Diabetes or peroxisome proliferator-activated receptor α agonist increases mitochondrial thioesterase I activity in heart

Kristen L. King,* Martin E. Young,† Janos Kerner,§ Hazel Huang,* Karen M. O'Shea,§ Stefan E. H. Alexson, Charles L. Hoppel,†† and William C. Stanley[1,*,§,§§]**

Department of Physiology and Biophysics,* Case Western Reserve University, Cleveland, OH 44106; Department of Pediatrics,† Baylor College of Medicine, Houston, TX 77030; Department of Nutrition,§ School of Medicine, Case Western Reserve University, Cleveland, OH 44106; Department of Laboratory Medicine,** Karolinska Institutet, Karolinska University Hospital at Huddinge, Stockholm, Sweden; Department of Pharmacology,†† Case Western Reserve University, Cleveland, OH 44106; and Department of Medicine,§§ Division of Cardiology, University of Maryland School of Medicine, Baltimore, MD 21201

Abstract Peroxisome proliferator-activated receptor α (PPARα) is a transcriptional regulator of the expression of mitochondrial thioesterase I (MTE-I) and uncoupling protein 3 (UCP3), which are induced in the heart at the mRNA level in response to diabetes. Little is known about the regulation of protein expression of MTE-I and UCP3 or about MTE-I activity; thus, we investigated the effects of diabetes and treatment with a PPARα agonist on these parameters. Rats were either made diabetic with streptozotocin (55 mg/kg ip) and maintained for 10–14 days or treated with the PPARα agonist fenofibrate (300 mg/kg/day) for 4 weeks. MTE-I and UCP3 protein expression, MTE-1 activity, palmitate export, and oxidative phosphorylation were measured in isolated cardiac mitochondria. Diabetes and fenofibrate increased cardiac MTE-I mRNA, protein, and activity (~4-fold compared with controls). This increase in activity was matched by a 6-fold increase in palmitate export in fenofibrate-treated animals, despite there being no effect in either group on UCP3 protein expression. Both diabetes and fenofibrate caused significant decreases in state III respiration of isolated mitochondria with pyruvate + malate as the substrate, but only diabetes reduced state III rates with palmitoylcarnitine. Both diabetes and specific PPARα activation increased MTE-I protein, activity, and palmitate export in the heart, with little effect on UCP3 protein expression.—King, K. L., M. E. Young, J. Kerner, H. Huang, K. M. O'Shea, S. E. Alexson, C. L. Hoppel, and W. C. Stanley. **Diabetes or peroxisome proliferator-activated receptor α agonist increases mitochondrial thioesterase I activity in heart. *J. Lipid Res.* 2007.** 48: **1511–1517.**

Supplementary key words cardiac • fatty acids • lipotoxicity

Fatty acids are the primary fuel for mitochondrial ATP production in the heart (1) and also act as ligands for the peroxisome proliferator-activated receptor α (PPARα), a ligand-activated nuclear receptor that increases the expression of key proteins that regulate cellular fatty acid uptake and oxidation (2). It was recently demonstrated that the mRNA expression for two mitochondrial proteins involved in fatty acid metabolism, mitochondrial thioesterase I (MTE-I) and uncoupling protein 3 (UCP3), is increased in the heart by either diabetes or activation of PPARα (3–5). UCP3 resides on the inner mitochondrial membrane and can export fatty acid anions across the inner mitochondrial membrane (6–8). MTE-I mRNA and protein are most strongly expressed in tissues with high rates of fatty acid oxidation, such as brown adipose tissue, liver, kidney, skeletal muscle, and heart (9, 10). MTE-I catalyzes the formation of fatty acid anions from long-chain acyl-CoA in the mitochondrial matrix, which are then exported to the cytosol, suggesting that UCP3 and MTE-I act together to help regulate the long-chain fatty acyl-CoA concentration in the mitochondrial matrix (3, 7, 11). Little is known about the effects of diabetes and PPARα activation on UCP3 and MTE-I expression or on mitochondria respiration and fatty acid export from the mitochondria.

Recent studies suggest that diabetes and the subsequent increase of plasma fatty acids can cause excessive accumulation of lipids in the heart, resulting in cardiac dysfunction and cardiomyopathy (12, 13), and that this condition can be mimicked by cardiac-specific overexpression of PPARα (14). Gerber, Aronow, and Matlib (3) recently

Manuscript received 16 August 2006 and in revised form 15 December 2006 and in re-revised form 26 March 2007.

Published, JLR Papers in Press, April 16, 2007.
DOI 10.1194/jlr.M600364-JLR200

Abbreviations: IFM, interfibrillar mitochondria; MTE-I, mitochondrial thioesterase I; P/O, ADP-to-oxygen ratio; PPARα, peroxisome proliferator-activated receptor α; RCR, respiratory control ratio; SSM, subsarcolemmal mitochondria; UCP3, uncoupling protein 3.

[1] To whom correspondence should be addressed.
e-mail: wstanley@medicine.umaryland.edu

observed that diabetes upregulates MTE-I protein expression and activity in the heart; however, it is not known whether selective activation of PPARα mimics this effect. In addition, the consequences of alterations in MTE-I protein expression on mitochondrial function are not well understood (11, 15). Altered mitochondrial respiration in liver, skeletal muscle, and heart has been observed in response to perturbations that alter UCP3 and/or MTE-I expression (e.g., treatment with fenofibrate or thyroid hormone, caloric restriction, fasting, or diabetes) (3, 16–22). Induction of MTE-I should facilitate the export of fatty acid anions and reduce the accumulation of toxic long-chain acyl-CoAs (3, 7, 23–25) and thus might help prevent the lipotoxic cardiomyopathy that can develop in diabetes (12, 13), with accelerated lipid uptake or impaired fatty acid metabolism (26–29).

The goal of this investigation was to determine whether the initiation of diabetes and selective PPARα activation increases the protein expression of MTE-I and/or UCP3, the mitochondrial activity of MTE-I, or the function of isolated mitochondria from the rat heart. We hypothesized that PPARα stimulation with diabetes, and pharmacologically with fenofibrate, would increase the protein expression and activity of MTE-I and the export of fatty acid anions from isolated mitochondria. Because diabetes decreases cardiac mechanical efficiency by increasing myocardial oxygen consumption (30), we also investigated whether diabetes or treatment with fenofibrate would decrease the ADP-to-oxygen ratio (P/O) or increase state IV respiration in the mitochondria (31, 32). Experiments were performed in established rat models of fenofibrate treatment and streptozotocin-induced diabetes that have previously shown upregulation of the mRNA of MTE-I and UCP3. It is well documented that cardiac mitochondria exist in two distinct populations, with the interfibrillar mitochondria (IFM) subpopulation having a higher respiratory capacity per milligram of mitochondrial protein than the subsarcolemmal mitochondria (SSM) subpopulation (33); thus, both populations were isolated and studied.

MATERIALS AND METHODS

Animals

Experiments were performed on male Wistar rats that were 16 weeks old at the time of euthanasia. Animal care was carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 85-23). The protocol was approved by the Institutional Animal Care and Use Committee at Case Western Reserve University.

Study design

Three series of studies were performed. In series I, diabetes was induced with streptozotocin (55 mg/kg ip) (n = 9) and maintained for 10–14 days; these animals were then compared with a parallel untreated control group of rats matched for age (n = 8). In the diabetic rats, plasma glucose was monitored to ensure that it was >250 mg/dl. In series II, rats were fed a standard rat chow or treated with the PPARα agonist fenofibrate [300 mg/kg/day in the chow (34)] (n = 12/group). The cardiac mitochondria from series I and II were used to measure respiration, the expression of UCP3 and MTE-I mRNA and protein, and the activity of MTE-I. In series III, rats were either untreated or treated with fenofibrate as in series II (n = 9/group), and the rate of fatty acid export and MTE-1 activity were measured in SSM. All groups were in a reverse light/dark cycle for at least 1 week before euthanasia (35) and were euthanized in the fed state 2 h after initiation of the dark phase to ensure peak changes in MTE-I and UCP3 mRNA (4). Rats were anesthetized with isoflurane (1.5–2.0%), the chest was open by sternotomy, and the left ventricle was removed. Cardiac mitochondria were isolated into SSM and IFM fractions as described below, and aliquots were frozen for Western blot and enzyme activities. A separate tissue sample for mRNA analysis was immediately frozen in liquid nitrogen.

Isolation of mitochondria from cardiac tissue

Tissue was placed in buffer A [100 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM $MgSO_4(7H_2O)$, and 1 mM ATP, pH 7.4] at 4°C. Cardiac mitochondria were then isolated according to Hoppel and colleagues (33, 36) with minor modifications. Tissue was finely minced and placed in buffer B (buffer A with 0.2% BSA), homogenized with a Polytron tissue processor (Brinkman Instruments, Westbury, NY) for 2.5 s at a rheostat setting of 6.5, and further homogenized with a Potter-Elvejhem homogenizer at a setting of 3. The homogenate was centrifuged at 600 *g*, with the supernatant being saved for SSM isolation and the pellet being saved for IFM isolation. For the IFM, the pellet underwent Polytron treatment and digestion with trypsin (5 mg/g wet weight) for 10 min at 4°C, and the mitochondria were collected as described above. Both fractions were centrifuged at 3,100 *g* and resuspended in 100 mM KCl, 50 mM MOPS, and 0.5 mM EGTA. Protein was determined using the method of Lowry et al. (37) with BSA as a standard.

Measurement of mitochondrial oxygen consumption

Mitochondrial oxygen consumption was measured using a Clark-type oxygen electrode at 30°C in 80 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM KH_2PO_4, and 1 mg/ml BSA. The substrates pyruvate + malate, palmitoylcarnitine + malate, succinate, durohydroquinone, and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine ascorbate were used to measure specific sites in the electron transport chain, state III (ADP-stimulated) respiration, state IV (ADP-limited) respiration, maximal oxidative phosphorylation with high ADP (2 mM), and uncoupled respiration with dinitrophenol. The respiratory control ratio (RCR) (state III/state IV) and P/O (ratio of oxygen consumed after the addition of a known amount of ADP) were also determined.

Measurement of mitochondrial palmitate export

Palmitate export was measured in isolated SSM from fenofibrate–treated rats according to Gerber, Aronow, and Matlib (3) The export of [1-^{14}C]palmitic acid was measured after incubating isolated mitochondria for 6 min with 20 μM [1-^{14}C]palmitoylcarnitine (specific activity, 10,000 dpm/nmol) at 37°C. The reaction was terminated as described previously (3), and the extramitochondrial fluid was separated by centrifugation and filtration (0.45 μm). Lipids were extracted and fatty acid moieties were separated by thin-layer chromatography, and the palmitic acid spot was visualized and counted (3).

RNA extraction and quantitative RT-PCR

RNA extraction and real-time quantitative RT-PCR were performed, as described previously, on frozen powdered left ven-

tricle tissue (38–40). Previously published rat sequences for the expression of MTE-I and UCP3 were found using GenBank, with specific quantitative assays designed for each (4, 5). The primer sequences were as follows: 5′-GTGACCTATGACATCATCAAGGA-3′ (forward), 5′-GCTCCAAAGGCAGAGACAAAG-3′ (reverse), and 5′-FAM-CTGGACTCTCACCTGTTCACTGACAACTTCC-TAMRA-3′ (probe) for UCP3; and 5′-TGTTGGGAACACCGTATGCT-3′ (forward), 5′-CCACGACATCCAAGAGACCA-3′ (reverse), and 5′-FAM-TTTGGTCATTTTGACTTTGTCTCTCAGAAGGG-TAMRA-3′ (probe) for MTE-1. The T7 polymerase method (Ambion) was used to make standard RNA using total RNA isolated from rat hearts. The correlation between the amount of standard and the number of PCR cycles required for the fluorescent signal to reach a detection threshold was linear over at least a 5 log range of RNA for all assays. The mRNA level for cyclophilin was quantitatively measured in each sample to control for sample-to-sample differences in RNA concentration. Because the expression of cyclophilin was not different among the groups, the PCR data are reported as the number of transcripts per nanogram of total RNA.

Western blot analysis

Western blot analysis for MTE-I and UCP3 was performed on frozen SSM and IFM aliquots in duplicate at 75 and 150 μg of total protein, respectively. The protein was separated by electrophoresis on 4–12% Bis-Tris SDS-PAGE gels and transferred onto a polyvinylidene difluoride membrane. Membranes were blocked with TBS-Tween containing 5% milk and then incubated with primary antibodies to MTE-I (1:2,000) and UCP3 (1:1,000; Alpha Diagnostic). A polyclonal antibody for MTE-I was raised in rabbits immunized with purified rat MTE-I as described previously (9). After treatment with the secondary antibody, the membranes were developed in an enhanced chemiluminescence substrate solution (Amersham) and bands were quantified using NIH software. Variability among gels was corrected for by loading a standard sample of isolated skeletal muscle mitochondria (100 μg of protein) in duplicate on each gel. Results are expressed as a fraction of the mean of the control SSM group. To verify that the blot at 30 kDa was UCP3, isolated cardiac mitochondria from UCP3 knockout and wild-type mice (a gift from Dr. E. Dale Abel, University of Utah) were run alongside samples from all treatment groups, and the expected band at 30 kDa for UCP3 was completely absent for the UCP3 knockout mouse (data not shown).

Measurement of MTE-I and citrate synthase activities

Citrate synthase activity was assessed on frozen SSM and IFM as described previously (41). MTE-I activity was measured spectrophotometrically on SSM and IFM with palmitoyl-CoA as the substrate by following the increase in absorbance at 412 nm in the presence of 5,5′-dithiobis 2-nitrobenzoic acid (22, 42).

Statistical analysis

Effects of treatment and differences between SSM and IFM were determined using a two-way ANOVA with the Bonferroni post hoc test. Differences in mRNA and plasma measurements were determined with a two-tailed *t*-test. Data are presented as means ± SEM, and $p < 0.05$ was considered significant.

RESULTS

Diabetes

Treatment with streptozotocin induced severe diabetes, as seen in the significantly higher plasma concentrations of glucose (42.4 ± 2.5 vs. 10.9 ± 0.6 mM) and fatty acids (2.87 ± 0.57 vs. 0.20 ± 0.06 mM) and lower insulin (22 ± 3 vs. 147 ± 59 pM).

mRNA and protein expression

Diabetes or activation of PPARα with fenofibrate resulted in significant increases in MTE-1 and UCP3 mRNA compared with control animals (**Fig. 1**). MTE-I protein content in SSM and IFM increased by >5-fold with diabetes or fenofibrate treatment compared with controls; however, there were no significant changes in UCP3 protein expression (**Fig. 2**). There were no differences between SSM and IFM in MTE-I or UCP3 protein content. Similar results for MTE-I and UCP3 protein expression were found when data were normalized to mitochondrial citrate synthase activity (data not shown).

Enzyme activities

Mitochondrial citrate synthase activity was unaffected by fenofibrate but decreased with diabetes in both the SSM (1.77 ± 0.18 vs. 2.31 ± 0.23 μmol/mg protein/min) and IFM (2.20 ± 0.17 vs. 2.96 ± 0.37 μmol/mg protein/min) ($p < 0.05$). MTE-I activity was increased by diabetes in both SSM (27.9 ± 4.4 vs. 6.1 ± 1.6 nmol/mg protein/min) and IFM (32.3 ± 3.8 vs. 8.8 ± 2.0 nmol/mg protein/min) ($p < 0.001$). Treatment with fenofibrate had a similar effect on MTE-I activity in both SSM (22.5 ± 2.9 vs. 8.2 ± 1.1 nmol/mg protein/min) and IFM (39.1 ± 3.0 vs. 11.1 ± 2.3 nmol/mg protein/min) ($p < 0.001$). Similar results were evident when MTE-I activity was normalized to mitochondrial citrate synthase activity (**Fig. 3**).

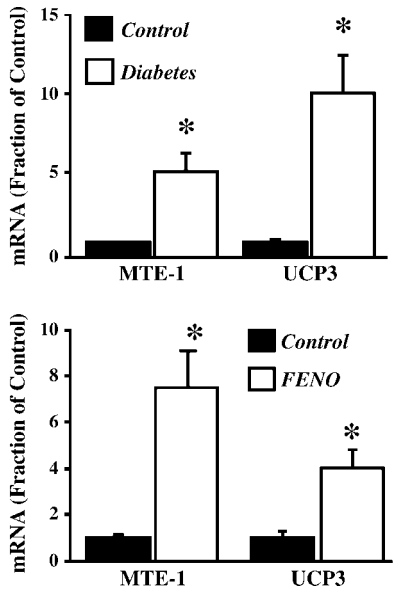

Fig. 1. Cardiac mRNA content for mitochondrial thioesterase I (MTE-I) and uncoupling protein 3 (UCP3) for control (n = 7) and diabetic (n = 8) animals (upper panel) and for control (n = 11) and fenofibrate-treated (FENO) (n = 12) animals (lower panel). Data are presented as means ± SEM. * $P < 0.001$ compared with the respective control group.

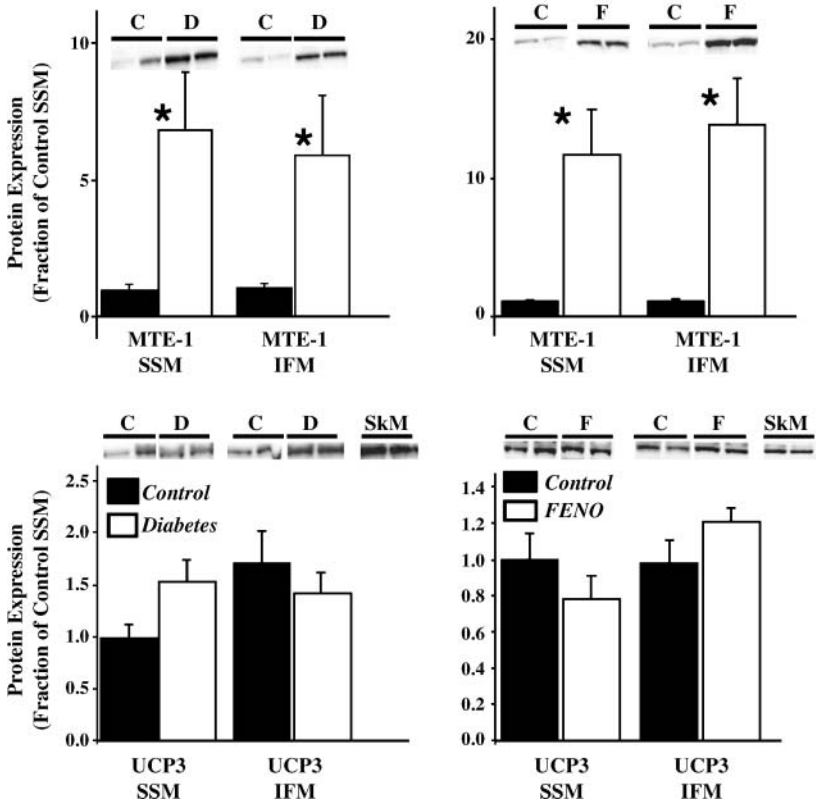

Fig. 2. MTE-I (upper panels) and UCP3 (lower panels) protein expression in isolated mitochondria for control (C) and diabetic (D) animals and for control (C) and fenofibrate-treated (F) animals. Results are expressed normalized to the subsarcolemmal mitochondria (SSM) for the respective control group. Data are presented as means ± SEM. * $P < 0.001$ between control (n = 7) and diabetic (n = 8) animals and between control (n = 10) and fenofibrate-treated (n = 9) animals for MTE-I protein in both SSM and interfibrillar mitochondria (IFM). Representative Western blots are shown above the bar graphs. SkM, skeletal muscle.

Export of palmitate from mitochondria

Activation of PPARα with fenofibrate significantly increased palmitate export from cardiac mitochondria compared with control animals (**Fig. 4**).

Mitochondrial respiration

Diabetes resulted in a significant decrease in state III and state IV respiration in SSM and IFM, with no change in RCR or P/O with pyruvate + malate as substrate (**Table 1**). State III respiration was reduced with palmitoylcarnitine as the substrate in diabetic animals compared with controls in both SSM and IFM, with no change in state IV respiration, RCR, or P/O. Treatment with fenofibrate had an effect similar to diabetes on state III and state IV rates with pyruvate + malate as substrate, with significant reductions in both, and no effect on the RCR or P/O (**Table 2**). In contrast, treatment with fenofibrate had no effect on any parameters when palmitoylcarnitine was used as a substrate (Table 2). The effect of diabetes on the respiratory function of electron transport chain complexes was investigated by measuring the maximal rates of respiration with succinate (complex II), durohydroquinone (complex III), and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine ascorbate (complex IV) (**Table 3**). Maximal rates were decreased significantly with all three substrates, and this effect was not relieved by uncoupling the mitochondria with the addition of dinitrophenol, suggesting a complex IV defect in diabetes but not ruling out possible additional abnormalities at complexes II and III.

DISCUSSION

The results of this study show that diabetes or activation of PPARα increases the protein expression and activity of MTE-I and the rate of fatty acid export from cardiac mitochondria. Neither diabetes nor specific activation of PPARα significantly affected the protein expression of UCP3 despite a >4-fold increase in mRNA for UCP3. These findings add support to the concept that MTE-I functions to regulate the concentration of long-chain fatty acyl-CoAs in the mitochondrial matrix through the generation and export of free fatty acid anions. Therefore, MTE-I appears to play a key role in the adaptation of cardiac mitochondria to the increase in long-chain fatty acids that occurs in diabetes.

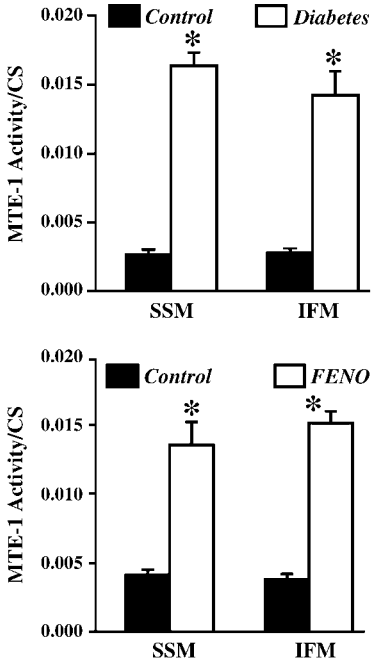

Fig. 3. MTE-I activity for control and diabetic animals (upper panel) and for control and fenofibrate-treated (FENO) animals (lower panel). MTE-I activity was measured in isolated SSM and IFM mitochondria and then normalized to citrate synthase (CS) activity. Data are presented as means ± SEM. * $P < 0.001$ between control (n = 7) and diabetic (n = 8) animals and between control (n = 8) and fenofibrate-treated (n = 9) animals for MTE-1 activity in both SSM and IFM.

These results showed a dramatic upregulation of MTE-1 mRNA, protein expression, and activity by diabetes, which was largely mimicked by pharmacological stimulation of PPARα, suggesting that increased plasma fatty acids in diabetes upregulate MTE-I via ligand activation of PPARα. Upregulation of MTE-I activity in cardiac mitochondria may help prevent the lipotoxic cardiomyopathy that can develop in diabetes (12, 13), as it would reduce the accumulation of toxic long-chain acyl-CoA in the mitochondrial matrix and facilitate the export of fatty acid anions (7, 11, 23–25). Upregulation of MTE-1 in diabetes should also prevent depletion of the free CoA pool and thus help maintain the oxidation of pyruvate and α-ketoglutarate. UCP3 is known to transport fatty acid anions across the inner mitochondrial membrane (43); however, the results of this study demonstrate that although MTE-I protein and activity, and palmitate export, are clearly upregulated with diabetes or PPARα activation, UCP3 protein is not (Fig. 2). Perhaps there is either a sufficient amount of UCP3 in the membrane to support the increased rate of fatty acid export observed after chronic treatment with fenofibrate or other protein(s) are involved in this process. Recent studies show that the plasma membrane fatty acid transport protein CD36 also resides in skeletal muscle mitochondria, suggesting the possibility that it could participate in this process in cardiac mitochondria (44–46).

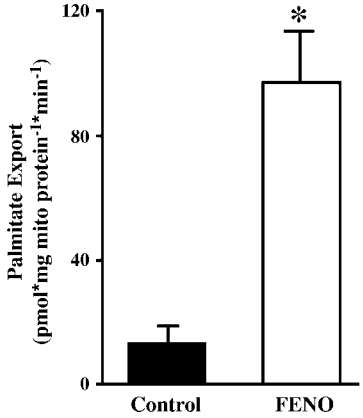

Fig. 4. Mitochondrial palmitate export rates for control and fenofibrate-treated (FENO) animals. Data are presented as means ± SEM; n = 9 for each group. * $P < 0.05$ compared with controls.

TABLE 1. Respiration of isolated cardiac mitochondria in control and diabetic rats

	SSM		IFM	
Variable	Control	Diabetic	Control	Diabetic
Pyruvate + malate				
State III	232 ± 30	146 ± 6[a]	357 ± 44[b]	161 ± 8[a]
State IV	77 ± 12	49 ± 4[a]	132 ± 19[b]	65 ± 5[a,b]
RCR	3.07 ± 0.11	3.06 ± 0.18	2.75 ± 0.07[b]	2.55 ± 0.17[b]
P/O	2.34 ± 0.11	2.49 ± 0.07	2.25 ± 0.12	2.36 ± 0.09
High ADP	185 ± 33	132 ± 9[a]	281 ± 44[b]	150 ± 18[a,b]
Palmitoylcarnitine				
State III	260 ± 43	160 ± 27[a]	372 ± 60[b]	227 ± 21[a,b]
State IV	76 ± 10	60 ± 11	119 ± 19[b]	88 ± 14[b]
RCR	3.35 ± 0.15	3.32 ± 0.36	3.17 ± 0.19	2.79 ± 0.28
P/O	2.23 ± 0.07	2.34 ± 0.12	2.08 ± 0.06	2.18 ± 0.14
High ADP	259 ± 48	149 ± 29[a]	359 ± 61	201 ± 18[a]

IFM, interfibrillar mitochondria; P/O, ADP-to-oxygen ratio; RCR, respiratory control ratio; SSM, subsarcolemmal mitochondria. Values are means ± SEM (n = 7 and 8 animals for control and diabetic groups, respectively). Respiratory rates are expressed as nA oxygen/min/mg mitochondrial protein.

[a] $P < 0.05$ between control and diabetic groups.

[b] $P < 0.05$ between SSM and IFM.

TABLE 2. Respiration of isolated cardiac mitochondria in control and fenofibrate-treated rats

	SSM		IFM	
Variable	Control	Fenofibrate-Treated	Control	Fenofibrate-Treated
Pyruvate + malate				
State III	189 ± 22	144 ± 8[a]	314 ± 49[b]	229 ± 16[a,b]
State IV	51 ± 8	44 ± 2[a]	91 ± 18[b]	66 ± 5[a,b]
RCR	3.8 ± 0.3	3.3 ± 0.2	3.9 ± 0.51	3.5 ± 0.16
P/O	2.45 ± 0.11	2.59 ± 0.05	2.38 ± 0.17	2.55 ± 0.07
Palmitoylcarnitine				
State III	204 ± 17	182 ± 10	334 ± 43[b]	298 ± 18[b]
State IV	54 ± 6	48 ± 2	81 ± 11[b]	74 ± 6[b]
RCR	3.9 ± 0.42	3.8 ± 0.16	4.6 ± 0.81	4.2 ± 0.32
P/O	2.33 ± 0.09	2.27 ± 0.06	2.24 ± 0.11	2.30 ± 0.07

Values are means ± SEM (n = 11 and 12 animals for control and fenofibrate-treated groups, respectively). Respiratory rates are expressed as nA oxygen/min/mitochondrial protein.

[a] $P < 0.05$, significant difference between treatment groups.

[b] $P < 0.05$ between SSM and IFM.

TABLE 3. Effect of diabetes on the respiratory function of electron transport chain complexes as measured from the maximal rates of respiration with succinate (complex II), durohydroquinone (complex III), and TMPD (complex IV)

	SSM		IFM	
Variable	Control	Diabetic	Control	Diabetic
Succinate				
High ADP	287 ± 41	192 ± 20	495 ± 64[a]	250 ± 21[b]
Uncoupling	304 ± 36	186 ± 21[b]	468 ± 65[a]	232 ± 21[a,b]
Durohydroquinone				
High ADP	724 ± 105	546 ± 74[b]	1,004 ± 140	654 ± 73[b]
Uncoupling	1,050 ± 147	804 ± 110[b]	1,482 ± 220	928 ± 93[b]
TMPD				
High ADP	998 ± 85	776 ± 111[b]	1,263 ± 247	867 ± 79[b]
Uncoupling	1,131 ± 107	890 ± 131[b]	1,404 ± 286	990 ± 98[b]

TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine. High ADP indicates the maximal rate of oxidative phosphorylation; uncoupling indicates uncoupled respiration with dinitrophenol. Values are means ± SEM (n = 7 and 8 animals for control and diabetic groups, respectively). Respiratory rates are expressed as nA oxygen/min/mitochondrial protein.

[a] $P < 0.05$ between SSM and IFM.

[b] $P < 0.05$ between control and diabetic groups.

Additional work is needed to determine the specific protein responsible for the export of the fatty acid anion generated by MTE-I in the matrix.

Although several previous studies have established that there is upregulation of the mRNA for UCP3 with fasting, diabetes, or stimulation of PPARα (5), less is known about the regulation of protein expression. The increase in UCP3 mRNA with severe diabetes or treatment with a PPARα agonist is >3-fold (4, 5); however, Murray et al. (15) recently observed a more modest increase of ~50% in UCP3 protein levels in cardiac mitochondria isolated from streptozotocin-induced and db/db diabetic mice and in mice treated with the PPARα agonist WY-14,643. Gerber, Aronow, and Matlib (3) observed a similar increase in UCP3 protein in streptozotocin-induced diabetic rats. In this study, we did not observe a significant increase in protein expression in either the diabetic or fenofibrate group; however, there was a strong trend for an increase in UCP3 in the SSM fraction of the diabetic animals (54% increase compared with the control group) (Fig. 2), and when only the SSM fraction was compared using a *t*-test, the difference was significant ($p < 0.03$). There was no significant increase in UCP3 protein expression in the IFM fraction of either group. In the studies by Murray et al. (15) and Gerber, Aronow, and Matlib (3), the procedure for the isolation of mitochondria would yield mainly SSM, suggesting that any increase in UCP3 is primarily in this fraction. Together, it appears that the increase in mRNA for UCP3 is not proportional to the increase in UCP3 protein expression and that the increase in MTE-I protein expression far exceeds the increase in UCP3.

The effects of diabetes on the expression of MTE-I were emulated by pharmacological activation of PPARα; however, mitochondrial respiration was impaired to a greater extent in diabetes, with a reduction in state III rates with both lipid and nonlipid substrates, whereas PPARα activation only affected nonlipid substrates (Tables 1, 2). Previous studies demonstrated a similar reduction in state III respiration with either glutamate and succinate (31, 32) or pyruvate (30) with diabetes. The results of this study extended these finding by demonstrating reduced respiratory function of electron transport chain complexes II–IV (Table 3). We hypothesized that state IV respiration would be increased, as demonstrated recently by Lashin and Romani (31, 32) in ketotic diabetic rats; however, we observed a significant decrease with pyruvate + malate but not with palmitoylcarnitine (Table 1), consistent with the well-documented decreased activity of pyruvate dehydrogenase in the diabetic heart (47). How et al. (30) did not find any difference in state IV respiration between normal and diabetic mice with either pyruvate or palmitoylcarnitine as substrate, yet they observed decreased cardiac mechanical efficiency (external power generation/myocardial energy expenditure) in working hearts at both low and high fatty acid concentrations. The mechanism for this effect remains unclear.

In conclusion, treatment with a PPARα agonist increases MTE-1 mRNA, protein expression, and activity and the rate of palmitate export from isolated cardiac mitochondria, suggesting that MTE-I functions to regulate the concentration of long-chain fatty acyl-CoA in the mitochondrial matrix through the generation and export of free fatty acid anions. Thus, upregulation of MTE-I activity may prevent the accumulation of long-chain fatty acyl-CoA in the mitochondrial matrix when the heart is exposed to high levels of fatty acids.

The authors thank Dr. Timothy Kern and Chieh Allen Lee for their help with the streptozotocin-induced diabetic rats and Dr. E. Dale Abel and Sihem Boudina of the University of Utah for the cardiac mitochondria from UCP3 knockout and wild-type mice. This work was supported by National Institutes of Health Grants HL-074237 and HL-074259.

REFERENCES

1. Stanley, W. C., F. A. Recchia, and G. D. Lopaschuk. 2005. Myocardial substrate metabolism in the normal and failing heart. *Physiol. Rev.* **85:** 1093–1129.
2. Huss, J. M., and D. P. Kelly. 2004. Nuclear receptor signaling and cardiac energetics. *Circ. Res.* **95:** 568–578.
3. Gerber, L. K., B. J. Aronow, and M. A. Matlib. 2006. Activation of a novel long-chain free fatty acid generation and export system in mitochondria of diabetic rat hearts. *Am. J. Physiol. Cell Physiol.* **291:** C1198–C1207.
4. Stavinoha, M. A., J. W. RaySpellicy, M. F. Essop, C. Graveleau, E. D. Abel, M. L. Hart-Sailors, H. J. Mersmann, M. S. Bray, and M. E. Young. 2004. Evidence for mitochondrial thioesterase 1 as a peroxisome proliferator-activated receptor-alpha-regulated gene in cardiac and skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* **287:** E888–E895.
5. Young, M. E., S. Patil, J. Ying, C. Depre, H. S. Ahuja, G. L. Shipley, S. M. Stepkowski, P. J. Davies, and H. Taegtmeyer. 2001. Uncoupling protein 3 transcription is regulated by peroxisome proliferator-activated receptor (alpha) in the adult rodent heart. *FASEB J.* **15:** 833–845.
6. Brand, M. D., and T. C. Esteves. 2005. Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3. *Cell Metab.* **2:** 85–93.
7. Himms-Hagen, J., and M. E. Harper. 2001. Physiological role of

UCP3 may be export of fatty acids from mitochondria when fatty acid oxidation predominates: an hypothesis. *Exp. Biol. Med. (Maywood).* **226:** 78–84.
8. Krauss, S., C. Y. Zhang, and B. B. Lowell. 2005. The mitochondrial uncoupling-protein homologues. *Nat. Rev. Mol. Cell Biol.* **6:** 248–261.
9. Svensson, L. T., S. E. Alexson, and J. K. Hiltunen. 1995. Very long chain and long chain acyl-CoA thioesterases in rat liver mitochondria. Identification, purification, characterization, and induction by peroxisome proliferators. *J. Biol. Chem.* **270:** 12177–12183.
10. Hunt, M. C., S. E. Nousiainen, M. K. Huttunen, K. E. Orii, L. T. Svensson, and S. E. Alexson. 1999. Peroxisome proliferator-induced long chain acyl-CoA thioesterases comprise a highly conserved novel multi-gene family involved in lipid metabolism. *J. Biol. Chem.* **274:** 34317–34326.
11. Hunt, M. C., and S. E. Alexson. 2002. The role acyl-CoA thioesterases play in mediating intracellular lipid metabolism. *Prog. Lipid Res.* **41:** 99–130.
12. Zhou, Y. T., P. Grayburn, A. Karim, M. Shimabukuro, M. Higa, D. Baetens, L. Orci, and R. H. Unger. 2000. Lipotoxic heart disease in obese rats: implications for human obesity. *Proc. Natl. Acad. Sci. USA.* **97:** 1784–1789.
13. Young, M. E., P. H. Guthrie, P. Razeghi, B. Leighton, S. Abbasi, S. Patil, K. A. Youker, and H. Taegtmeyer. 2002. Impaired long-chain fatty acid oxidation and contractile dysfunction in the obese Zucker rat heart. *Diabetes.* **51:** 2587–2595.
14. Finck, B. N., X. Han, M. Courtois, F. Aimond, J. M. Nerbonne, A. Kovacs, R. W. Gross, and D. P. Kelly. 2003. A critical role for PPARalpha-mediated lipotoxicity in the pathogenesis of diabetic cardiomyopathy: modulation by dietary fat content. *Proc. Natl. Acad. Sci. USA.* **100:** 1226–1231.
15. Murray, A. J., M. Panagia, D. Hauton, G. F. Gibbons, and K. Clarke. 2005. Plasma free fatty acids and peroxisome proliferator-activated receptor {alpha} in the control of myocardial uncoupling protein levels. *Diabetes.* **54:** 3496–3502.
16. Lanni, A., F. Mancini, L. Sabatino, E. Silvestri, R. Franco, G. De Rosa, F. Goglia, and V. Colantuoni. 2002. De novo expression of uncoupling protein 3 is associated to enhanced mitochondrial thioesterase-1 expression and fatty acid metabolism in liver of fenofibrate-treated rats. *FEBS Lett.* **525:** 7–12.
17. Okere, I. C., M. P. Chandler, T. McElfresh, J. Rennison, V. Sharov, H. N. Sabbah, K. Y. Tserng, B. D. Hoit, P. Ernsberger, M. E. Young, et al. 2006. Differential effects of saturated and unsaturated fatty acid diets on cardiomyocyte apoptosis, adipose distribution, and serum leptin. *Am. J. Physiol. Heart Circ. Physiol.* **291:** H38–H34.
18. Morgan, E. E., J. H. Rennison, M. E. Young, T. A. McElfresh, T. A. Kung, K. Y. Tserng, B. D. Hoit, W. C. Stanley, and M. P. Chandler. 2006. Effects of chronic activation of peroxisome proliferator-activated receptor-alpha or high-fat feeding in a rat infarct model of heart failure. *Am. J. Physiol. Heart Circ. Physiol.* **290:** H1899–H1904.
19. Bevilacqua, L., J. J. Ramsey, K. Hagopian, R. Weindruch, and M. E. Harper. 2005. Long-term caloric restriction increases UCP3 content but decreases proton leak and reactive oxygen species production in rat skeletal muscle mitochondria. *Am. J. Physiol. Endocrinol. Metab.* **289:** E429–E438.
20. McLeod, C. J., A. Aziz, R. F. Hoyt, Jr., J. P. McCoy, Jr., and M. N. Sack. 2005. Uncoupling proteins 2 and 3 function in concert to augment tolerance to cardiac ischemia. *J. Biol. Chem.* **280:** 33470–33476.
21. Silvestri, E., M. Moreno, A. Lombardi, M. Ragni, P. de Lange, S. E. Alexson, A. Lanni, and F. Goglia. 2005. Thyroid-hormone effects on putative biochemical pathways involved in UCP3 activation in rat skeletal muscle mitochondria. *FEBS Lett.* **579:** 1639–1645.
22. Moreno, M., A. Lombardi, P. de Lange, E. Silvestri, M. Ragni, A. Lanni, and F. Goglia. 2003. Fasting, lipid metabolism, and triiodothyronine in rat gastrocnemius muscle: interrelated roles of uncoupling protein 3, mitochondrial thioesterase, and coenzyme Q. *FASEB J.* **17:** 1112–1114.
23. Shrago, E. 2000. Long-chain acyl-CoA as a multi-effector ligand in cellular metabolism. *J. Nutr.* **130 (Suppl. 2S):** 290–293.
24. Chua, B. H., and E. Shrago. 1977. Reversible inhibition of adenine nucleotide translocation by long chain acyl-CoA esters in bovine heart mitochondria and inverted submitochondrial particles. Comparison with atractylate and bongkrekic acid. *J. Biol. Chem.* **252:** 6711–6714.
25. Shug, A. L., E. Shrago, N. Bittar, J. D. Folts, and J. R. Koke. 1975. Acyl-CoA inhibition of adenine nucleotide translocation in ischemic myocardium. *Am. J. Physiol.* **228:** 689–692.
26. Chiu, H. C., A. Kovacs, D. A. Ford, F. F. Hsu, R. Garcia, P. Herrero, J. E. Saffitz, and J. E. Schaffer. 2001. A novel mouse model of lipotoxic cardiomyopathy. *J. Clin. Invest.* **107:** 813–822.
27. Chiu, H. C., A. Kovacs, R. M. Blanton, X. Han, M. Courtois, C. J. Weinheimer, K. A. Yamada, S. Brunet, H. Xu, J. M. Nerbonne, et al. 2005. Transgenic expression of fatty acid transport protein 1 in the heart causes lipotoxic cardiomyopathy. *Circ. Res.* **96:** 225–233.
28. Yagyu, H., G. Chen, M. Yokoyama, K. Hirata, A. Augustus, Y. Kako, T. Seo, Y. Hu, E. P. Lutz, M. Merkel, et al. 2003. Lipoprotein lipase (LpL) on the surface of cardiomyocytes increases lipid uptake and produces a cardiomyopathy. *J. Clin. Invest.* **111:** 419–426.
29. Finck, B. N., J. J. Lehman, T. C. Leone, M. J. Welch, M. J. Bennett, A. Kovacs, X. Han, R. W. Gross, R. Kozak, G. D. Lopaschuk, et al. 2002. The cardiac phenotype induced by PPARalpha overexpression mimics that caused by diabetes mellitus. *J. Clin. Invest.* **109:** 121–130.
30. How, O. J., E. Aasum, D. L. Severson, W. Y. Chan, M. F. Essop, and T. S. Larsen. 2006. Increased myocardial oxygen consumption reduces cardiac efficiency in diabetic mice. *Diabetes.* **55:** 466–473.
31. Lashin, O., and A. Romani. 2003. Mitochondria respiration and susceptibility to ischemia-reperfusion injury in diabetic hearts. *Arch. Biochem. Biophys.* **420:** 298–304.
32. Lashin, O., and A. Romani. 2004. Hyperglycemia does not alter state 3 respiration in cardiac mitochondria from type-I diabetic rats. *Mol. Cell. Biochem.* **267:** 31–37.
33. Palmer, J. W., B. Tandler, and C. L. Hoppel. 1977. Biochemical properties of subsarcolemmal and interfibrillar mitochondria isolated from rat cardiac muscle. *J. Biol. Chem.* **252:** 8731–8739.
34. Ogata, T., T. Miyauchi, S. Sakai, M. Takanashi, Y. Irukayama-Tomobe, and I. Yamaguchi. 2004. Myocardial fibrosis and diastolic dysfunction in deoxycorticosterone acetate-salt hypertensive rats is ameliorated by the peroxisome proliferator-activated receptor-alpha activator fenofibrate, partly by suppressing inflammatory responses associated with the nuclear factor-kappa-B pathway. *J. Am. Coll. Cardiol.* **43:** 1481–1488.
35. Stavinoha, M. A., J. W. RaySpellicy, M. L. Hart-Sailors, H. J. Mersmann, M. S. Bray, and M. E. Young. 2004. Diurnal variations in the responsiveness of cardiac and skeletal muscle to fatty acids. *Am. J. Physiol. Endocrinol. Metab.* **287:** E878–E887.
36. Moghaddas, S., M. S. Stoll, P. E. Minkler, R. G. Salomon, C. L. Hoppel, and E. J. Lesnefsky. 2002. Preservation of cardiolipin content during aging in rat heart interfibrillar mitochondria. *J. Gerontol. A Biol. Sci. Med. Sci.* **57:** B22–B28.
37. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
38. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162:** 156–159.
39. Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams. 1996. Real time quantitative PCR. *Genome Res.* **6:** 986–994.
40. Gibson, U. E., C. A. Heid, and P. M. Williams. 1996. A novel method for real time quantitative RT-PCR. *Genome Res.* **6:** 995–1001.
41. Srere, P. A. 1969. Citrate synthase. *Methods Enzymol.* **13:** 3–5.
42. Alexson, S. E., and J. Nedergaard. 1988. A novel type of short- and medium-chain acyl-CoA hydrolases in brown adipose tissue mitochondria. *J. Biol. Chem.* **263:** 13564–13571.
43. Jaburek, M., M. Varecha, R. E. Gimeno, M. Dembski, P. Jezek, M. Zhang, P. Burn, L. A. Tartaglia, and K. D. Garlid. 1999. Transport function and regulation of mitochondrial uncoupling proteins 2 and 3. *J. Biol. Chem.* **274:** 26003–26007.
44. Bezaire, V., C. R. Bruce, G. J. Heigenhauser, N. N. Tandon, J. F. Glatz, J. J. Luiken, A. Bonen, and L. L. Spriet. 2006. Identification of fatty acid translocase on human skeletal muscle mitochondrial membranes: essential role in fatty acid oxidation. *Am. J. Physiol. Endocrinol. Metab.* **290:** E509–E515.
45. Campbell, S. E., N. N. Tandon, G. Woldegiorgis, J. J. Luiken, J. F. Glatz, and A. Bonen. 2004. A novel function for fatty acid translocase (FAT)/CD36: involvement in long chain fatty acid transfer into the mitochondria. *J. Biol. Chem.* **279:** 36235–36241.
46. Holloway, G. P., V. Bezaire, G. J. Heigenhauser, N. N. Tandon, J. F. Glatz, J. J. Luiken, A. Bonen, and L. L. Spriet. 2006. Mitochondrial long chain fatty acid oxidation, fatty acid translocase/CD36 content and carnitine palmitoyltransferase I activity in human skeletal muscle during aerobic exercise. *J. Physiol.* **571:** 201–210.
47. Stanley, W. C., G. D. Lopaschuk, and J. G. McCormack. 1997. Regulation of energy substrate metabolism in the diabetic heart. *Cardiovasc. Res.* **34:** 25–33.