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Journal of Nutritional Biochemistry 23 (2012) 1384–1393

Journal of Nutritional Biochemistry

Fish oil and 3-thia fatty acid have additive effects on lipid metabolism but antagonistic effects on oxidative damage when fed to rats for 50 weeks☆

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Received 23 February 2011; received in revised form 5 August 2011; accepted 18 August 2011

Abstract

The 3-thia fatty acid tetradecylthioacetic acid (TTA) is a synthetic modified fatty acid, which, similar with dietary fish oil (FO), influences the regulation of lipid metabolism, the inflammatory response and redox status. This study was aimed to penetrate the difference in TTA's mode of action compared to FO in a long-term experiment (50 weeks of feeding). Male Wistar rats were fed a control, high-fat (25% w/v) diet or a high-fat diet supplemented with either TTA (0.375% w/v) or FO (10% w/v) or their combination. Plasma fatty acid composition, hepatic lipids and expression of relevant genes in the liver and biomarkers of oxidative damage to protein were assessed at the end point of the experiment. Both supplements given in combination demonstrated an additive effect on the decrease in plasma cholesterol levels. The FO diet alone led to removal of plasma cholesterol and a concurrent cholesterol accumulation in liver; however, with TTA cotreatment, the hepatic cholesterol level was significantly reduced. Dietary FO supplementation led to an increased oxidative damage, as seen by biomarkers of protein oxidation and lipoxidation. Tetradecylthioacetic acid administration reduced the levels of these biomarkers confirming its protective role against lipoxidation and protein oxidative damage. Our findings explore the lipid reducing effects of TTA and FO and demonstrate that these bioactive dietary compounds might act in a different manner. The experiment confirms the antioxidant capacity of TTA, showing an improvement in FO-induced oxidative stress.

Keywords: Reactive oxygen species; Oxidative damage; UCP; β-oxidation

Abbreviations: AASA, α-aminoadipic semialdehyde; *Acaca*, acetyl-coenzyme A carboxylase α; *Acadm*, acyl-coenzyme A dehydrogenase, medium chain; *Acadvl*, acyl-coenzyme A dehydrogenase, very long chain; ACOX1, acyl-CoA oxidase 1; *Arbp*, acidic ribosomal protein, P0; CPT, carnitine palmitoyltransferase; CEL, *N*ε-carboxyethyl-lysine; CML, *N*ε-carboxymethyl-lysine; DBI, double-bond index; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; GSA, γ-glutamic semialdehyde; *Hadha*, hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, α-subunit; MDAL, *N*ε-malondialdehyde-lysine; PL, phospholipids; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TTA, tetradecylthioacetic acid; UCP, uncoupling protein.

☆ Grants and funding: This study was financially supported by grants from NordForsk, grant 070010, MitoHealth; the Research Council of Norway, grant 190287/110; and the European Community's Seventh Framework Program (FP7/2007-2013), grant 201668, AtheroRemo. Work carried out at the Department of Experimental Medicine was supported in part by R+D grants from the Spanish Ministry of Science and Innovation (AGL2006-12433 and BFU2009-11879/BFI), the Spanish Ministry of Health (RD06/0013/0012 and PI081843), the Autonomous Government of Catalonia (2009SGR735), and COST B35 Action of the European Union. D.C. received a fellowship from the Spanish Health Institute Carlos III. The authors declare no conflict of interest.

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doi:10.1016/j.jnutbio.2011.08.006

1. Introduction

The body's inability to handle excess energy intake leads to metabolic abnormalities such as hyperglycemia and dyslipidemia. The coexistence of these risk factors with overweight and hypertension represents the metabolic syndrome [1,2]. Thus, the discovery and testing of dietary supplements that can improve lipid metabolism and maintain homeostasis, and consequently, prevent the development of risk factors of metabolic syndrome, is of great importance.

Both fish oil (FO) and tetradecylthioacetic acid (TTA) have been widely used in *in vivo* experiments. FO has been shown to inhibit lipogenesis and exert a hypolipidemic effect by lowering plasma cholesterol and triacylglycerol (TAG) levels [3], as well as having an anti-inflammatory effect beneficial against both atherosclerosis [4] and arthritis [5,6]. The effects of FO are attributed to ω−3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are thought to bind and activate peroxisome proliferator-activated receptors (PPARs) [7]. They act upon ligand activation by controlling networks of target genes and therefore serve as lipid sensors because they can be triggered by metabolic derivates of fatty acids in the body.

Tetradecylthioacetic acid, a structurally modified 16-carbon saturated fatty acid (SFA) with a sulfur atom inserted in the third position of the alkyl chain, has been documented as a pan-PPAR ligand in several cell lines [8–10]. Tetradecylthioacetic acid has a particularly high affinity for PPARα [9–11] and exerts its hypolipidemic effect by inducing gene expression of enzymes involved in hepatic fatty acid β-oxidation. It thereby reduces the availability of fatty acids for very-low-density-lipoprotein synthesis and secretion and lowers plasma TAG and cholesterol levels. In addition, TTA has been shown to have an important role in diminishing of inflammation [12–16].

Both physiological processes and externally induced oxidative stress reactions can lead to formation of reactive oxygen species (ROS) in the body. It is well-known that dietary FO supplementation can increase lipoxidative damage due to the high amount of unsaturated fatty acids [17–19]. Tetradecylthioacetic acid has a potent capacity to attenuate the oxidative stress and protect the cellular membrane lipids from oxidative damage. Based on this, we hypothesized that a dietary TTA supplementation to the high-fat diet would be able to reduce the oxidative stress induced by FO.

Thus, the main focus in this 50-week-long *in vivo* experiment was to investigate the effects of TTA and FO, given separately or in combination, on body weight gain, plasma and liver lipid levels, as well as fatty acid composition in plasma and several genes encoding important enzymes involved in fatty acid metabolism. Further, despite the similar ability to act through PPAR-activation, we revealed principal differences in the mechanisms of action of TTA and FO. Finally, we confirmed that TTA can act as an antioxidant and prevent the FO-caused oxidative damage.

2. Methods

2.1. Animals and diets

This animal study was conducted according to the Guidelines for the Care and Use of Experimental Animals, and the protocol was approved by the Norwegian State Board of Biological Experiments with Living Animals. Eight- to 10-weeks-old male Wistar rats, weighing 200–250 g, were obtained from Taconic Europe (previously Möllegaard and Bomholtgaard, Ry, Denmark). Throughout the experiment, the rats were housed in Makrolon III cages in an open system and kept under standard laboratory conditions with temperature 22°C±1°C, dark/light cycles of 12/12 h, relative humidity 55%±5% and 20 air changes per hour. The animals were housed five per cage and had free access to food and water during the study. They were acclimatized under these conditions with standard chow for 1 week before the experiment started. All rats were divided into four groups. The first group of animals (control group) was fed a high-fat diet with 25% fat, consisting of 23% lard and 2% soybean oil. The second group (TTA group) was fed a high-fat diet supplemented with TTA (0.375%). The third group (FO group) was fed a diet supplemented with 10% FO (EPAX 4020 TG) (12.6% lard and 2% soy oil). The fourth group (TTA+FO group) was fed both diet supplements. The amount of ω−3 fatty acids in FO-containing diets was 8% (where the EPA content was 4.5% and DHA was 2.3%). All diets were isocaloric in their energetic value (4900 kcal).

The animals were part of a larger study described elsewhere, and all animals underwent a jejunogastric reflux surgical procedure (manuscript in preparation). An additional 2-month feeding experiment on male Wistar rats with and without surgery was performed to determine if the procedure had an effect on the nutritional state of the animals. There was no difference in body weight or plasma lipids between the groups (data not shown), and thus it could be assumed that the surgery had no adverse effect on nutritional uptake.

Tetradecylthioacetic acid was synthesized as previously described [20]. The rats were anaesthetized with isofluorane (Forane; Abbott Laboratories, Abbott Park, IL) inhalation under nonfasting conditions. Blood was drawn by cardiac puncture and collected in BD Vacutainer tubes containing EDTA (Becton-Dickinson, Plymouth, UK), and the organs were immediately removed and frozen in liquid nitrogen.

2.2. Quantification of lipids and fatty acids

Plasma and liver lipids were measured on the Hitachi 917 system (Roche Diagnostics, Mannheim, Germany). Quantification of TAGs and total cholesterol in plasma and liver were obtained by using kits from Roche Diagnostics. Choline-containing phospholipids (PLs) in plasma and liver were measured by PAP150 from bioMérieux (Lyon, France). Hepatic lipids were analyzed in cytoplasmic extracts. Lipids from plasma and liver were extracted before the fatty acid composition was analyzed as described previously [21,22].

From the fatty acid profile, the double-bond index (DBI) of lipid susceptibility to oxidative modification [23] was calculated: DBI=[(1×∑mol% monoenoic)+(2×∑mol% dienoic)+(3×∑mol% trienoic)+(4×∑mol% tetraenoic)+(5×∑mol% pentaenoic)+(6×∑mol% hexaenoic)].

2.3. Enzyme activities

Fresh liver tissue samples were homogenized in ice-cold sucrose medium and centrifuged. The resulting three postnuclear fractions, a mitochondrial-enriched fraction (M), a peroxisome-enriched fraction (L) and a cytosolic fraction (S), were isolated as previously described and frozen at −80°C [24]. Then the activities of fatty acyl-CoA oxidase 1 (ACOX1) [25], carnitine palmitoyltransferase II (CPT-II) [26], 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMG-CoA synthase) [27] and fatty acid synthase (FAS) [28,29] were measured.

2.4. Gene expression analysis

Liver samples were frozen in liquid nitrogen immediately after dissection and stored at −80°C. Total cellular RNA was purified from 20- to 30-mg tissue using RNeasy Mini Kit (Qiagen). RNA was quantified spectrophotometrically (NanoDrop 1000; NanoDrop Technologies, Boston, MA), and the quality was evaluated by capillary electrophoresis (Agilent 2100 Bioanalyzer; Agilent Technologies, Palo Alto, CA). For each sample, 400 ng total RNA was reversely transcribed in 20-μl reactions using Applied Biosystem's High Capacity cDNA Reverse Transcription Kit with RNase inhibitor according to the manufacturer's description. Real-time polymerase chain reaction was performed with custom-made 384-well microfluidic plates [Taq-Man

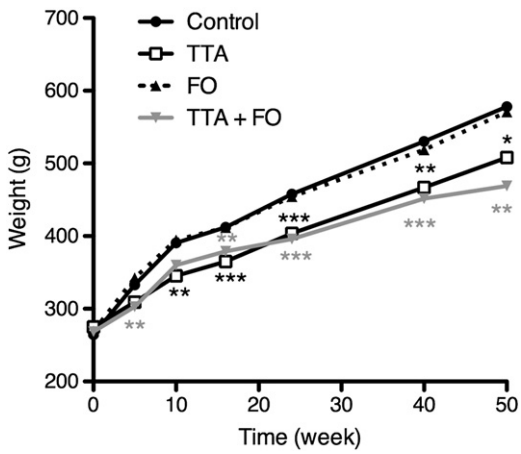

Fig. 1. Average weight of rats on high-fat diets supplemented with 3-thia fatty acid TTA and/or FO for 50 weeks. Data represent means (n=10). Values that were significantly different from control by t test are indicted by black (TTA) or gray (TTA+FO) asterisks (*P<.05, **P<.01, ***P<.001).

Low Density Arrays (TLDA), Applied Biosystems, Foster City, CA]. The genes selected were acyl-coenzyme A dehydrogenase, medium chain (*Acadm*), acyl-coenzyme A dehydrogenase, very long chain (*Acadvl*), carnitine palmitoyltransferase 1A and 2 (*Cpt1a* and *Cpt2*, respectively), acetyl-coenzyme A carboxylase α (*Acaca*), uncoupling protein 2 and 3 (*Ucp2* and *Ucp3*, respectively), glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, α-subunit (*Hadha*). All probes and primers were obtained from Applied Biosystems. The reference gene was chosen according to the MIQE guidelines [30,31]. Three different control genes were included: *18s* (Kit-FAM-TAMRA, reference RT-CKFT-18s from Eurogentec, Seraing, Belgium), *Gapdh* (Rodent GAPDH Control Reagents, part 4308313) and *Arbp* (from Applied Biosystems). In a comparative analysis using the programs geNorm and Normfinder, *Arbp* was found to be the best. The expression value of each gene in each sample was normalized against this endogenous control.

2.5. Oxidative damage markers

Glutamic semialdehyde (GSA), aminoadipic semialdehyde (AASA), carboxymethyl-lysine (CML), carboxyethyl-lysine (CEL) and malondialdehyde-lysine (MDAL) concentrations in total proteins from liver homogenates and mitochondrial fraction were measured by gas chromatography/mass spectrometry (GC/MS) as previously described [18]. Samples containing 0.5 mg of protein were delipidated using chloroform/methanol (2:1, v/v), and proteins were precipitated by adding 10% trichloroacetic acid (final concentration) and subsequent centrifugation. Protein samples were reduced overnight with 500 mM $NaBH_4$ (final concentration) in 0.2 M borate buffer, pH 9.2, containing 1 drop of hexanol as an antifoam reagent. Proteins were then reprecipitated by adding 1 ml of 20% trichloroacetic acid and subsequent centrifugation. The following isotopically labeled internal standards were then added: [$^2H_8$]lysine (d_8-Lys; CDN Isotopes); [$^2H_4$]CML (d_4-CML), [$^2H_4$]CEL (d_4-CEL) and [$^2H_8$]MDAL (d_8-MDAL), prepared as described [32,33]; [$^2H_5$]5-hydroxy-2-aminovaleric acid (for GSA) and [$^2H_4$]6-hydroxy-2-aminocaproic acid (for AASA) were prepared as described [34]. The samples were hydrolyzed at 155°C for 30 min in 1 ml of 6 HCl and then dried in vacuo. The *N,O*-trifluoroacetyl methyl ester derivatives of the protein hydrolyzate were prepared as previously described [34]. Gas chromatography/mass spectrometry analyses were carried out on a Hewlett-Packard model 6890 gas chromatograph equipped with a 30-m HP-5MS capillary column (30 m×0.25 mm×0.25 μm) coupled to a Hewlett-Packard model 5973A mass selective detector (Agilent, Barcelona, Spain). The injection port was maintained at 275°C; the temperature program was 5 min at 110°C, then 2°C/min to 150°C, then 5°C/min to 240°C, then 25°C/min to 300°C and finally held at 300°C for 5 min. Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and nondeuterated standards. Analytes were detected by selected ion monitoring GC/MS. The ions used were lysine and d_8-lysine, *m/z* 180 and 187, respectively; 5-hydroxy-2-aminovaleric acid and d_5-5-hydroxy-2-aminovaleric acid (stable derivatives of GSA), *m/z* 280 and 285, respectively; 6-hydroxy-2-aminocaproic acid and d_4-6-hydroxy-2-aminocaproic acid (stable derivatives of AASA), *m/z* 294 and 298, respectively; CML and d_4-CML, *m/z* 392 and 396, respectively; CEL and d_4-CEL, *m/z* 379 and 383, respectively; and MDAL and d_8-MDAL, *m/z* 474 and 482, respectively. The amounts of products were expressed as the ratio of micromole of GSA, AASA, CML, CEL, or MDAL/mol of lysine.

2.6. Statistical analysis and presentation of data

The results are presented as mean values with their standard deviations for 7 to 10 rats per group. The data were evaluated by one-way ANOVA and Tukey test or by unpaired Student *t* test with the level of statistical significance set at $P<.05$ (GraphPad Prism version 4.01; GraphPad, San Diego, CA). Single outliers (significance level >0.05) were removed using Grubb test (www.Graphpad.com).

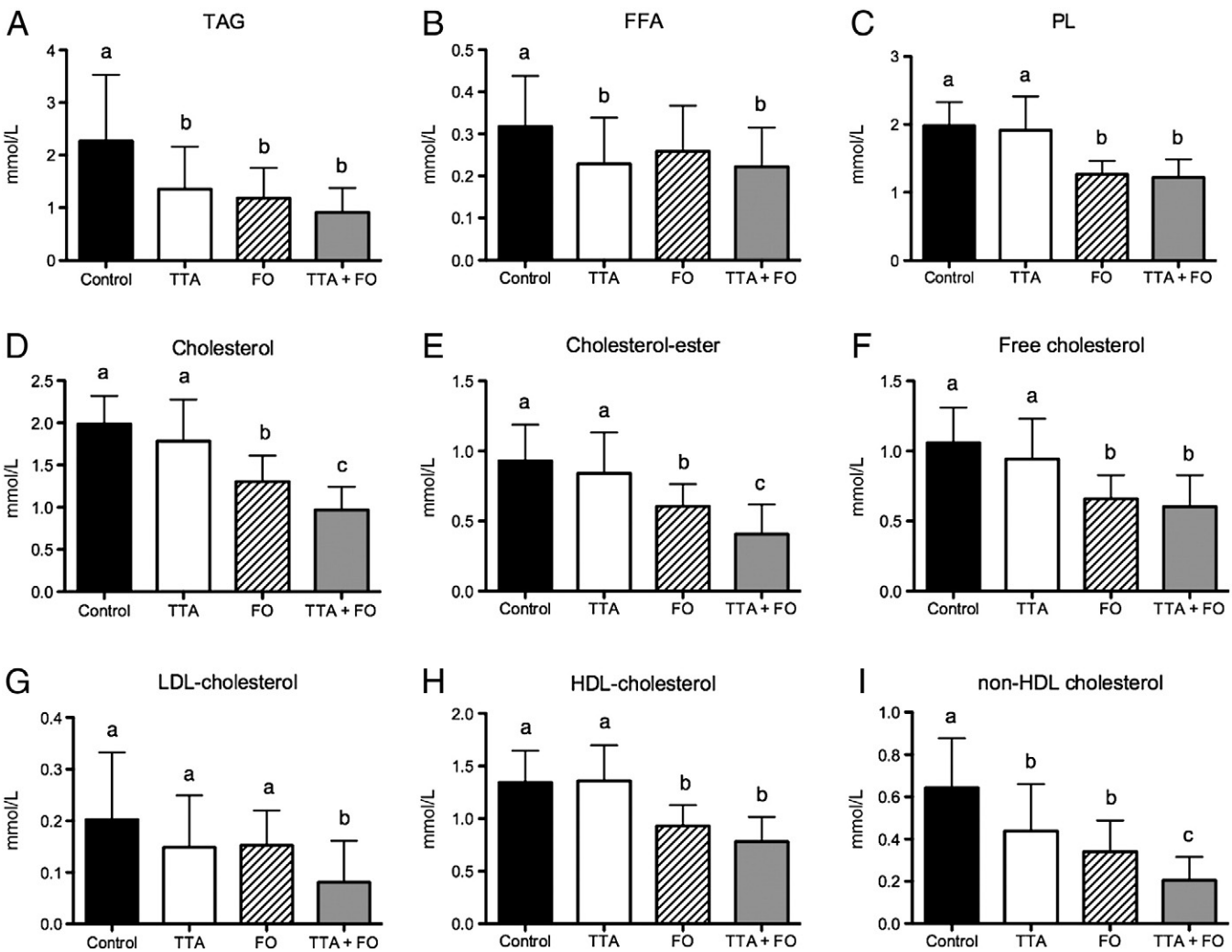

Fig. 2. Plasma lipid concentrations in male Wistar rats after 50 weeks of high-fat diets supplemented with 3-thia fatty acid TTA or FO. Values are means±S.D. (n=31–38). Results were analyzed by ANOVA, and means with the same letter are not significantly different from each other (Tukey–Kramer test, $P<.05$).

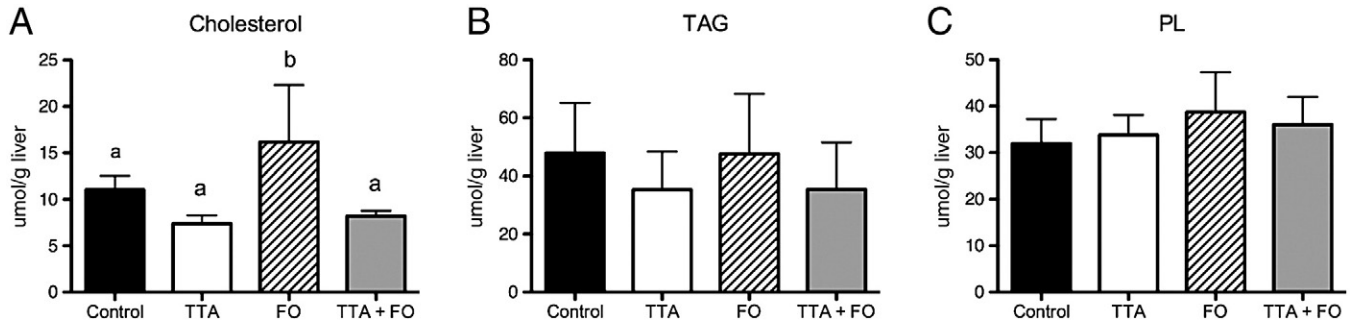

Fig. 3. Liver lipid concentrations in male Wistar rats after 50 weeks of high-fat diets supplemented with 3-thia fatty acid TTA or FO. Values are means±S.D. (n=10–11). Results were analyzed by ANOVA and means with the same letter are not significantly different from each other (Tukey–Kramer test, P<.05).

3. Results

3.1. Animal body weight

The addition of FO to a high-fat diet (25%) had no effect on the growth rate of rats, while the combined treatment led to a significant reduction in weight gain compared to the control group already after 5 weeks of treatment (Fig. 1). Moreover, the diet that contained both bioactive lipids seemed to reduce body weight more than TTA alone after 50 weeks of treatment but not significantly (P=.23). The weight loss caused by TTA stabilized over time. In this study, all groups demonstrated the same average feed intake. Thus, animals from TTA and TTA+FO groups had a reduced body weight compared to control group animals with the same energy intake over a 50-week period.

3.2. Plasma lipids

We studied the impact of TTA and FO on key parameters in lipid metabolism. Analysis of plasma lipids at the end point of the experiment demonstrated that all treatments had a substantial lipid-lowering potential (Fig. 2). The plasma TAG level was significantly improved by all the treatments (Fig. 2A). Only TTA and the combination of TTA and FO were able to lower the free fatty acids (FFAs, Fig. 2B). In contrast, FO and the combination with TTA were able to reduce plasma PL levels by 30%, while TTA had no effect compared to the control diet (Fig. 2C).

FO treatment alone or in combination with TTA was effective in lowering of the total plasma cholesterol, cholesterol esters and free cholesterol (Fig. 2D–F). While the FO-containing diet alone reduced cholesterol by 35% compared to the control high-fat diet, the cotreatment with TTA and FO reduced it by 49%, indicating an additive effect of the supplements (Fig. 2D). Likewise, a significant decrease in the cholesterol ester level was obtained after TTA+FO treatment compared to either treatment alone (Fig. 2E). The additive effect of TTA and FO was mainly on LDL cholesterol (Fig. 2G) and non-HDL cholesterol (Fig. 2I).

3.3. Liver lipids

The hepatic cholesterol concentrations had a tendency to decrease after administration of TTA and TTA+FO (Fig. 3) but increased significantly with FO treatment compared to control. In addition, in rats fed a TTA diet, the total hepatic TAG concentration was 26% lower than in control. In contrast, FO treatment did not influence the TAG concentration in liver and caused an insignificant rise in PL levels.

3.4. Fatty acid composition in plasma

The fatty acid composition in plasma is shown in Table 1. Compared to control animals, the plasma levels of SFAs and monounsaturated fatty acids (MUFAs) were marginally changed by TTA treatment alone, except for an increase in C20:3n−9 (P<.0005). In contrast to that, FO supplementation caused the significant reduction in amounts of SFAs (P<.0005), MUFAs (P<.0005) and n−6 PUFAs (P<.0005) in plasma from FO-treated animals. ω−3 PUFAs

Table 1
Plasma fatty acid composition of male Wistar rats after 50 weeks of feeding with high-fat (25% w/v) diets supplemented with TTA, FO or TTA+FO

	Dietary supplementation			
	Control	TTA	FO	TTA+FO
SFAs	35.20±0.88^{a}	34.98±1.36^{a}	30.82±2.06^{b}	32.66±1.65^{c}
C16:0	21.22±1.49^{a}	22.41±1.23^{b}	17.72±1.28^{c}	20.35±1.44^{a}
C18:0	12.12±1.43^{a}	10.78±0.81^{b}	11.27±1.26^{b}	10.40±0.96^{b}
MUFAs	24.65±4.75^{a}	25.87±3.73^{a}	16.79±1.62^{b}	20.28±2.74^{c}
C16:1n−7	1.05±0.29^{a,b}	1.20±0.65^{b}	0.77±0.13^{c}	0.91±0.18^{a,c}
C18:1n−7	1.96±0.28^{a}	1.65±0.29^{b}	1.49±0.14^{c}	1.50±0.17^{b,c}
C20:1n−7	0.17±0.08	0.13±0.08^{a}	0.19±0.09^{b}	0.16±0.07
C22:1n−7	0.03±0.01^{a}	0.02±0.01^{b}	0.04±0.02^{a}	0.03±0.01^{a}
C16:1n−9	0.26±0.06^{a}	0.36±0.07^{b}	0.24±0.03^{a}	0.32±0.06^{c}
C18:1n−9 (OA)	20.46±4.23^{a}	21.70±3.49^{a}	12.99±1.37^{b}	16.29±2.50^{c}
C22:1n−9	0.02±0.00^{a}	0.02±0.01^{a}	0.04±0.01^{b}	0.04±0.01^{b}
C24:1n−9	0.27±0.11^{a}	0.46±0.14^{b}	0.48±0.11^{b}	0.53±0.14^{b}
n−9 PUFAs				
C20:3n−9 (MA)	0.23±0.03^{a}	0.85±0.33^{b}	0.12±0.01^{c}	0.13±0.02^{c}
n−6 PUFAs	36.26±4.61^{a}	33.76±3.59^{b}	22.72±2.63^{c}	22.38±2.50^{c}
C18:2n−6 (LA)	17.94±1.76^{a}	15.16±3.22^{b}	12.32±1.03^{c}	13.07±0.97^{c}
C18:3n−6	0.22±0.05^{a}	0.46±0.10^{b}	0.11±0.02^{c}	0.22±0.04^{a}
C20:2n−6	0.26±0.04^{a}	0.20±0.04^{b}	0.16±0.03^{c}	0.16±0.03^{c}
C20:3n−6	0.56±0.12^{a}	1.11±0.22^{b}	0.37±0.08^{c}	0.74±0.18^{d}
C20:4n−6 (AA)	16.76±4.69^{a}	16.57±3.69^{a}	9.45±1.89^{b}	7.95±1.83^{b}
C22:4n−6	0.34±0.07^{a}	0.14±0.03^{b}	0.07±0.02^{c}	0.05±0.01^{c}
C22:5n−6	0.16±0.04^{a}	0.12±0.06^{b}	0.22±0.04^{c}	0.17±0.02^{a}
n−3 PUFAs	3.55±0.5^{a}	2.30±1.03^{a}	29.48±4.19^{b}	22.90±3.14^{c}
C18:3n−3 (ALA)	0.61±0.16^{a}	0.45±0.16^{b}	0.59±0.14^{a}	0.50±0.17^{b}
C18:4n−3	0.02±0.01^{a}	0.01±0.02^{a}	0.54±0.19^{b}	0.53±0.22^{b}
C20:4n−3	0.12±0.02^{a}	0.09±0.02^{b}	0.39±0.06^{c}	0.30±0.05^{d}
C20:5n−3 (EPA)	0.02±0.01^{a}	0.01±0.02^{a}	16.52±3.38^{b}	12.58±2.82^{c}
C22:5n−3 (DPA)	0.47±0.11^{a}	0.17±0.08^{b}	1.32±0.30^{c}	0.79±0.10^{d}
C22:6n−3 (DHA)	2.01±0.49^{a}	1.28±0.58^{a}	9.84±2.27^{b}	8.02±1.03^{c}
TTA	ND	1.36±0.38^{a}	ND	1.04±0.17^{b}
TTA:1n−8	ND	0.79±0.28^{a}	ND	0.55±0.25^{b}
n−3 PUFA/n−6 PUFA ratio	0.10±0.01^{a}	0.07±0.03^{a}	1.34±0.37^{b}	1.04±0.22^{c}
DBI	1.52±0.16^{a}	1.43±0.13^{b}	2.38±0.15^{c}	2.04±0.11^{d}
ω−3 index	2.33±0.52^{a}	1.58±0.94^{a}	26.36±3.83^{b}	20.59±2.85^{c}

Data are given as mol%±S.D. (n=31–38).
Results were analyzed by ANOVA, and means with the same letter are not significantly different from each other (Tukey–Kramer test, P<.05).
AA, arachidonic acid; ALA, α-linolenic acid, LA, linolenic acid; ND, not detectable; OA, oleic acid.

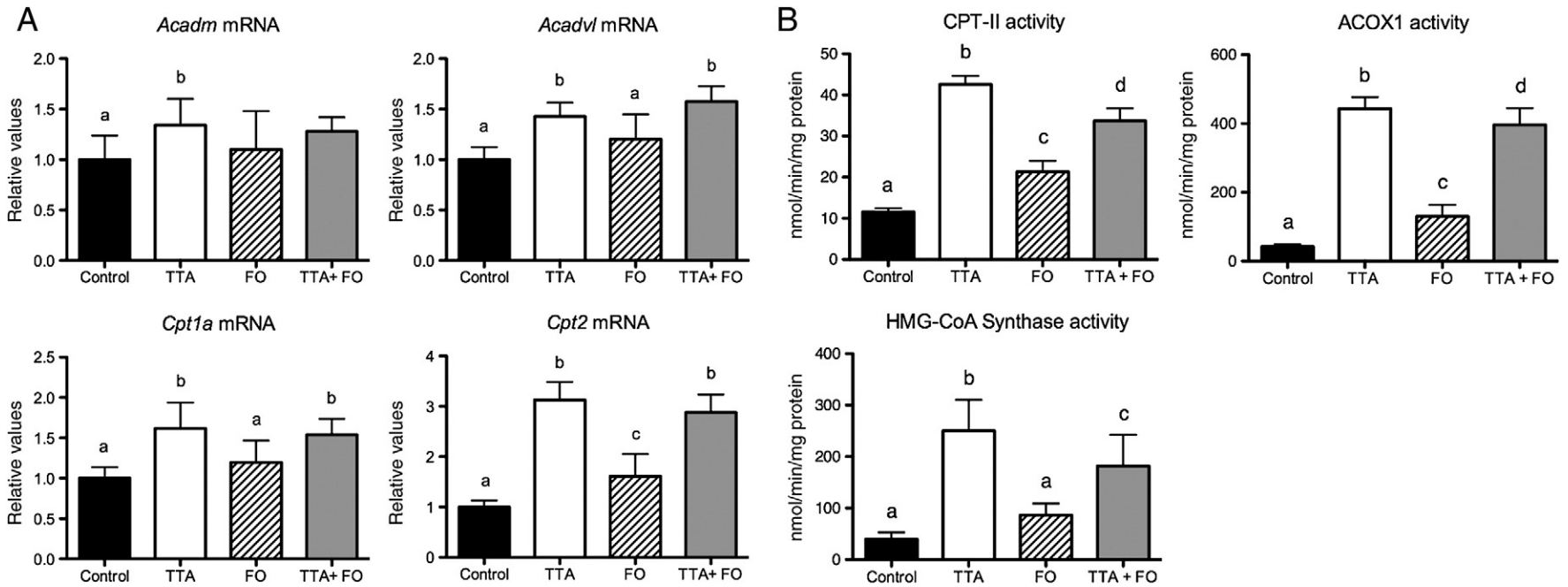

Fig. 4. Effect of 3-thia fatty acid TTA and FO on hepatic fatty acid β-oxidation. (A) Gene expression of *Acadm*, *Acadvl*, *Cpt1a*, and *Cpt2*. Data are shown as relative values. (B) Activity of ACOX1, measured in postnuclear fractions, CPT-II and HMG-CoA synthase, measured in crude mitochondrial fractions. Values are means±S.D. (n=8–10). Results were analyzed by ANOVA, and means with different letters are significantly different from each other (Tukey–Kramer test, P<.05).

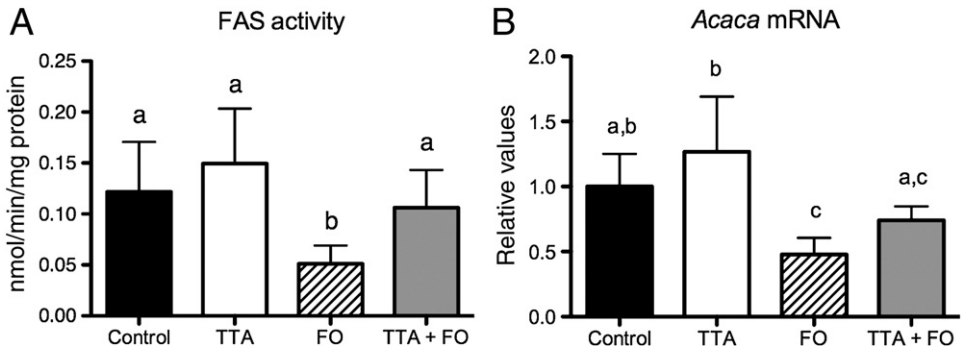

Fig. 5. Effect of 3-thia fatty acid TTA and FO on hepatic FAS activity (A) and hepatic gene expression of *Acaca* (B). Values are means±S.D. (n=8–10). Results were analyzed by ANOVA, and means with different letters are significantly different from each other (Tukey–Kramer test, P<.05).

[C20:5n−3 (EPA), C22:5n−3 (docosapentaenoic acid, DPA), C22:6n−3 (DHA)] were decreased after TTA treatment [35,36], leading to a significantly reduced DBI. We also found a significant (P<.0005) reduction of ω−3 PUFA in TTA+FO-treated animals compared to FO treatment alone. However, the combined treatment increased the n−3/n−6 PUFA ratio, as well as the ω−3 index (DHA+EPA) (Table 1), by over 10-fold compared to TTA alone.

3.5. Genes and enzymes involved in fatty acid metabolism

Several of the PPAR-controlled genes involved in the mitochondrial fatty acid β-oxidation pathway, such as *Acadm*, *Acadvl*, *Cpt1a*, and *Cpt2* were induced in liver by the TTA diet (Fig. 4A). The FO diet seemed to give an induction of PPAR-activated genes; however, only *Cpt2* was significantly increased.

Hepatic ACOX1, CPT-II and HMG-CoA synthase activities indicated a high induction of fatty acid β-oxidation after 50 weeks of TTA diet (Fig. 4B). The addition of FO caused the less pronounced effect on hepatic enzyme activities in combination group compared to TTA group.

The activity of lipogenic enzyme FAS was significantly reduced by FO administration (Fig. 5A). Tetradecylthioacetic acid gave an insignificant increase in FAS activity and counteracted the FO effect in the combined treatment.

We also investigated the messenger RNA (mRNA) expression of *Acaca* (Fig. 5B). This gene encodes the enzyme acetyl-coenzyme A carboxylase, which catalyzes carboxylation of acetyl-CoA to malonyl-CoA. *Acaca* mRNA level in liver tended to be up-regulated by TTA compared to control group. The FO-supplemented group had a significantly lower *Acaca* mRNA expression than both TTA group and control group (P<.001). Also, in the TTA+FO group, *Acaca* expression was significantly lower than in the TTA group (P<.01).

3.6. Differential effects of TTA and FO on Ucp mRNA expression

In the liver samples, differential effects of TTA and FO were observed on the gene expression of *Ucp2* and *Ucp3* mRNA, which are suggested to act as regulators of mitochondrial energy metabolism and ROS. Both TTA and TTA+FO diets resulted in the down-regulation of hepatic *Ucp2* mRNA levels, in contrast to FO alone (Fig. 6A). We revealed a substantial up-regulation (1500×) of *Ucp3* in rats fed a TTA-supplemented diet (Fig. 6B). The combination of TTA with FO also led to an up-regulation of *Ucp3* mRNA levels, while the FO diet had no significant effect on this mitochondrial uncoupling protein.

3.7. Oxidative damage in homogenate of whole liver and mitochondria

We evaluated the relationship between different oxidative biomarkers (representing different oxidative damage pathways) between whole-liver *vs.* mitochondrial fraction (Fig. 7).

γ-Glutamic semialdehyde is a marker that is related to direct metal catalysed oxidation on proline and arginine residues in proteins. Interestingly, the diet supplementation with FO led to non-significantly higher levels of GSA than in the control group in both mitochondrial extract and whole-liver homogenate, which indicated an increase in protein oxidative damage (Fig. 7A). In contrast to this, the dietary supplementation of TTA significantly reduced protein oxidative damage in mitochondrial extracts obtained from TTA- and TTA+FO-fed animals.

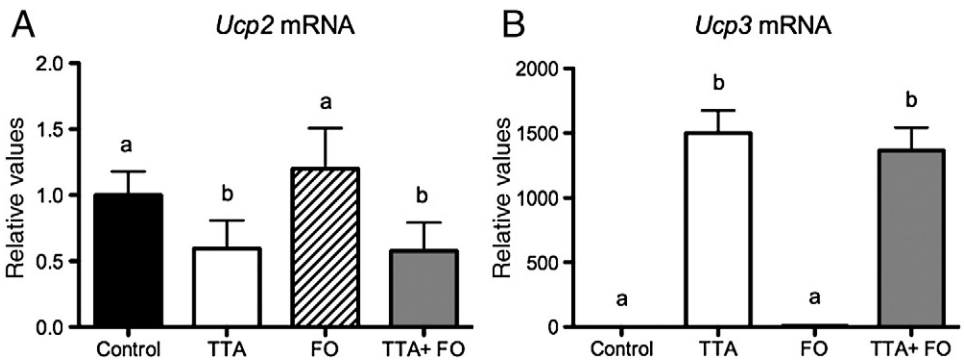

Fig. 6. Effect of 3-thia fatty acid TTA and FO on the hepatic gene expression of mitochondrial uncoupling proteins *Ucp3* (A) and *Ucp2* (B). Values are means±S.D. (n=10). Results were analyzed by ANOVA, and means with different letters are significantly different from each other (Tukey–Kramer test, P<.05).

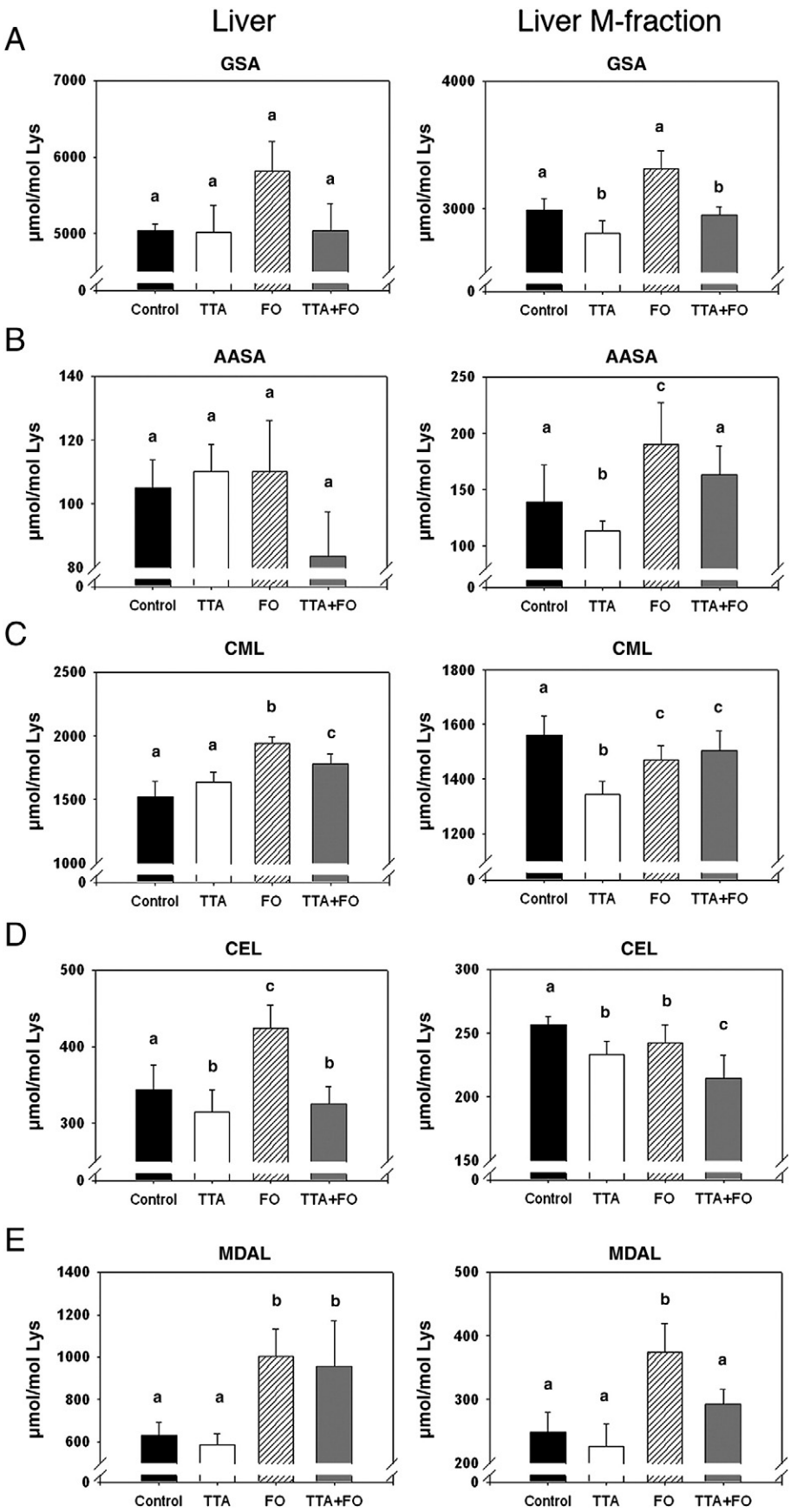

Fig. 7. Protein oxidative damage amongst different dietary interventions. GSA, AASA, CEL, MDAL and CML measured in liver (left) and liver mitochondrial homogenate (liver M-fraction, right). Values are means±S.D. (n=7 or 8 animals in each group). Results were analyzed by ANOVA, and means with different letters are significantly different from each other (Tukey–Kramer test, P<.05).

The other marker of protein oxidative damage, α-AASA, was also significantly higher in mitochondrial samples from the FO group than in animals from the control group and groups treated with TTA or the combination (Fig. 7B). The TTA supplementation led to reduction of protein oxidative damage mitochondrial extract.

The nonenzymatic glycation and subsequent irreversible oxidation of proteins cause formation of glycoxidation products. *N*ε-Carboxymethyl-lysine is known to be formed from the oxidation of both carbohydrates and lipids, making it a biomarker of general oxidative stress. The whole-liver samples from the FO group showed significantly increased lipoxidation and TTA diminished this potentially deleterious effect of FO in animals from the TTA+FO group (Fig. 7C). In mitochondrial extracts, both FO and TTA treatment resulted in reduced levels of CML compared to the control group.

*N*ε-Carboxyethyl-lysine is a homolog of CML, which is formed by the reaction of lysine residues in proteins with methylglyoxal as well as with triose phosphates and other sugars. Our results showed that FO increased oxidative (nonenzymatic) sources of methylglyoxal, while the addition of TTA into the diets reduced CEL levels in whole-liver homogenates (Fig. 7D). In mitochondrial extracts, both bioactive compounds resulted in lower levels of CEL compared to control group. The cotreatment with TTA and FO demonstrated significantly lowered CEL compared with control, TTA, and FO groups in mitochondrial extract.

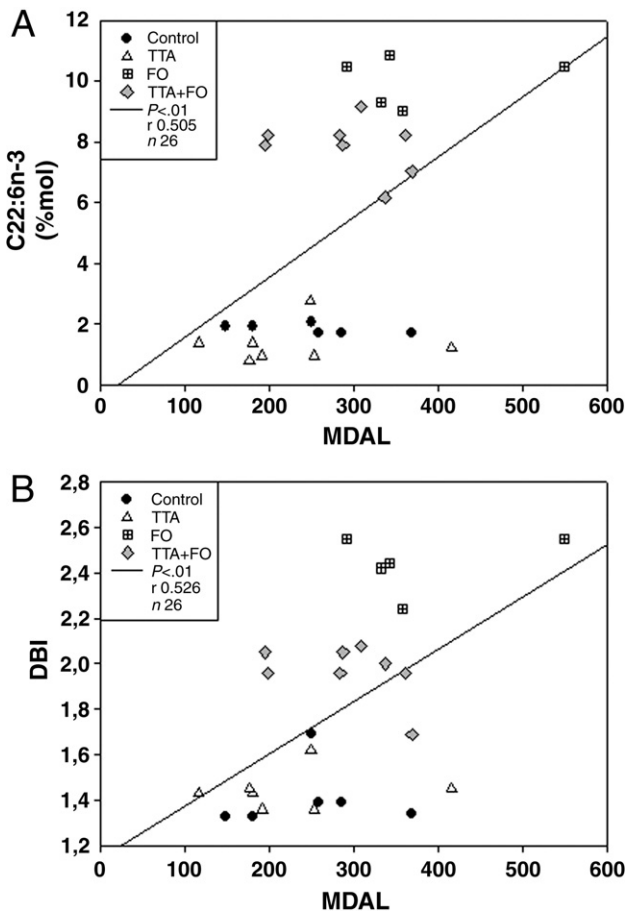

Fig. 8. Correlations for the fatty acid composition in plasma and the lipoxidative damage marker MDAL measured in mitochondrial extracts. (A) Correlation between DHA (C22:6*n*−3) and MDAL. C22:6*n*−3 values are shown as mole percent of the total lipid content. (B) Correlation between the DBI and MDAL. DBI values are expressed as ∑% of unsaturated fatty acids×number of double bonds per unsaturated fatty acid. MDAL values are expressed in μmol MDAL/mol Lys.

*N*ε-Malondialdehyde-lysine is a biomarker of lipoxidation. FO supplementation showed an increased oxidative damage compared to control group in both whole-liver and mitochondrial homogenate samples (Fig. 7E). We found that TTA cotreatment diminished the FO-induced lipoxidative damage in mitochondrial homogenates.

Furthermore, there is a significant positive correlation between MDAL concentration and the DHA content, as well as the DBI (Fig. 8).

4. Discussion

Contrary to earlier performed animal experiments on the effects of bioactive lipids such as TTA and ω−3 PUFAs, the distinguishing factor of our study was its duration of 50 weeks. The extended duration gave us the possibility to evaluate and compare the effectiveness of combined treatment *vs.* mono supplementation with TTA or FO. The prolonged beneficial effects observed were changes in expression of genes involved in fatty acid metabolism and activities of corresponding key enzymes, improvement of plasma lipid parameters and liver lipid composition and not the least weight reduction. A 50-week period of feeding with TTA resulted in a significantly reduced body weight gain during the whole study period, apparently reflecting increased energy expenditure due to TTA. In agreement with other studies, FO did not affect the overall body weight of male Wistar rats given a high-fat diet [37]. Further, the rise in activities of CPT-II, HMG-CoA synthase and ACOX1 (Fig. 4B) combined with the enhanced expression of hepatic *Cpt1*, *Cpt2*, *Acadm* and *Acadvl* (Fig. 4A) as well as *UCP3* (Fig. 6) suggests that TTA both increases the β-oxidation and induces the energy consumption via intensification of uncoupling in the liver (45).

The diets had a differential effect on the lowering of plasma lipid levels. While the TTA diet mainly lowered TAG, FFAs and LDL cholesterol in this long-term treatment, FO supplementation showed substantially more lowering of TAG, PLs, cholesterol and HDL cholesterol than TTA alone. The combined supplementation with TTA and FO demonstrated the additive effect of both components on the decrease in several plasma lipids and especially in a significantly enhanced reduction in plasma cholesterol.

In agreement with the results obtained by Duan at al. [38], our 50-weeks-long experiment showed that high-fat feeding can induce elevation in hepatic TAG levels (Fig. 3B). The accumulation of TAG was probably due to inability of the liver to handle the increased import of plasma lipids and indicated the development of liver steatosis. The diet with TTA showed a potential to improve the hepatic TAG and cholesterol levels, whereas the FO diet did not. This could be due to the high induction of hepatic β-oxidation by TTA that enhances lipid metabolism and consequently results in pronounced removal of lipids from both plasma and liver [13]. In addition, TTA has previously been shown to drain lipids from specific adipose tissue depots [39]. It might be possible that both FO and TTA diets are able to increase cholesterol and TAG transport to the liver, but animals given the FO diet are less able to metabolize the excess hepatic lipid levels. The stimulated activities of CPT-II and ACOX1 were much more pronounced with TTA than with FO feeding (Fig. 4B). Thus, TTA seemed to lower plasma lipid levels by an increased hepatic β-oxidation, while FO redistributed lipids without affecting the total lipid level and body weight.

We have revealed a tendency to decrease in the hepatic cholesterol concentrations after administration of TTA and TTA+FO, whereas FO supplementation alone gave a significant increase in hepatic cholesterol compared to controls. Consequently, the observed lowering effect of hepatic cholesterol by TTA+FO combination might be due to the TTA effect.

In this long-term experiment, TTA supplementation caused several changes in the plasma fatty acid composition, particularly, the decrease in ω−3 PUFA content (Table 1). The observed reduction in ω−3 PUFA levels can be explained by TTA-induced stimulation of

mitochondrial and peroxisomal fatty acid oxidation (36). Furthermore, the $n-9$ fatty acid, mead acid (MA, C20:3$n-9$), was up-regulated in the plasma of TTA-fed rats (approximately fourfold). Notably, both FO and the combined treatment led to a twofold reduction of MA compared to control. As an increased production of the nonessential MA may be linked to a poor PUFA status in humans [40], the FO-induced decrease in MA supports a similar regulation of MA in rats.

The addition of both TTA and FO to the high-fat diet induced genes linked to fatty acid metabolism and increased the corresponding enzyme activity levels. Tetradecylthioacetic acid was far more potent than FO, and the combination of TTA and FO did not further increase the effect. The pattern of enzyme activity implicated in fatty acid metabolism was similar to previous findings [19,36,39], demonstrating that the metabolic effects of FO and TTA supplementation are long-lasting.

Tetradecylthioacetic acid and FO seem to affect the factors involved in lipogenesis in a different manner. The significant reduction in hepatic FAS activity in rats fed the FO diet could contribute to the registered changes in plasma lipid content. In contrast, the TTA diet showed an insignificant increase in hepatic FAS activity ($P=.24$). Moreover, the FO diet significantly decreased *Acaca* expression in liver compared to TTA. Thus, comparing the effect of combination of both supplements with the effect of TTA treatment, the reduction of *Acaca* mRNA level could be a result of the competing modes of action of TTA and FO.

The study with Wistar rats fed a low-fat TTA-supplemented diet for 7 days showed an enhanced *Ucp2* mRNA expression in liver [41], and it has been suggested that this early, transient induction in hepatic *Ucp2* may contribute to increased energy expenditure. However, our long-term experiment and the 7-week study performed by Wensaas *et al.* [39] demonstrated a significant decrease in *Ucp2* expression caused by TTA-supplemented diets.

Ucp3 is usually not expressed in the liver, except in response to PPAR activation during situations with a high level of fatty acid metabolism [42]. The increase in hepatic *Ucp3* mRNA expression caused by TTA treatment alone (over 1500-fold) and the combined treatment (over 1300-fold) was the most striking results in our experiment. As discussed previously, the stimulated hepatic expression of *Ucp3* due to TTA administration could be related to an increased metabolism, possibly also through energy uncoupling (Fig. 6B). Altogether, these findings indicate that the induction of *Ucp2* becomes less pronounced over a longer period, while the induction of *Ucp3* is a prolonged effect by TTA.

A hypothesis about a possible protective role of endogenous UCP3 against oxidative damage by ROS in skeletal muscle mitochondria was proposed and tested by Brand *et al.* [43]. They discovered that down-regulation of *Ucp3* was associated with increased oxidative damage through higher ROS generation, while the *Ucp3* up-regulation was not. Based on findings from our study and in accordance with previous observations [44,45], we could propose that such pronounced effects might represent an adaptive response to protect the mitochondria and their host cells from oxidative stress in situations characterized by intensive lipid metabolism and consequently further support the theory of the defensive role of *Ucp3* against lipid-induced mitochondrial damage.

In agreement with previous data [46–48], we confirm that dietary FO supplementation increased lipoxidative damage. Our experiment revealed marked changes in CML and MDAL, which are markers related to lipoxidative status, in both liver homogenate and mitochondrial extracts. Since liver is a crucial organ in lipid metabolism, the fatty acid profile in liver is sometimes assumed to reflect the fatty acid composition in plasma. Therefore, it is not unreasonable to think that the profile might reflect the membrane lipid composition of liver cells. From that point of view and being demonstrated by a 1.5-fold increase of DBI for FO-treated animals compared with the control group (Fig. 8), one can postulate that the different degree of unsaturation in liver membranes may account for the increased damage observed and could achieve enough amplification without ROS production being raised. In other words, FO diet is not necessarily responsible for an increased ROS production but for a major ROS susceptibility, since membrane composition is slightly altered and the degree of unsaturation is increased. Finally, both CML and MDAL were reduced by addition of TTA into the diets. Thus, TTA was able to partially prevent those FO-induced changes. This fully agrees with the hypothesis of TTA as an antioxidant.

The results also demonstrate that a dietary manipulation comprising changes in the amount of fatty acids induce a significant change in the steady state levels of GSA and AASA, markers of direct oxidative damage. Reinforcing the implication of mitochondria in this change, only mitochondrial proteins show those changes, whereas liver homogenates did not. Fish oil effects could be due to increased free radical production as recently demonstrated in other tissues and experimental models, implicating Ca^{+2} transit between mitochondria and endoplasmic reticulum [49–51]. Most interestingly, the fact that TTA significantly lowers direct oxidative damage supports the notion that TTA increases mitochondrial efficiency (irrespectively of FO) and may reproduce beneficial effects of caloric restriction.

To conclude, this study provides evidence that the long-term effects of TTA are similar to those seen in experiments of shorter duration. We report that TTA and FO are efficient dietary supplements in improvement of plasma lipid profile, despite differences in their mechanisms of action. While TTA increased energy expenditure and lipid metabolism, FO seemed to lower plasma lipid levels mainly by a redistribution of lipids, as well as a reduced fatty acid synthesis. Through our experiment, we confirmed that, during long-term FO diets, the proper supplementation of antioxidants, such as TTA, is essential.

Acknowledgments

The authors are grateful to Kari Williams, Liv Kristine Øysæd, and Svein Krüger for excellent technical contribution and to the staff at the Laboratory Animal Facility, University of Bergen. We thank Professor Jon Skorve for helpful discussions.

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