1	Fatty acid intake		1.7 <sup>d</sup>	0.82±0.01	1.88±0.04 <sup>d</sup>	1.8 <sup>d</sup>	0.64±0.01
<i>Septin 6</i>	NM_019942_1	Cell division		2.1 <sup>d</sup>			1.4	
<i>mKiaa0195</i>	BC007157_1	Unknown		4.0 <sup>d</sup>			1.1	
<i>cPges</i>	AB024935	Prostaglandin metabolism	Yes		0.33±0.01	0.65±0.01 <sup>d</sup>		
<i>Ptp4a1</i>	AK078120	Cell differentiation	Yes		0.19±0.01	0.39±0.01 <sup>d</sup>		
<i>Arkadia</i>	AK036351	Cytokine signalling	Yes					
<i>Hgrg8</i>	AK031616	High glucose response	Yes					
<b>Depression by sHFF-F2 diet</b>								
<i>Actin beta</i>	NM_007393_1	Cytoskeleton		3.3 <sup>d</sup>	0.44±0.01	0.20±0.02 <sup>d</sup>	2.1 <sup>d</sup>	
<i>Scd1</i>	NM_009127_1	Lipogenesis		2.5 <sup>d</sup>	0.29±0.04	0.17±0.05 <sup>d</sup>	3.2 <sup>d</sup>	
<i>Dgat2</i>	NM_026384_1	Lipogenesis		2.2 <sup>d</sup>			1.0	
<i>Resistin like α</i>	NM_020509_1	Insulin sensitivity		2.5 <sup>d</sup>			1.8	
<i>Gnas</i>	NM_010309_1	Hormonesignalling		2.5 <sup>d</sup>			1.4	
<i>Npc2</i>	NM_023409_1	Lipid transport		2.5 <sup>d</sup>			2.5 <sup>d</sup>	
<i>P-lysozyme</i>	NM_013590_1	Non-specific immunity		2.5 <sup>d</sup>			2.4 <sup>d</sup>	
<i>Ubiquitin c</i>	NM_019639_1	Proteolysis		1.2			2.3 <sup>d</sup>	
<i>A730008L03Rik</i>	NM_021393	Unknown		0.9			3.1 <sup>d</sup>	

<sup>a</sup>The analysis was designed to detect genes upregulated by the sHFF-F2 diet

<sup>b</sup>The most discriminating genes with significantly different levels of expression in at least one of the tissues are listed (see Results and Materials and methods)

<sup>c</sup>Analysis was performed only where indicated; data are means±SEM ( $n=12$ )

<sup>d</sup>Significant effect of diet

AU arbitrary units; *mt-Atp6* and *mt-Atp8* subunits 6 and 8 of mitochondrial ATPase; *cPges* cytosolic prostaglandin E<sub>2</sub> synthase; *Gnas*α-subunits of stimulatory G-protein; *Hgrg8* high glucose-regulated protein 8; *mt-Nd1* and *mt-Nd4* subunits 1 and 4 of mitochondrial NADH dehydrogenase (complex I); *OXPPOS* oxidative phosphorylation; *Npc2* Niemann–Pick type C2 protein; *Ptp4a1* protein tyrosine phosphatase 4a1

See Results for other abbreviations. The animals described in Table 1 were used



**Table 3** Quantification of the protein components of the oxidative phosphorylation system in sHff and sHff-F2 diet-fed mice

Fat depot	Diet	SDHA	MT-CO1	COX6	ATP5A1
Epididymal fat	sHff	0.25±0.04	0.37±0.07	0.28±0.02	0.30±0.06
	sHff-F2	0.48±0.06 <sup>a</sup>	0.68±0.08 <sup>a</sup>	0.67±0.14 <sup>a</sup>	0.44±0.03 <sup>a</sup>
Dorsolumbar fat	sHff	0.42±0.04 <sup>b</sup>	0.34±0.06	0.43±0.08	0.33±0.07
	sHff-F2	0.46±0.07	0.32±0.07 <sup>b</sup>	0.38±0.07	0.39±0.07

Individual antigens were quantified in crude cell membranes isolated from adipose tissues using immunoblots and their content was expressed in AU using isolated mouse liver mitochondria as a standard (see Fig. 1)

Data are means±SEM ( $n=9$ ). The effect of diet in fat depots (two-way ANOVA) was different, except for ATP5A1

<sup>a</sup>Significant differences between diets

<sup>b</sup>Significant differences between fat depots

variation in gene expression between the groups and the 0.5% of genes that contributed most to the variation (the discriminating genes) were subsequently selected. Finally, from the selected genes only those genes were considered that exerted significantly different (*t*-test) levels of expression in at least one of the tissues analysed.

#### Quantitative real-time RT-PCR

This analysis (qRT-PCR) was performed using a PCR kit (QuantiTect SYBR Green; Qiagen, Hilden, Germany) and LightCycler (Hoffman-La Roche, Basel, Switzerland). All samples were treated with DNAase. Dilution of cDNA samples before PCR quantification of mitochondrially encoded genes was 100-fold higher compared with nuclear-encoded genes. Levels of all transcripts were correlated with that of the gene for cyclophilin- $\beta$  since quantity of this transcript is not affected by the dietary treatments [8] and is expressed in arbitrary units (AU). Lasergene software (DNASar, Madison, WI, USA) was used to design oligonucleotide primers (see ESM Table 3).

#### Quantification of mitochondrial proteins

Frozen tissues were immersed in a buffer containing 0.25 mol/l sucrose, 50 mmol/l Tris and 5 mmol/l EDTA (pH 7.5), and homogenised for 7 min on ice using a Teflon/glass homogeniser. Tissue fragments were removed by centrifugation for 10 min at 4°C and 600 g and crude cell membranes were separated from the supernatant by centrifugation for 35 min at 4°C and 100,000 g. Samples of the crude cell membranes were analysed using SDS-PAGE and immunoblots [26]. Polyclonal rabbit antibody [26] was used (1:5,000) to detect cytochrome *c* oxidase subunits I and VI (MT-CO1 and COX6). Monoclonal antibody was used to detect the 70 kDa subunit of complex II (SDHA; 1:2,000; Molecular Probes A-11142, Eugene, OR, USA) and subunit  $\alpha$  of F<sub>1</sub>-ATPase (ATP5A1; 1:200,000, lot 20D6 [27]). Peroxidase-labelled goat anti-mouse IgG (1:1,000, A8924; Sigma, St Louis, MO, USA) or goat anti-rabbit IgG (1:1,000, F0382; Sigma) was used as a secondary antibody. Antigens were visualised by luminescence and detected on a LAS 1000 CCD camera system (Fuji, Tokyo, Japan). Liver mitochondria isolated from chow diet-fed mice were

used as standard to quantify the relative content of each antigen.

#### Oxidation and synthesis of fatty acid in fragments of adipose tissue

Fatty acid oxidation was measured as the amount of radioactivity released into the medium from fragments (50 mg tissue chopped into about ten pieces using a razor blade) prelabelled with [9,10(*n*)-<sup>3</sup>H]oleic acid [28]. Synthesis of fatty acids was measured as before [22] except that the medium also contained 1 mM pyruvate and <sup>3</sup>H<sub>2</sub>O (18.5 MBq/ml) was used instead of radioactive glucose.

#### Statistics

The data were evaluated by one-way (diet) or two-way ANOVA with one grouping factor (diet) and one trial factor (depot). Logarithmic or square root transformation was used to stabilise variance in cells when necessary. The Spearman correlation coefficient was used to evaluate the relationship between transcript levels. The level of significance for all tests was set at  $p=0.05$ .

## Results

#### Screening for genes regulated by EPA and DHA in white adipose tissue

To identify the genes specifically regulated in white adipose tissue by marine-derived *n*-3 PUFA, mice were maintained for 4 weeks on the sHff diet, rich in ALA and free of DHA or EPA, while the other mice were fed a similar diet, except that 44% of dietary lipids were replaced by EPA/DHA concentrate (sHff-F2 diet). In accordance with previous results [8], animals fed the sHff diet maintained stable body weight during 4 weeks of the treatment, while in the sHff-F2 group the mean body weight declined by 11±2%. The weight of epididymal fat was 30±3% lower than in the sHff group (mean±SEM of four independent experiments,  $n=7-12$ ), while the weight of dorsolumbar fat did not change significantly. As observed before [8], food consumption was not affected (Table 1).

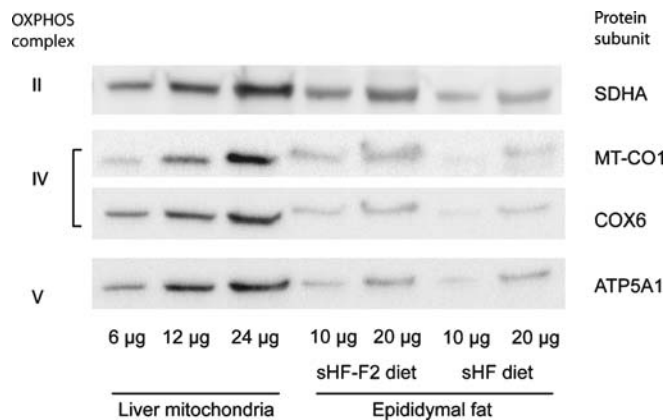
First, cDNA PCR subtraction was used to identify genes upregulated in the sHFF-F2-fed mice, starting from total RNA prepared from epididymal pads pooled from mice fed either the sHFF or the sHFF-F2 diet. In total, 52 clones were identified corresponding to 19 differentially expressed genes, six of them having known functions (Table 2): genes for cytochrome *c* oxidase subunit III (*mt-Co3*) and subunit 6 of ATPase (*mt-Atp6*), i.e. the key components of the mitochondrial oxidative phosphorylation system, encoded by mitochondrial DNA, and four nuclear genes engaged in regulatory pathways.

Secondly, oligonucleotide microarray analysis was applied to RNA samples isolated from both epididymal and dorsolumbar fat. Tissues from individual animals fed either sHFF or sHFF-F2 diet (Table 1) were processed and analysed separately. Only 0.5% of the genes most discriminating in both directions (see Materials and methods) between the sHFF and sHFF-F2 diets were considered (Table 2). In accordance with the results of cDNA PCR subtraction, the majority of the most upregulated genes in both fat depots of the sHFF-F2-fed mice belong to the mitochondrial genome and code for components of the oxidative phosphorylation system (complex I, cytochrome *c* oxidase and complex V, ATP synthase). Also, the nuclear gene for adipisin, the adipocyte hormone engaged in the control of fatty acid intake [29], was upregulated. Conversely, the most down-regulated genes in the sHFF-F2-fed animals were those supporting lipogenesis and encoding stearoyl-CoA desaturase (*Scd1* [30]), in both epididymal and subcutaneous fat depots, and acyl-CoA:diacylglycerol acyltransferase (*Dgat2*), in the former depot only (Table 2).

Thirdly, seven of the 18 differentially expressed genes were verified using qRT-PCR (Table 1). The differences in the expression of the gene for *mt-Co1* were more pronounced in the epididymal than in the dorsolumbar fat depot (Table 2).

#### Specific induction of mitochondrial proteins in epididymal fat by the sHFF-F2 diet

Subunits of the mitochondrial oxidative phosphorylation complexes were quantified in crude cell membranes prepared from white adipose tissue using immunoblots (Fig. 1, Table 3). In epididymal fat, levels of SDHA, MT-CO1, COX6 and ATP5A1 were elevated approximately 2-fold by the sHFF-F2 diet compared with the sHFF diet, resulting, in the case of MT-CO1, in higher levels in epididymal than in dorsolumbar fat in the sHFF-F2-fed animals. In the dorsolumbar fat, no effect of the diet on any of the analysed antigens was observed. In contrast to the differential effect of the sHF-F2 diet on the quantity of SDHA and ATP5A1 in two fat depots, qRT-PCR analysis ( $n=7-9$ ) revealed significant induction of transcripts for these genes by sHF-F2 diet in both epididymal fat (*Sdha*,  $1.35 \pm 0.16$  vs  $2.71 \pm 0.35$  AU; *Atp5a1*,  $1.82 \pm 0.08$  vs  $3.97 \pm 0.75$  AU) and subcutaneous fat (*Sdha*,  $1.96 \pm 0.19$  vs  $2.75 \pm 0.26$  AU; *Atp5a1*,  $1.44 \pm 0.23$  vs  $3.31 \pm 0.54$  AU).

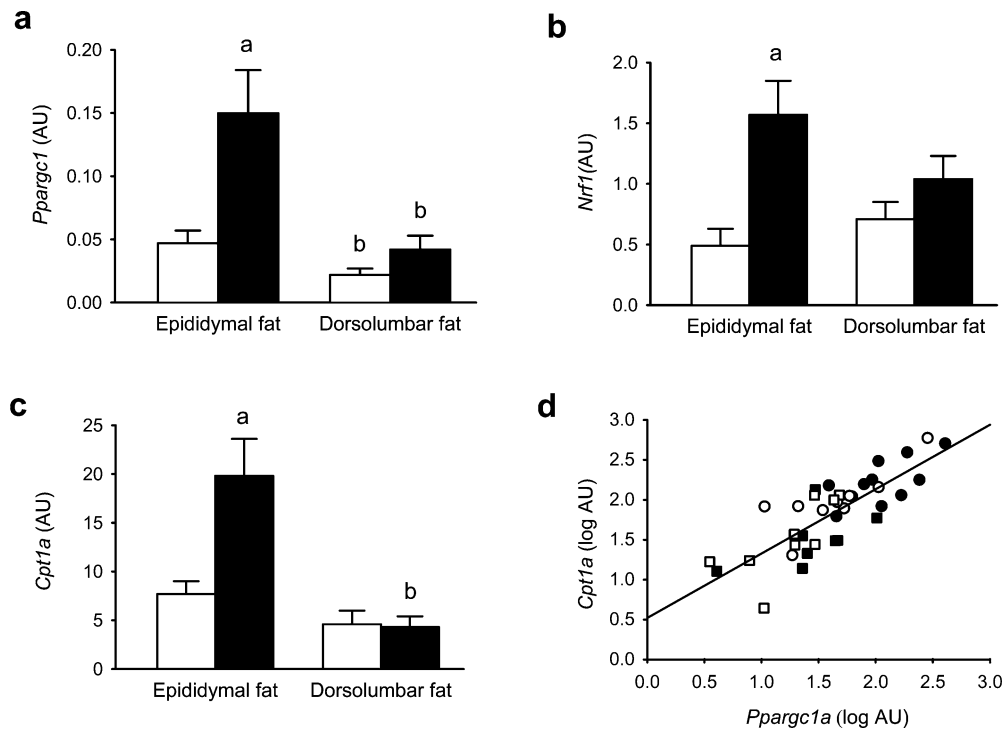


**Fig. 1** Immunoblotting of mitochondrial proteins in epididymal fat of the mice fed sHFF and mice fed sHFF-F2 diets. The analysis was performed using crude cell membranes isolated from adipose tissue. The amount of protein in each lane is indicated. Isolated mouse liver mitochondria were used as a standard (see Table 3)

#### Induction of *Ppargc1a*, *Nrf1*, *Cpt1a*, fatty acid oxidation and depression of fatty acid synthesis in white adipose tissue by the sHFF-F2 diet

We hypothesised that the induction of PPARGC1A, as well as NRF1 [18, 19], which is a downstream target for PPARGC1A and orchestrates mitochondrial biogenesis [19], is involved in the effect of EPA/DHA concentrate on white adipose tissue. qRT-PCR analysis revealed approximately 3.4-fold induction of both *Ppargc1a* and *Nrf1* transcripts in epididymal fat by the sHFF-F2 diet compared with the sHFF diet (Figs. 2a and b). Both genes tended to be upregulated also in dorsolumbar fat, but the effects were much smaller and not statistically significant. Also, CPT1, which is necessary for the translocation of fatty acid into mitochondria, represents a downstream target for PPARGC1A [31]. qRT-PCR analysis revealed that the liver isoform of the gene encoding CPT1 (*Cpt1a*) was upregulated by the sHFF-F2 diet in epididymal but not in dorsolumbar fat (Fig. 2c). Positive correlations between the expressions of genes encoding PPARGC1A and CPT1A within identical tissues and diets were observed, while correlations between the expression of genes encoding PPARGC1A and NRF1 were not statistically significant (Fig. 2d and legend). The upregulation of the gene for CPT1A suggests stimulation of fatty acid oxidation in the epididymal fat of the sHFF-F2 mice. Indeed, oxidation of oleate was 1.5- to 1.8-fold higher in epididymal fat of the sHFF-F2 compared with the sHFF mice, when the rate of oxidation was related either to tissue weight, protein, or DNA (Fig. 3). In dorsolumbar fat, similar rates were observed in both diet groups (Fig. 3). Fatty acid synthesis was lower in epididymal fat of sHFF-F2 than sHFF mice ( $83 \pm 7$  vs  $114 \pm 12$  dpm of  $^3\text{H}_2\text{O}$  incorporated into saponifiable fatty acids per mg of tissue DNA;  $n=7$ ).

Levels of acyl-CoA oxidase 1 (*Acox1*) mRNA, the marker of peroxisomal fatty acid oxidation, were significantly higher in mice fed the sHFF-F2 diet than in those fed the sHFF diet, in both epididymal ( $0.27 \pm 0.05$  vs  $0.14 \pm 0.02$  AU)



**Fig. 2** Gene expression in adipose tissue. **a–c** Using qRT-PCR, transcript levels were evaluated in total RNA isolated from adipose tissue depots of mice fed the sHFf diet (white bars) or the sHFf-F2 diet (black bars). For all transcripts, the effect of diet in fat depots was different (two-way ANOVA). Significant differences between diets; significant differences between fat depots. Data are means $\pm$ SEM ( $n=9–15$ ). **d** Correlation between *Ppargc1a* and *Cpt1a* mRNA

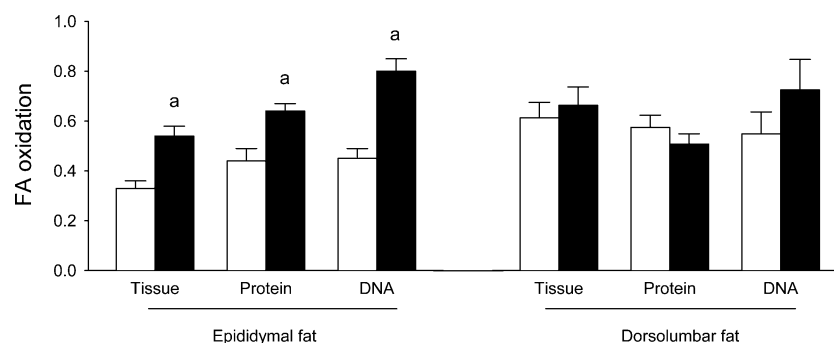
levels in epididymal fat of the mice fed sHFf (white circles) and sHFf-F2 (black circles) and in dorsolumbar fat of the mice fed sHFf (white squares) and sHFf-F2 (black squares). Spearman correlation coefficients for each set of data were 0.72–0.83. Corresponding values for the correlation between *Ppargc1a* and *Nrfl* transcripts were 0.38–0.62. Only the correlations between *Ppargc1a* and *Cpt1a* transcripts (**d**) were statistically significant ( $p<0.01$ )

and dorsolumbar ( $0.09\pm 0.02$  vs  $0.04\pm 0.01$  AU) adipose tissue. Thus, in contrast to the gene encoding CPT1A, similar stimulation of transcription of the gene for ACOX1 was observed in both fat depots of sHFf-F2 mice ( $n=13$ ).

In contrast to white adipose tissue, especially the epididymal fat, no significant changes in the expression of *Ppargc1a*, *Nrfl* and *Cpt1a* were observed in liver (not shown). Only the levels of *Acox1* mRNA were significantly higher in mice fed the sHFf-F2 than in those fed the sHFf diet ( $32\pm 1.5$  vs  $21\pm 1.3$  AU;  $n=5$ ).

Upregulation of the markers of mitochondrial biogenesis and  $\beta$ -oxidation and suppression of lipogenic genes in adipocytes from mice fed the composite diet cHF-F1

These experiments were designed to verify whether mitochondrial biogenesis and  $\beta$ -oxidation are stimulated when induction of obesity by the cHF diet is reduced by replacement of only 15% of dietary lipids by EPA/DHA concentrate (cHF-F1 diet). In accordance with our previous



**Fig. 3** Oxidation of oleate in fat depots of mice fed sHFf (white bars) or sHFf-F2 (black bars). Activity is expressed as pmol fatty acids $\cdot$ h $^{-1}\cdot$ mg $^{-1}$  tissue (*Tissue*), pmol fatty acids $\cdot$ h $^{-1}\cdot$ mg $^{-1}$  protein $\cdot$ 10 $^{-2}$  (*Protein*), or pmol fatty acids $\cdot$ h $^{-1}\cdot$  $\mu$ g $^{-1}$  DNA (*DNA*). There was a different effect of diet in fat depots (two-way ANOVA). Data are means $\pm$ SEM ( $n=9$ ). The tissue

DNA concentration was  $0.78\pm 0.08$  and  $0.76\pm 0.11$   $\mu$ g DNA/mg tissue in epididymal fat of sHFf and sHFf-F2 mice, respectively. Corresponding values for dorsolumbar fat were  $1.27\pm 0.16$  and  $1.05\pm 0.20$ . Significant difference between diets

**Table 4** Gene expression in adipocytes isolated from epididymal fat of mice fed cHF or cHF-F1 diet

Diet	Ppargc1a	mt-Co3	Cpt1a	Scd1	Ucp2	Ucp3
cHF	0.14±0.03	9.25±1.02	1.40±0.32	1.57±0.18	0.87±0.11	0.82±0.02
cHF-F1	0.51±0.09 <sup>a</sup>	12.81±0.64 <sup>a</sup>	2.32±0.61 <sup>a</sup>	0.40±0.08 <sup>a</sup>	0.40±0.09 <sup>a</sup>	1.01±0.03

Data are means±SEM ( $n=7$ )

<sup>a</sup>Significant difference between diets

study [8], a significant 22±7% reduction of body weight gain was observed in the cHF-F1-fed mice compared with the cHF-fed mice after 5 weeks of the treatment. The weight of epididymal fat in the cHF-F1-fed mice was 24±3% lower than in the cHF group (mean±SEM of three independent experiments,  $n=10-13$ ), while the weight of dorsolumbar fat was not significantly different. As observed before [8], food consumption was not affected (see Table 1 for data from a typical experiment).

In animals fed the cHF and cHF-F1 diets (Table 1), gene expression was studied using RNA prepared from the adipocytes isolated from epididymal fat (Table 4). Expression of the genes for PPARGC1A, MT-CO3 and CPT1A was enhanced by the cHF-F1 diet compared with the cHF diet, while the gene encoding SCD1 was down-regulated. Since PPARGC1A can induce the expression of the gene for mitochondrial UCP1 [18, 20] and marine-derived *n-3* PUFA have been shown to induce expression of this gene in brown fat [14], the *Ucp1* transcript was also measured. However, it was barely detectable (levels were at least four orders of magnitude lower than in interscapular brown fat) and no induction of the *Ucp1* transcript by cHF-F1 diet was observed (not shown). Levels of *Ucp2* transcript were lower in adipocytes from the cHF-F1-fed than the cHF-fed mice, while levels of *Ucp3* transcript were similar in the two groups of mice (Table 4).

#### Direct effect of *n-3* PUFA on adipocytes

3T3-L1 adipocytes differentiated in cell culture were incubated for 24 h with various fatty acids: oleate alone, oleate plus ALA, or oleate plus DHA, and *Ppargc1a* and *Nrf1* transcripts were quantified (Fig. 4). Compared with oleate, both ALA and DHA significantly increased the levels of both transcripts. The stimulation by DHA seemed to be more pronounced than that by ALA, but the dif-

ference was not statistically significant. Similar results were obtained in primary cultures of adipocytes (not shown).

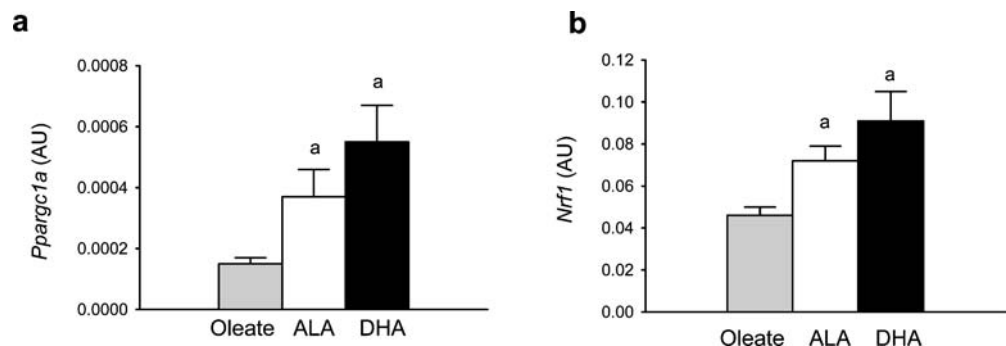
#### Discussion

The results of this study demonstrate the involvement of white adipose tissue, in addition to the liver, brown fat and other organs, in the mechanism whereby marine-derived *n-3* PUFA reduce adiposity and body weight. Our finding that EPA/DHA concentrate can specifically stimulate expression of *Ppargc1a* and *Cpt1a*, increase the content of mitochondrial proteins and induce  $\beta$ -oxidation in epididymal but not dorsolumbar fat is consistent with the preferential reduction of adipose tissue accumulation in the abdomen.

The idea that changes induced in white adipocytes by EPA and DHA could contribute to the reduction of obesity is strongly supported by the upregulation of genes encoding PPARGC1A and MT-CO3 and downregulation of the gene encoding SCD1 in adipocytes prepared from epididymal fat of the cHF-F1-fed mice. Under these conditions (mouse fed the cHF-F1 diet), only 15% of dietary fat was replaced by EPA/DHA concentrate, resulting in a substitution of about 10% of the fat for EPA/DHA. This led to a significant reduction in weight gain compared with mice fed the cHF diet.

Increased expression of *Nrf1* is consistent with the upregulation of genes for mitochondrial proteins by EPA/DHA. The majority of these genes (i.e. *mt-Co1*, *mt-Co3*, *mt-Nd1*, *mt-Nd4*, *mt-Atp6* and *mt-Atp8*) are encoded by mitochondrial DNA. However, immunoblotting analysis revealed that the biosynthesis of nuclear-encoded mitochondrial proteins (COX6, SDHA and ATP5A1) is also upregulated. Expression of genes encoding PPARGC1A and NRF1 is more stimulated in epididymal than in dorsolumbar

**Fig. 4** Effect of *n-3* PUFA on gene expression in 3T3-L1 cells. Postconfluent cells were treated for 24 h with oleate, oleate plus ALA, or oleate plus DHA (see Materials and methods), and transcripts were quantified using qRT-PCR. Data are means±SEM ( $n=12$ ). Significant differences versus oleate





fat, and striking fat depot-specific differences were observed in the effect of EPA/DHA on the expression of the gene encoding CPT1A, fatty acid oxidation activity and the quantity of oxidative phosphorylation proteins. In contrast, EPA/DHA induced transcripts for most of the mitochondrial proteins as well as the marker of peroxisomal fatty acid oxidation, *Acox1*, to a similar extent in both fat depots. These results suggest distinct post-transcriptional control in different fat depots of the expression of the genes for mitochondrial proteins and the involvement of mitochondria rather than peroxisomes in the stimulatory effect of EPA and DHA on fatty acid oxidation in epididymal fat.

Our in vitro experiments suggest that *n*-3 PUFA can modulate gene expression in white adipose tissue by direct interaction with adipocytes. PUFA of the *n*-3 series and their metabolites, eicosanoids [6], are ligands [32] of various isoforms of the peroxisome proliferator-activated receptor (PPAR [33–35]). All PPARs ( $\alpha$ ,  $\gamma$  and  $\delta$ ) physically interact with the gene encoding PPARGC1A [18, 34, 35], which is upregulated by EPA/DHA in adipocytes. A recent study indicates that treatment of *ob/ob* mice with rosiglitazone, a PPAR $\gamma$  ligand of the thiazolidinedione family and a widely used antidiabetic drug, increases mitochondrial mass, palmitate oxidation and the expression of genes encoding PPARGC1A, CPT1A and UCP1 in epididymal fat [36]. Except for the upregulation of the gene for UCP1, all these effects are similar to those elicited in white adipose tissue by EPA/DHA, suggesting that both *n*-3 PUFA and rosiglitazone induce similar metabolic changes in white adipose tissue cells, while increasing mitochondrial oxidative capacity and stimulating glucose uptake into adipocytes. However, in contrast to EPA/DHA, thiazolidinediones also support an adipogenic programme [36]. Surprisingly, rosiglitazone induces the accumulation of *n*-3 PUFA in white adipose tissue of diabetic mice [37]. Whether the mechanism of the effect of rosiglitazone depends on the accumulation of *n*-3 PUFA in adipocytes remains to be clarified.

Forced expression of the gene for PPARGC1A in both 3T3-F442A (references, see [18]) and human adipocytes [20] induces the conversion of adipocytes to energy-dissipating cells, including the recruitment of the gene for UCP1. Low levels of transgenic UCP1 in white adipose tissue may protect mice against obesity [17] while stimulating oxidation of fatty acids [28] and mitochondrial biogenesis [23], and depressing lipogenesis [22] in adipocytes. Also, leptin [38, 39],  $\beta_3$ -adrenergic agonists [40] and bezafibrate [41] have similar effects in rodents [38, 41], including induction of the genes for CPT1A [39, 41] and PPARGC1A [38] in white adipose tissue. Apparently, various stimuli can convert adipocytes into fat-burning cells and depress the accumulation of body fat, even without induction of UCPs in these cells, as documented by the effect of EPA/DHA. Lack of recruitment of UCP1 in the EPA/DHA-treated mice may be explained by the absence of other cofactors or hormones required in addition to PPARGC1A [18, 20]. The absence of UCP1 also suggests that the increase in fatty acid oxidation occurs in white rather than brown fat cells. This idea is supported by the induction of the gene for

CPT1A, which is preferentially expressed in white adipose tissue in mice [42]. Because of the low activity of CPT1A, the rate of  $\beta$ -oxidation in white adipose tissue is normally low and fatty acids are directed to esterification [42, 43]. Thus, the enhancement of fatty acid oxidation in white adipose tissue by EPA/DHA concentrate could depend on the increase in the expression of the gene encoding CPT1A. Decreased production of malonyl CoA due to suppression of lipogenesis may result in further augmentation of fatty acid oxidation in mitochondria via the CPT1A-mediated mechanism [44].

It was found recently that expression of the gene encoding PPARGC1A is reduced in the adipose tissue of morbidly obese subjects [45] and inversely correlated with adipose cell mass [46], and that *Ppargc1a* polymorphisms segregate with obesity [47] and type 2 diabetes [48]. Onset of obesity in *ob/ob* mice is associated with downregulation of genes coding for mitochondrial proteins [36], and heat production by white adipose tissue cells from obese humans is lower than in lean individuals [49]. All these findings are compatible with the hypothesis that changes in fuel partitioning and reduced oxidation of lipids in white adipocytes contribute to the development of obesity and type 2 diabetes.

In conclusion, we show for the first time that dietary EPA and DHA preferentially upregulate genes for mitochondrial proteins, including their regulatory genes *Ppargc1a* and *Nrf1*, and increase  $\beta$ -oxidation while depressing lipogenesis in abdominal fat. This metabolic switch is induced independently of ALA intake and could reduce the development of obesity. Relatively minor changes in the composition of lipids in an obesity-promoting diet exert surprisingly pronounced effects. Our findings have important implications for the design of novel dietary and pharmacological strategies for the prevention and treatment of obesity and the metabolic syndrome.

**Acknowledgements** We thank J. Bemova, S. Hornova, D. Salkova, V. Fialova, C. Vink for technical assistance, J. Vorlicek for statistical analysis, and Drs A. Kotyk and V. Mohamed-Ali for critical reading of the manuscript. This work was supported by the Grant Agency of the ASCR (KJB5011410 and KJB5011303), the Czech Science Foundation (303/05/2580 and 303/03/0749), the MEYS (LL00A079), research projects AVOZ 50110509 and 1M6837805002 (Czech Republic), Pronova Biocare a.s. (Norway) and the Ministry of Agriculture, Nature Management and Food Control (The Netherlands).

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