Changed Energy State and Increased Mitochondrial β-Oxidation Rate in Liver of Rats Associated with Lowered Proton Electrochemical Potential and Stimulated Uncoupling Protein 2 (UCP-2) Expression

EVIDENCE FOR PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-α INDEPENDENT INDUCTION OF UCP-2 EXPRESSION

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Lowering of plasma triglyceride levels by hypolipidemic agents is caused by a shift in the liver cellular metabolism, which become poised toward peroxisome proliferator-activated receptor (PPAR) α-regulated fatty acid catabolism in mitochondria. After dietary treatment of rats with the hypolipidemic, modified fatty acid tetracetylthioacetic acid (TTA), the energy state parameters of the liver were altered at the tissue, cell, and mitochondrial levels. Thus, the hepatic phosphate potential, energy charge, and respiratory control coefficients were lowered, whereas rates of oxygen uptake, oxidation of pyridine nucleotide redox pairs, β-oxidation, and ketogenesis were elevated. Moderate uncoupling of mitochondria from TTA-treated rats was confirmed, as the proton electrochemical potential (ΔpH) was 15% lower than controls. The change affected the Δψ component only, leaving the ΔpH component unaltered, suggesting that TTA causes induction of electrogenic ion transport rather than electroforetic fatty acid activity. TTA treatment induced expression of hepatic uncoupling protein 2 (UCP-2) in rats as well as in wild type and PPARα-deficient mice, accompanied by a decreased double bond index of the mitochondrial membrane lipids. However, changes of mitochondrial fatty acid composition did not seem to be related to the effects on mitochondrial energy conductance. As TTA activates PPARα, we discuss how this subtype might compensate for deficiency of PPARα. The overall changes recorded were moderate, making it likely that liver metabolism can maintain its function within the confines of its physiological regulatory framework where challenged by a hypolipemic agent such as TTA, as well as others.

Administration of 3-thia fatty acids to rats leads to hypolipidemia. The metabolism and biological effects of these non-oxidizable fatty acid analogues, of which tetracetylthioacetic acid (TTA) is the most studied, have been reviewed (1–4). A considerable body of evidence points to shifts in the liver cellular metabolism, resulting in channeling of fatty acids to an enhanced mitochondrial β-oxidation, at the expense of triacylglycerol synthesis. Simultaneously, there is up-regulation of the inner carnitine palmitoyltransferase II, 2,4-dienoyl-CoA reductase, and mitochondrial 3-hydroxy-3-methyl-CoA synthase. The outer carnitine palmitoyltransferase-I is not affected, suggesting that the rate control of β-oxidation and ketogenesis resides in steps beyond acyl group translocation into the matrix (4–6). Modulation of lipid metabolism with TTA seems at least in part to be related to the role of TTA as a regulator for members of the peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors. TTA has been demonstrated to function as a ligand and activator of the PPAR subtypes PPARα, PPARδ, and PPARγ (4, 7–9). PPARα is the predominant subtype in the liver where it controls transcription of genes involved in fatty acid metabolism, such as the genes for peroxisomal acyl-CoA oxidase and fatty acid transport protein, which are up-regulated after TTA treatment (7, 9).

Mitochondrial uncoupling by fatty acids has been widely demonstrated during the last decades. Energy coupling is impaired when protons and other ions are allowed to pass through the inner membrane without the production of ATP. Consequently, the stored energy from the mitochondrial proton gradient intended for ATP synthesis is converted to heat. Wojtczak et al. (10) have demonstrated protonophoric behavior in vitro of high concentrations of 3-thia fatty acids toward the mitochondrial inner membrane. The concentration range causing rapid transbilayer movement of acyl chains was on par with that of normal, unipolar long chain fatty acids like palmitic or oleic acids. A similar behavior has been shown to apply to other hypolipidemic fatty acid analogues, such as β,β'-methyl-substituted-hexadecane-α,ω-dioic acid (11). The molecular basis for

1 The abbreviations used are: TTA, tetracetylthioacetic acid; Δψ, proton electrochemical potential; Δψ, membrane potential (electrical potential difference); ΔpH, pH difference; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PPAR, peroxisome proliferator-activated receptor; UCP, uncoupling protein; TTP, tetraphenylphosphonium; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
fatty acid-mediated uncoupling of respiration remains unclear, but both passive and protein-mediated mechanisms appear to be involved. Skulachev (12) introduced the hypothesis of fatty acid cycling, assuming spontaneous translocation (flip-flop) of the protonated form of the fatty acid in one direction (toward matrix) and a transfer of the anionic form in the other direction, mediated by some inner membrane proteins. Putative candidates for such proteins are the ADP/ATP-PFAR− and the uncoupling proteins (UCPs) (12, 13). UCP homologues form a family of mitochondrial carriers that are capable of depleting the proton gradient. The UCP subtypes, UCP-1, UCP-2, and UCP-3, differ in respect to tissue distribution and probably also function. UCP-1 appears to be solely expressed in brown adipose tissue where it mediates thermogenesis, whereas UCP-2 and UCP-3 are more widely expressed. The functions of UCP-2 and UCP-3 are still unclear, but a mild uncoupling of respiration could prevent the accumulation of oxygen radicals and/or control the NAD+/NADH ratio and consequently regulate metabolic pathways such as ketogenesis and lipogenesis (14, 15). The activities of the UCPs are induced by free fatty acids (16). Furthermore, mono- and polyunsaturated fatty acids, but not saturated fatty acids, were found to increase UCP-2 expression in hepatocytes possibly via a PFARα-mediated pathway (17). Others have found that PFARα mediates in vivo regulation of hepatic ucp-2 gene expression and that PFARγ has the same property in brown adipose tissue (18).

These observations suggest the possibility that PFAR activation and increased β-oxidation rate in liver mitochondria of rats fed TTA might be associated with increased proton conductance across the membrane. We have investigated whether this occurs in vivo after long term feeding of TTA to rats, by measuring energy state parameters at the tissue level, the cellular level, as well as at the level of isolated mitochondria, and if so to assess the extent to which such a mechanism might contribute to increased fatty acid oxidation.

**EXPERIMENTAL PROCEDURES**

**Materials**—TTP was synthesized as described previously (19). [U-14C]-sucrose, [1-14C]l-palmitoyl-l-carnitine, [1-14C]l-palmitoyl-CoA, [1-3H]-ulinin, [1-3H]tetrathenophosphonous (TTP), [14C]5,5'-dimethylx- azoline-2,4-dione, and dextran T-40 were obtained from Amersham Biosciences. Unlabeled species of the same compounds were purchased from Sigma. Saponin was from Fluka-Chemika-Biochemica, Switzerland. Other chemicals were of the highest purity commercially available.

**Animals**—Male Wistar rats were obtained from Mellegaard Breeding Laboratory, Eiby, Denmark. They were housed in pairs in wire cages and maintained on a 12-h cycle of light and dark at 20 ± 3 °C. The rats had free access to pellet food and water, and they were acclimatized to these conditions for at least 1 week prior to the experiment. Each test and control group consisted of at least 4 animals. If not otherwise stated, palmitic acid and TTA were separately dissolved in acetone and sprayed on pellets to an amount of 3 g/kg of pellets, resulting in an approximate daily dose of 300 mg/kg body weight (estimated consumption as stated, palmitic acid and TTA were separately dissolved in acetone and sprayed on pellets to an amount of 3 g/kg of pellets, resulting in an approximate daily dose of 300 mg/kg body weight (estimated consumption as stated, palmitic acid and TTA were separately dissolved in acetone and sprayed on pellets to an amount of 3 g/kg of pellets, resulting in an approximate daily dose of 300 mg/kg body weight (estimated consumption as given by Christiansen & Fournier, France) or 1.7% TTA. At termination the animals were anesthetized with a subcutaneous injection of Hypnorm DormicumTM from Sigma. Saponin was from Fluka Chemica-Biochemica, Switzerland.

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Changes in contents and composition of nucleotides in livers of rats given TTA versus palmitic acid as dietary supplement

Nucleotides and inorganic phosphate were determined after freeze clamping of livers in vivo as described under “Experimental Procedures,” and corrected for the amounts due to blood contaminating the tissue. Contents are given as nanomoles/g wet weight.

**TABLE I**

| Nucleotide components | Dietary supplement |
|-----------------------|-------------------|
|                       | Palmitic acid     | TTA               |
| AMP + ADP + ATP       | 3898 ± 34         | 3769 ± 131        |
| GDP + EP             | 479 ± 34          | 425 ± 17          |
| NAD + NADH           | 781 ± 36          | 1421 ± 101        |
| NADP + NADPH         | 338 ± 23          | 457 ± 18          |
| Inorganic phosphate  | 3500 ± 613        | 3678 ± 174        |

| Tissue energy parameters | Palmitic acid | TTA |
|--------------------------|--------------|----|
| [NAD]/[NADH]             | 27.1 ± 1.22  | 58.4 ± 4.3 |
| [NADP]/[NADPH]           | 0.40 ± 0.12  | 0.60 ± 0.06 |
| Phosphorylation state    | 497 ± 69     | 340 ± 35     |
| Energy charge            | 0.73 ± 0.03  | 0.64 ± 0.06  |

* NS, not significant for differences between TTA- and palmitic acid-treated rats; n = 5.
* * p < 0.05.
† * p < 0.01.

Incubation was performed in uncapped tubes at 25 °C. For measurement of the transmembrane pH difference (ΔpH), labeled TTP was exchanged for 0.18 μCi of [14C]5,5-dimethylxazolidine-2,4-dione/ml + 0.6 μCi of [3H]inositol. Experiments were started by addition of substrate, either 40 nmol of palmitoyl-L-carnitine/mg of mitochondrial protein, in the presence of 1 mM malate, pH 7.4, or 2.5 mM Tris succinate, pH 7.4, in the presence of 10 μg of rotenone. At 3 and 5 min appropriate samples were withdrawn and added to tubes previously charged with a silicon oil layer (ρ = 1.5) above a layer of 10% (w/v) perchloric acid (ρ = 1.2), followed by centrifugation for 1 min at 15,000 rpm (Eppendorf microcentrifuge). For the ΔpH experiments, the disappearance of [14C] from the upper, incubation medium layer was recorded by subjecting aliquots of that layer to scintillation counting, whereas for the ΔΔpH experiments, aliquots were withdrawn from both the upper as well from the bottom (perchloric acid) layers, and subjected to dual-channel scintillation counting. Determination of intra-mitochondrial volume was made by recording the transmembrane distributions of [14C]sucrose and [3H]O2 (32). The sucrose impermeable space of liver mitochondria isolated from animals given dietary palmitate was 1.27 ± 0.11 versus 1.28 ± 0.14 μg/ml of protein for the TTA-treated ones (n = 12). No correction was applied for possible overestimation of ΔpH because of passive TTP binding since the measured ΔpH was always higher than the figure where deviation from Nernst behavior has been demonstrated (34). Parallel, polarographic incubations were used to verify that a steady state rate of oxygen uptake existed within the time frame of withdrawal of aliquots.

Mitochondrial Fatty Acid Composition—TTA suspended in 0.5% carboxymethylcellulose was administered to rats by orogastric intubation (150 mg/kg body weight) once daily for 10 days. Control animals received carboxymethylcellulose only. PPARγ−/− and PPARγ+/− mice were given the diet described above. Lipids were extracted from the liver mitochondrial fractions of rats and mice, transesterified with BF3-methanol, and analyzed essentially as described in Ref. 35. The methyl esters of fatty acids were analyzed on a GC 8000 (Hewlett-Packard) using a column comprised of a highly polar SP 2340 phase with film thickness 0.25 mm (Supelco). Natural occurring fatty acids were positively identified by comparison to known standards (Larodan Fine Chemicals, and probe, 5'-TGGACATGAGTCTCAG-3' and probe, 5'-TGGACATGAGTCTCAG-3', and probe, 5'-TGGACGCTTGTCCTAG-3', and probe, 5'-TGGACGCTTGTCCTAG-3', and probe, 5'-TGGACGCTTGTCCTAG-3', and probe, 5'-TGGACGCTTGTCCTAG-3', and probe, 5'-TGGACGCTTGTCCTAG-3', and probe, 5'-TGGACGCTTGTCCTAG-3'. The GAPDH was used as endogenous control for normalization of cDNA amounts. This analysis was also performed on isolated hepatocytes (performed as described above) that were purified by centrifugation on a 45% Percoll cushion to minimize the influence from Kuffer cells (17).

Western Analysis—Protein from extracts were separated by SDS-PAGE and transferred to nitrocellulose membrane (Hybond ECL, Amersham) according to standard techniques. Blots were probed with a polyclonal goat antibody to UCP-2 (sc-6525, Santa Cruz Biotechnology Inc.) and goat horseradish peroxidase-conjugated anti-rabbit antibody (Bio-Rad).

Statistics and Presentation of Results—The data are presented as mean ± S.D., and differences were evaluated by a two-sample Student’s t test (two-tailed distribution) where relevant. p < 0.05 was regarded as statistical significant.

**RESULTS**

Energy Parameters in Rat Liver—To investigate whether the hypolipidemia caused by supplementing rat diets with 3-thia fatty acids is associated with perturbation of the liver energy state, we established a 7-day regime of feeding animals TTA compared with a set of control animals receiving palmitic acid (which does not cause hypolipidemia), TTA treatment resulted in a lowering of energy state parameters such as phosphate potential by 30% and energy charge by 13%, compared with control livers (Table I). Simultaneously, the liver NAD+/NADH and NADP+/NADPH redox pairs became more oxidized. Whereas the total amounts of adenine nucleotides or inorganic phosphate remained unaffected by the dietary treatment, the amounts of nicotinamide adenine dinucleotide almost doubled and that of nicotinamide adenine dinucleotide phosphate increased by 30%, strongly suggesting that TTA stimulates the oxidative pathways for pyridine nucleotides in liver or, alternatively, inhibits glycohydrolases, which converts NADH to NADPH.

Oxidative Capacities of Primary Hepatocytes from TTA-treated Rats—Hepatocytes prepared from animals given TTA versus palmitic acid in their diets would be expected to expose facets of a mechanism for lowering the liver energy state. Accordingly, a study was undertaken of cellular production of acid-soluble products from labeled fatty acid substrates (as a measure of β-oxidation) as well as of fatty acid-stimulated ketogenesis and respiratory rates. As shown in Table II, TTA feeding caused increases in rates of cellular β-oxidation by 1.4–1.9-fold, eicosapentaenoic acid (EPA) being the better substrate among the fatty acids supplied. Stimulation by TTA feeding on ketogenesis in cells was more pronounced for palmitic acid (2-fold) and EPA (1.9-fold) than for docosahexaenoic acid (DHA; 1.5-fold) as sources of carbon. The fatty acid-stimulated rates of oxygen uptake responded similarly (1.3-fold-
increase) to TTA feeding of source animals. The oxygen uptake rates measured in the uncoupled state (preincubation with FCCP) were almost identical with any fatty acid substrate, regardless of feeding regime, indicating that the capacity of the mitochondrial respiratory chain had not been altered by the diet supplement. However, as a consequence there was some indication of a lowering of energy transducing activity in cells from TTA-treated rats compared with controls. Thus, respiratory control ratios for all substrates were significantly lower: from 2.8 to 1.9 for palmitic acid, from 2.8 to 2.0 for EPA, and from 2.9 to 2.2 for DHA. These changes, mitochondrial in origin, may plausibly be interpreted as increased proton conductance, leading to lower rates of ATP production.

To test for possible lack of citric acid cycle intermediates in mitochondria, hepatocytes were preincubated with malate. This addition affected neither β-oxidation nor oxygen uptake rates with palmitic acid as substrate but, surprisingly, did cause a stimulation of ketogenesis 7-fold in cells from palmitic acid-treated rats against 4.4-fold for the TTA-fed rat cells. As neither O2 uptake rates, nor rates of β-oxidation were correspondingly elevated in the presence of added malate, carbon from malate would appear to be channeled to ketone bodies, possibly from pyruvate via malic enzyme (the precise source of carbon for ketogenesis under these conditions is under investigation).

Effect of TTA as Diet Supplement on the Energy Metabolism of Isolated Rat Liver Mitochondria—One probable cause of the low energy state of the liver in rats subjected to dietary treatment with TTA would be deficient ATP production. Consequently, liver mitochondria isolated from the two groups of animals were examined for energy transducing properties. TTA treatment stimulated the oxygen uptake rates of mitochondria oxidizing palmitoyl-CoA or palmitoyl-l-carnitine by 25 and 20%, respectively (Table III). The measured rates of O2 uptake were not different from those recorded where the mitochondria had been preincubiated with 0.3 nmol of oligomycin/mg of mitochondrial protein, and were thus regarded as representing state 4. However, the chosen concentration of added malate (5 mM) may have been excessive as replenishment of lost citric acid cycle intermediates and competed with the products of β-oxidation as source of reducing equivalents for the respiratory chain. Thus, in the presence of 0.5 mM palmitic acid, malate uptake rates with fatty acyl substrates were lower, but stimulation of state 4 in mitochondria from TTA-treated rats became even more pronounced (50% increase). Oxidation rates with succinate (+ rotenone) were approximately equal with mitochondria from the two groups of rats. Yet, the overall parameters of energy transduction, respiratory control, and ADP/O ratios, decreased significantly by 10% with mitochondria from TTA-treated rats versus controls regardless of the substrate used, suggesting that an increase in proton conductance had taken place. A similar effect has been detected in liver mitochondria of mice subjected to long term dietary treatment with TTA (36). In parallel experiments, rates of β-oxidation from palmitoyl-CoA had increased by 40% (not shown) over controls, in agreement with previous studies (6, 37).

Energy transduction in rat liver mitochondria from the two groups of rats was investigated by measuring proton electrochemical potentials (Δp) under conditions where O2 uptake rates were in a steady state. From Fig. 1A it appears that mitochondria from TTA-treated rats, respiring on succinate (+ rotenone) or palmitoyl-l-carnitine, maintained a Δp 15% lower than that of control mitochondria. Furthermore, this change affected the Δψ component only (Fig. 1B), leaving the ΔpH component unaltered. Thus, although the O2 uptake rates with fatty acyl substrates had increased (Table III), this increase was clearly insufficient to compensate for the loss of Δψ. It follows that TTA as a dietary supplement for rats results in an increase in proton conductance across liver mitochondrial membranes.

Also addressed was the question of whether the concentration of nonesterified TTA, which obtains in the liver under the given feeding conditions, would be sufficiently high to acutely cause the observed loss of Δψ. According to Ref. 38 rats receiving 300 mg of TTA/kg of body weight as a dietary supplement yield a total liver concentration of 20 nmol of nonesterified TTA/g wet tissue. Calculations to express this figure in terms of nanomoles/mg of mitochondrial protein were as outlined by Fleischer et al. (39), using values of 0.12 g of total protein/g wet liver and 0.25 g of mitochondrial protein/g of total protein that yields the figure of 30 mg of mitochondrial protein/g wet liver. The amount of nonesterified TTA in the liver of treated animals would therefore be of the order of 20/30 = 0.67 nmol/mg of mitochondrial protein. In accordance with this, experiments were made in which mitochondria from control animals were preincubiated with 0.6 nmol of TTA/mg of protein for 10 min prior to measurements of energy transduction parameters. As shown in Table III and Fig. 1 this amount of TTA did not measurably affect any parameter, findings that practically exclude the possibility that the observed lowering of Δψ was directly effected by the endogenous, nonesterified TTA present.

Mitochondrial Fatty Acid Composition—TTA can be converted to CoA thioester-like natural fatty acids (40) and is incorporated into different lipid classes including phospholipid, glycerophospholipid, and triacylglycerol. The fatty acid composition of phospholipids and glycerophospholipids of liver mitochondria from TTA-treated and control rats is shown in Table II. As expected, the composition of fatty acids was similar in the two groups of rats, except for the occurrence of Δ9,12- and Δ11,14-docosadienoic acids in the phospholipid fraction of the liver mitochondria of rats treated with TTA. These fatty acids were not found in controls, and the levels in TTA-fed rats were greater than those in control mitochondria.

\[ \text{TABLE II} \]

Oxidation of fatty acids by primary hepatocytes as a function of dietary treatment with palmitic acid or TTA

Experiments were performed as described under "Experimental Procedures." Production of acid-soluble radioactivity from [1-14C] fatty acid is given as nanomoles of fatty acid consumed/h/2 × 10\(^6\) cells at 30 °C. Ketogenesis is given as production of β-hydroxybutyrate as micromoles/h/2 × 10\(^6\) cells at 37 °C, and rates of oxygen consumption are given as nanogram atoms of oxygen/h/2 × 10\(^6\) cells at 37 °C. The concentrations of FCCP and malate were 5 μM and 10 mM, respectively. PA, palmitic acid.

| Substrate                   | Acid-soluble products | Dietary supplement | Ketogenesis                | Oxygen uptake rate |
|-----------------------------|-----------------------|--------------------|----------------------------|--------------------|
|                             | Palmitic acid         | TTA                | Palmitic acid              | TTA                |
| PA                           | 0.64 ± 0.11           | 1.14 ± 0.12ab      | 0.63 ± 0.018               | 1.28 ± 0.07ab      |
| PA + FCCP                    | 0.67 ± 0.12           | 0.85 ± 0.13        | 4.36 ± 0.17                | 5.64 ± 0.21        |
| PA + malate + FCCP           | 2.30 ± 0.7            | 4.38 ± 0.31b       | 0.523 ± 0.05               | 0.98 ± 0.05        |
| EPA                          | 1.95 ± 0.3            | 2.67 ± 0.41f       | 0.653 ± 0.05               | 1.01 ± 0.07f       |
| EPA + FCCP                   | 1.57 ± 0.01           | 1.51 ± 0.01        | 0.793 ± 0.01               | 1.01 ± 0.01        |
| DHA                          | 1.57 ± 0.01           | 1.51 ± 0.01        | 0.793 ± 0.01               | 1.01 ± 0.01        |
| DHA + FCCP                   | 1.57 ± 0.01           | 1.51 ± 0.01        | 0.793 ± 0.01               | 1.01 ± 0.01        |

\[ ^a p < 0.01 \]

\[ ^b p < 0.05 \] for differences between palmitic acid- and TTA-treated rats, n = 4.
Mitochondrial β-Oxidation, Energy State, and Uncoupling

TABLE III
Energy transducing parameters and rates of oxygen uptake of isolated mitochondria from palmitate- and TTA-treated rats

| Substrate                     | Palmitic acid | Dietary supplement |
|-------------------------------|---------------|--------------------|
|                               | Oxygen uptake rate | ADP/O | Oxygen uptake rate | ADP/O |
|                               | State 4 + FCCP |        | State 4 + FCCP |        |
| Palmitoyl-CoA + 5 mM malate   | 69.5 ± 4.6    | 86.4 ± 3.2        |
| Palmitoyl-CoA + 5 mM malate + TTA | 70.2 ± 3.1    |        | 61.1 ± 2.5        |
| Palmitoyl-CoA + 0.5 mM malate | 40.5 ± 0.9    |        | 24.8 ± 3          |
| Palmitoyl-l-carnitine + 5 mM malate | 20.6 ± 3      | 5.1    | 98.8 ± 6          |
| Palmitoyl-l-carnitine + 5 mM malate + TTA | 21.8 ± 1.8 | 5.1 | 2.0 ± 0.12 |
| Succinate                     | 9.2 ± 6.5     | 10.5 ± 2.4        |
| Succinate + TTA               | 20.4 ± 3.4    | 121.5 ± 3.8       |

To ascertain a true state 4 respiration, parallel experiments run in the presence of 0.2 μg of oligomycin/mg mitochondrial protein did not alter the recorded rate of oxygen uptake.

p < 0.05 for differences between palmitate- and TTA-treated rats; n = 5.

The main finding in this investigation was the lowered energy state of the liver in rats receiving a diet containing the hypolipidemic fatty acid TTA as compared with control rats receiving palmitic acid. This conclusion is evident from data on the phosphorylation state and energy charge pertaining in the liver in vivo.

The measured phosphorylation state (Table I) may not be a proper reflection of the effective “phosphate potential” as it

IDS (38, 41). To test the hypothesis that TTA action on Δψ could be mediated by alterations of fatty acid composition, mitochondria were isolated from TTA-treated rats and controls. The treatment markedly changed the total fatty acid composition in liver mitochondria (Table IV). TTA accumulated in the mitochondria while the levels of the saturated fatty acids 14:0, 15:0, and 17:0 decreased. The total amount of monounsaturated fatty acids was increased, especially the Δ9-monounsaturated fatty acids. The Δ9-desaturated product of TTA was also detected. TTA treatment altered the levels of polyunsaturated fatty acids of the n-3 and n-6 families. The amounts of 18:2 n-6, 18:3 n-3, and 20:5 n-3 were significantly lowered, whereas 18:3 n-6 and 20:3 n-6 were increased. Altogether, these changes resulted in lowering of the double bond index. It is worth noting that the levels of mitochondrial arachidonic acid (20:4 n-6) as well as 22:6 n-3 were unchanged after administration of TTA.

Expression of UCP-2—Members or the UCP family of proteins are able to deplete the mitochondrial proton gradient by allowing transmembrane proton transfer without the production of ATP. The biochemical activities and biological functions of the recently identified UCP-2 and UCP-3 proteins are not well known, but studies have suggested that they play roles in energy expenditure for adaptation of cellular metabolism to an excessive supply of substrates to regulate the ATP level, NAD+/NADH ratio, and various metabolic pathways. UCP-2 may also exert a protective role against formation of free radicals (15). In light of the hypothesized function of UCP-2 in energy regulation, and the central role of the liver in overall energy metabolism, we proposed that hepatocyte UCP-2 would be regulated by TTA. Indeed, TTA treatment of rats stimulated UCP-2 mRNA expression in the liver (Fig. 2A), whereas expression of UCP-1 was not detected (data not shown). Previously, it has been shown that Kupffer cells are responsible for a major portion of the UCP-2 expression in rat liver (42). We therefore measured the UCP-2 mRNA expression in isolated and purified primary hepatocytes from control rats and TTA-treated rats. According to Armstrong and Towle (17) such a procedure allows us to measure UCP-2 in the hepatocyte. Our results clearly showed that the mRNA level of UCP-2 was elevated in hepatocytes from TTA-treated animals (Fig. 2A). This demonstrates that the enhanced UCP-2 mRNA level observed in liver tissue from animals treated with TTA at least in part can be explained by an increased UCP-2 expression in the hepatocytes. Induction of UCP-2 expression was also found at the protein level in liver from TTA-treated rats (Fig. 2B).

Effect of TTA on UCP-2 Expression and Mitochondrial Fatty Acid Composition in Wild Type and PPARα-deficient Mice—To evaluate more conclusively that the effects of TTA are at the level of PPARα activation, as opposed to effects because of incorporation into mitochondrial membrane lipids, a study was performed in wild type and PPARα-deficient mice. A non-fatty acid PPAR activator, fenofibrate, was chosen as a reference control to differentiate effects because of TTA versus simple PPAR activation. Fig. 2C shows that UCP-2 mRNA was increased more than 6-fold in wild type mice given fenofibrate. This effect was completely abolished in PPARα-deficient mice, demonstrating that fenofibrate induces UCP-2 mRNA expression via PPARα, confirming the results reported by Kelly et al. (18). It is noteworthy that TTA induced UCP-2 expression both in wild type and PPARα-deficient mice, suggesting that UCP-2 induction also may be mediated by alternative pathways.
tents of pyridine nucleotides could indicate increased biosynthesis of these components from nicotinamide, and it is tempting to speculate that this resulted from the TTA-stimulated peroxisomal and mitochondrial proliferation (44) causing up-regulated expression of enzymes and other proteins that bind pyridine nucleotides, which might reflect adaptation for increased β-oxidation flux. The regulation of pyridine nucleotide synthesis in the liver is not completely understood, but is said to function under hormonal control (see Ref. 45).

If lowered phosphorylation state of the tissue was caused by impaired mitochondrial energy transduction, then parameters illustrating this effect should respond accordingly. Thus, fatty acid-stimulated rates of oxygen uptake in hepatocytes as well as isolated mitochondria had increased by 30% as a result of TTA treatment. Simultaneously, mitochondria exhibited lowered respiratory control- and ADP/O ratios. That the change in state 4 respiration was a true reflection of altered energy transduction received strong support from lowered mitochondrial proton electrochemical potentials measured under steady-state substrate oxidation (Fig. 1), indicative of higher proton conductance (48) because it is characterized by non-proportionality between the respiratory rate in state 4 and the corresponding Δp under conditions where mitochondria maintain a high Δψ. This condition does clearly not apply in the present experiments, in which the range of measured membrane potentials (90–120 mV) was below the threshold value for the non-ohmic leak (49). Therefore, we do not consider this a valid

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### TABLE IV

Mitochondrial fatty acid composition after treatment with TTA

| Fatty acid | Control | TTA |
|-----------|---------|-----|
| 14:0      | 0.33 ± 0.08 | 0.29 ± 0.03a |
| 15:0      | 0.19 ± 0.02 | 0.13 ± 0.02a |
| 16:0      | 23.5 ± 0.6  | 24.1 ± 1.2  |
| 17:0      | 0.39 ± 0.03 | 0.29 ± 0.02a |
| 18:1 n-9  | 0.13 ± 0.02 | 0.22 ± 0.07a |
| 18:1 n-9  | 5.8 ± 0.2   | 8.6 ± 0.9a  |
| 18:2 n-6  | 20.4 ± 0.8  | 18.3 ± 0.9a |
| 18:3 n-6  | 0.10 ± 0.02 | 0.15 ± 0.03 |
| 20:3 n-6  | 0.78 ± 0.06 | 1.4 ± 0.2a  |
| 20:4 n-6  | 16.5 ± 1.0  | 16.2 ± 0.8  |
| 18:3 n-3  | 0.19 ± 0.02 | 0.12 ± 0.02a |
| 20:5 n-3  | 0.72 ± 0.15 | 0.30 ± 0.09a |
| 22:6 n-3  | 5.9 ± 0.5   | 4.6 ± 1.1   |
| TTA ND    | ND        | ND          |
| TTA:1 n-9 | ND        | 0.20 ± 0.03 |
| MUFA      | 11.8 ± 0.7 | 14.3 ± 1.0a |
| Δ9 MUFA   | 7.6 ± 0.3  | 10.4 ± 0.9a |
| SFA       | 42.2 ± 0.7 | 43.4 ± 1.3  |
| DBIa       | 166.6 ± 4.1 | 154.8 ± 5.6a |

* Significantly different from control, p < 0.05.

ND, not detectable.

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Fig. 1. Proton electrochemical potentials of isolated liver mitochondria from rats given palmitic acid or TTA in the diet: steady state 4 oxidation. Measurements were performed at 25 °C as given under “Experimental Procedures.” Numbers on the abscissa refer to the following substrates and treatments: I, 10 mM succinate (+ rotenone), palmitic acid feeding; 2, 10 mM succinate (+ rotenone) + 0.6 nmol of TTA/mg of protein, palmitic acid feeding; 3, 10 mM succinate (+ rotenone), TTA feeding; 4, 50 mM palmitoyl-CoA/mg of protein, palmitic acid feeding; 5, 50 mM palmitoyl-CoA/mg of protein + 0.6 nmol of TTA/mg of protein, palmitic acid feeding; 6, 50 mM palmitoyl-CoA/mg of protein, TTA feeding. A, Δp = Δψ − 59 ΔψH, where numbers refer to mean Δp for each column. B, components of Δp. Columns are given as mV ± S.D. (n = 5). *, p < 0.01.

applies to the mitochondria because of binding of ADP to cytosolic enzymes and protein structures. Thus, measurements by Veech et al. (43) of components of the combined glycerol dehydration-3-phosphate dehydrogenase and 3-phosphoglycerate kinase of rat liver have indicated that [ADP]free is less than 1/20 of the total tissue ADP. These authors also concluded from measurements of the tissue [pyruvate]/[lactate] that changes in [NADH]/[NADH] were consequential to changes in the phosphorylation state, and as the components of the lactate dehydrogenase and malic enzyme appear to maintain a near equilibrium the tissue [NADPH]/[NADPH] would be proportional (24) to the [NADH]/[NAD]. In this context, the findings of a lowered phosphorylation state and a more oxidized state of both pyridine nucleotide redox pairs in livers of rats receiving TTA would be readily explainable. Present experiments, performed with 12-h fasted animals, did not examine rates of gluconeogenesis and it is therefore not known whether the TTA treatment elevates rates above those of controls although lowered figures for the calculated energy charge might be indicative of an increase in the rate of ATP utilization or decreased ATP generation. The marked elevation of the total tissue con-
Mitochondrial β-Oxidation, Energy State, and Uncoupling

Fig. 2. UCP-2 expression is increased in liver of TTA-treated rats. A, UCP-2 mRNA expression was measured in liver from male Wistar Rats that were given soy oil (control) or soy oil with TTA (150 or 300 mg/kg body weight per day) by orogastric intubation once daily for 10 days. Determinations were carried out three times for each sample, and the level of UCP-2 was normalized to the GAPDH value. The data is presented as %fold induction compared with control ± S.D. (n = 4). Primary hepatocytes were prepared and purified from animals receiving control diet or diet with TTA (approximate dose of 300 mg/kg/day) for 7 days as described under “Experimental Procedures.” The columns represent means of two experiments. B, UCP-2 protein was determined by Western analysis in liver extracts from rats given control, fish oil, or TTA treatment. C, UCP-2 mRNA level was analyzed in livers from wild type (WT) and PPARα-deficient (KO) mice receiving control diet or a diet with fish oil, TTA, or fenofibrate. Results are expressed as mean ± S.D. (n = 6). *p < 0.01.

explanation for the observed leak rate in liver mitochondria of TTA-treated animals. At this low level of Δψ any loss of Δψ should be compensated by a corresponding increase in ΔpH unless an electrophoretic mechanism for proton re-entry was brought into action. We would therefore suggest that the TTA treatment of rats results in expression of a respiration-dependent electrogenic ion transport system in the mitochondrial membrane, allowing a moderate degree of uncoupling.

The question of the cause of the increased proton leak in liver mitochondria of the TTA-treated rats remains. The well-known protonophoric effect of fatty acids on the inner membrane (14, 50, 51) does not apply to the uncoupling that is induced by TTA, because only the Δψ component, and not ΔpH, is affected. This is in contrast to what was observed when TTA was provided at a concentration of 30 nmol/mg of protein to liver mitochondria in vitro, which affected both components (10). Calculations (see above) have shown that the content of nonesterified TTA in the liver after TTA dietary treatment is ~0.7 nmol/mg of mitochondrial protein, probably even lower because of marked mitochondrial proliferation (44). Addition of this low amount of TTA to mitochondria from control animals in vitro failed to affect any measurable change in energy transduction parameters (Table III and Fig. 1).

If dietary treatment with TTA directly inflicts aberrations to mitochondrial membranes, likely targets would be changes in phospholipid components. Indeed, TTA and its Δ9-desaturated product are incorporated into mitochondrial lipids in rat liver (Table IV), even into phospholipids such as phosphatidylethanolamine, cardiolipin, and phosphatidylcholine.2 According to Brookes et al. (52) variations in the fatty acid composition of mitochondrial membrane phospholipids reconstituted in liposomes did not appear to accommodate large differences in proton conductance. In isolated mitochondria, however, the same authors found that proton leak correlated with several phospholipid fatty acid compositional parameters, including unsaturation index, 14:0, 18:1 n-9, 18:3 n-3, 18:3 n-6, and percent of monounsaturated fatty acids (53). Other reports support the understanding that certain aspects of phospholipid composition appear to be strongly correlated to proton conductance (54). Fatty acid composition in rat liver mitochondria was changed after TTA administration (Table IV). The mitochondrial content of 14:0, 17:0, and 20:5 n-3, but not 22:6 n-3, was decreased, probably because of the increased rate of mitochondrial β-oxidation (Table II) (55-57) and replacement of saturated and unsaturated fatty acids with TTA and Δ9-desaturated TTA. This is in agreement with data from Table II and our earlier findings that 20:5 n-3 is a better substrate for fatty acid oxidation than 22:6 n-3 (56, 58). Free fatty acids may uncouple respiration, e.g. via a flip-flop mechanism. The insertion of sulfur in the hydrocarbon chain of TTA makes the fatty acid more hydrophilic than a normal fatty acid, which probably restrains flip-flop across the hydrophobic phase. In support, addition of physiological amounts of TTA (38) to isolated mitochondria did not increase state 4 oxygen consumption, indicating that the mitochondrial membrane proton conductance remained unchanged.

In recent years, protein components, including the novel family of UCPs (UCP-1, UCP-2, and UCP-3) have become implicated as membrane vehicles for proton leakage (15, 59). It was of considerable interest to observe that UCP-2 mRNA and protein levels were increased in the liver of rats and mice under dietary treatment with TTA (Fig. 2). UCP-2 expression has been detected both in Kupffer cells (42) and in hepatocytes (17). It was confirmed that TTA-mediated UCP-2 induction takes place in hepatocytes by assessing the expression in isolated and purified hepatocytes (Fig. 2). For EPA (20:5 n-3), oleic acid (18:1 n-9), and arachidonic acid (20:4 n-6), such behavior involves regulation through a prostaglandin/PPARα-mediated pathway (17). However, in contrast to the PPARα selective drug fenofibrate, TTA induced UCP-2 expression to an equal level in wild type and PPARα-deficient mice (Fig. 2) although the mitochondrial fatty acid composition was differently changed (Table V), especially concerning the level of TTA itself, which accumulated to a much higher degree in mitochondria of PPARα-deficient than wild type animals. These data indicate that UCP-2 is up-regulated by TTA through a PPARα-independent pathway that is unlikely to involve incorporation of TTA into mitochondrial lipids and accompanied changes of fatty acid composition. TTA is a ligand for PPARδ (8, 60) and

2 H. J. Grav, P. Bohov, E. Hvattum, and R. K. Berge, unpublished experiments.
recently it has been published that this PPAR subtype might play a role in the regulation of muscle lipid homeostasis (61). Thus, it is likely that in liver of PPARα-deficient mice, the level of PPARα can compensate for the deficiency of PPARα. As mitochondrial fatty acid metabolizing proteins are encoded by PPARα, and possibly PPARγ, target genes, we propose that PPARα and PPARγ may play a role in determining the metabolic shift, i.e. increased β-oxidation flux and lowered energy state. Elevated expression of the hepatic UCP-2 mRNA is of special interest in this context, in light of the proposal that the UCP-2 protein participates in proton leak mechanisms. The possibility that UCP-2 is involved in electron transport in the mitochondrial membrane, allowing a moderate degree of uncoupling, should be considered.

TTA improves insulin sensitivity and reduces adiposity (4, 9). Drainage of fatty acids by the liver, relieving the fatty acid pressure on adipose tissue and muscles when fatty acids inhibit glucose uptake and oxidation, is an important effect of TTA. Uncoupling of hepatocyte mitochondria by long chain fatty acids under ketogenic conditions may allow production of ketone bodies at rates that are not limited by liver ATP consumption and requirements. TTA increased liver fatty acid oxidation and ketogenesis (Table II), accompanied by stimulated UCP-2 expression (Fig. 2). Thus, these results indicate a possible role of liver UCP-2 in the control of energy status. Accordingly, the UCP-2 protein may be involved in adaptation of lipid metabolism to an excessive supply of fatty acids to regulate the ATP level, the NADH/NAD+ ratio, and various metabolic pathways such as ketogenesis (15).

It has also been suggested that UCP-2 is involved in regulation of free radical formation within the cell. Induction of UCP-2 might decrease the redox pressure by preventing the escape of reactive oxygen species from the electron transport chain (62). Indeed, TTA is reported to have antioxidant effects in vitro and in vivo, including decrease in plasma lipid peroxides and malondialdehyde (63–65). In agreement with other suggestions (63), these observations provide indirect evidence for UCP-2 playing a role in management of redox homeostasis.

In summary, long term dietary treatment of rats with 3-thia fatty acid results in lowering of the liver energy state while lipid metabolism become poised toward increased mitochondrial fatty acid oxidation accompanied by increased rates of ketogenesis and oxygen uptake, over and above that which is characteristic of the fasted state in control animals. Simultaneously, the liver mitochondria acquire a definitive proton leakage, characterized by a partial loss of the proton electrochemical potential maintained during substrate oxidation in vitro. Whatever the vehicle or mechanism responsible for membrane leakiness, be it induced ion electrogenic transport, induced UCP-2 expression, fatty acid-dependent uncoupling, or compositional change in membrane phospholipids, increased rates of proton re-entry would support higher rates of β-oxidation. Elevated β-oxidation flux, aided by up-regulated carnitine palmitoyltransferase-II (4), would preferentially channel reducing equivalents to the respiratory chain at the expense of the citric acid cycle (66, 67). Simultaneously, there is up-regulation of the mitochondrial hydroxymethylglutaryl-CoA synthase, resulting in a 60% increase in plasma β-hydroxybutyrate (6), suggesting that the liver β-hydroxybutyrate dehydrogenase should possess an additional capacity for re-oxidation of NADH during fatty acid oxidation. The changes in fatty acid catabolism because of long term dietary treatment with TTA is judged to be sufficiently moderate to warrant the conclusion that liver lipid metabolism remains within the confines of its normal regulatory network.

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Table V

| Fatty acid | Wild type mice | Control | TTA | PPARα-deficient mice | Control | TTA |
|-----------|----------------|---------|-----|----------------------|---------|-----|
| 12:0      | 0.32 ± 0.03    | 0.08 ± 0.01* | 0.12 ± 0.03 | 0.16 ± 0.05 |
| 14:0      | 0.42 ± 0.05    | 0.10 ± 0.02* | 0.24 ± 0.09 | 0.38 ± 0.19 |
| 15:0      | 0.10 ± 0.02    | 0.07 ± 0.01* | 0.08 ± 0.01 | 0.09 ± 0.02 |
| 16:0      | 0.23 ± 1.09    | 0.40 ± 0.6 | 19.7 ± 1.6 | 12.8 ± 2.0* |
| 16:1 n-9  | 0.15 ± 0.37    | 0.35 ± 0.09* | 0.18 ± 0.07 | 0.25 ± 0.05* |
| 18:1 n-9  | 20.1 ± 4.2     | 14.3 ± 2.2 | 1.6 ± 0.2 | 13.7 ± 2.8 |
| 18:2 n-6  | 16.2 ± 1.7     | 17.5 ± 1.4 | 27.3 ± 2.4 | 22.0 ± 2.8* |
| 18:3 n-6  | 0.27 ± 0.05    | 0.15 ± 0.02* | 0.17 ± 0.04 | 0.46 ± 0.12* |
| 20:3 n-6  | 1.17 ± 0.14    | 1.74 ± 0.26* | 0.77 ± 0.20 | 0.25 ± 0.09* |
| 20:4 n-6  | 13.5 ± 4.1     | 18.0 ± 1.1 | 15.3 ± 1.9 | 13.1 ± 2.8 |
| 19:3 n-6  | 0.27 ± 0.07    | 0.09 ± 0.02* | 0.56 ± 0.19 | 0.44 ± 0.34 |
| 20:5 n-3  | 0.14 ± 0.02    | 0.20 ± 0.02 | 0.26 ± 0.06 | 0.12 ± 0.04* |
| 22:6 n-3  | 5.2 ± 1.6      | 7.4 ± 0.8 | 8.7 ± 0.9 | 7.2 ± 1.6 |
| TTA       | ND*            | 0.88 ± 0.39 | ND  | 8.4 ± 3.1 |
| TTA:1 n-9 | ND             | 0.02 ± 0.01 | ND  | 0.13 ± 0.08 |
| MUFA      | 28.0 ± 7.5     | 19.2 ± 2.6* | 11.6 ± 3.4 | 19.3 ± 3.7* |
| Δ9 MUFA   | 23.3 ± 6.7     | 15.7 ± 2.9* | 8.9 ± 2.5 | 15.9 ± 3.5* |
| SFA       | 43.4 ± 3.1     | 34.1 ± 1.3 | 34.2 ± 1.1 | 32.2 ± 3.2 |
| DBI       | 155.4 ± 19.9   | 180.8 ± 5.0* | 190.6 ± 8.8 | 176.4 ± 10.8* |

* Significantly different from control, p < 0.05.

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OF UCP-2 EXPRESSION INDEPENDENT INDUCTION

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