Activation of a novel long-chain free fatty acid generation and export system in mitochondria of diabetic rat hearts

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Gerber, Lamar K., Bruce J. Aronow, and Mohammed A. Matlib. 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, 2006. First published July 19, 2006; doi:10.1152/ajpcell.00246.2006.—A number of reports indicate that a long-chain free fatty acid export system may be operating in mitochondria. In this study, we sought evidence of its existence in rat heart mitochondria. To determine its potential role, we also sought evidence of its activation or inhibition in streptozotocin (STZ)-induced diabetic rat heart mitochondria. If confirmed, it could be a novel mechanism for regulation of long-chain fatty acid oxidation (FAO) in mitochondria. To obtain evidence of its existence, we tested whether heart mitochondria presented with palmitoyl-carnitine can generate and export palmitate. We found that intact mitochondria indeed generate and export palmitate. We have also found that the rates of these processes are markedly higher in STZ-diabetic rat heart mitochondria, in which palmitoyl-carnitine oxidation is also increased. Since mitochondrial thioesterase-1 (MTE-1) hydrolyzes acyl-CoA to CoA-SH + free fatty acid, and uncoupling protein-3 (UCP-3), reconstituted in liposomes, transports free fatty acids, we examined whether these proteins are also increased in STZ-diabetic rat heart mitochondria. We found that both of these proteins are indeed increased. Gene expression profile analysis revealed striking expression of mitochondrial long-chain fatty acid transport and oxidation genes, accompanying overexpression of MTE-1 and UCP-3 in STZ-diabetic rat hearts. Our findings provide the first direct evidence for the existence of a long-chain free fatty acid generation and export system in mitochondria and its activation in STZ-diabetic rat hearts in which FAO is enhanced. We suggest that its activation may facilitate, and inhibition may limit, enhancement of FAO.

LONG-CHAIN FATTY ACIDS are the major energy substrates of cardiac muscle (23). Their β-oxidation in mitochondria generates ~70% of ATP. The rate of oxidation is normally closely matched with supply so that very little lipid accumulates in cardiac myocytes. When chronic oversupply exceeds the capacity of oxidation or the oxidation process is compromised, intracellular lipid metabolites accumulate, which leads to arrhythmia and contractile dysfunction (20, 36). Intracellular lipid droplets in cardiac myocytes have been commonly observed in obese and type 2 diabetic patients postmortem and animal models (28, 37, 41). This condition has been termed “lipotoxic heart disease” (41), or “lipotoxic cardiomyopathy” (4). Appearance of lipid droplets is a symptom of depressed long-chain fatty acid oxidation (FAO) and accumulation of fatty acid metabolites in cytosol and in mitochondria. Oversupply of long-chain fatty acids and the depressed oxidation observed in Zucker diabetic fatty (ZDF) rats result in excessive production of fatty acid metabolites in cardiac myocytes (39, 41). A decrease in the CoA-SH/acetyl-CoA ratio or the accumulation of intermediary metabolites of β-oxidation in mitochondria can cause feedback inhibition of the β-oxidation pathway at several steps (7, 38). Excessive acyl-CoA can inhibit ADP/ATP transport into mitochondria (29), promote myocyte apoptosis (22, 30, 37), and cause membrane leaks (25). Currently, there is no intervention available to prevent fatty acid metabolite accumulation in cardiac myocytes. If a mechanism can be found to modulate β-oxidation and to prevent accumulation of the fatty acid metabolites, it may be effective in preventing lipotoxic cardiomyopathy in obesity and type 2 diabetes. The presence of a putative fatty acid export system in mitochondria has been suggested by some investigators (12, 15, 27), but there has been no direct evidence of its existence in any tissue. If it exists, its activation may prevent cardiotoxic fatty acid metabolite accumulation by preventing feedback inhibition of β-oxidation of long-chain fatty acids in mitochondria.

The supply and oxidation of long-chain fatty acids have been shown to increase in streptozotocin (STZ)-induced type 1 diabetic rat hearts (26), but it has not been clear how feedback inhibition of β-oxidation and fatty acid metabolites accumulation are prevented during heightened long-chain FAO. We surmise that if a system exists that performs these tasks, it would be prominent in STZ-diabetic rat heart mitochondria. The objective of the present study is to examine the premise that a system exists that converts acyl-carnitine via acyl-CoA to free fatty acid, which is then exported from mitochondria and that this process is activated in STZ-diabetic rat hearts.

MATERIALS AND METHODS

Materials. Rats were purchased from Harlan Laboratories, Indianapolis, IN. TRIzol reagent was purchased from GIBCO BRL, Life Technologies, Grand Island, NY. Affymetrix rat genome chip U34A was purchased from Affymetrix, Santa Clara, CA. The antiserum against mitochondrial thioesterase-1 (MTE-1) was obtained as a gift from Dr. Stefan E. H. Alexson, Karolinska Institute, Sweden. The antiserum against uncoupling protein-3 (UCP-3) and UCP-2 were obtained from Alpha Diagnostic International, San Antonio, TX. The antiserum against UCP-1 was obtained from Chemicon, Temecula, CA. [1-14C]palmitoyl-carnitine, [1-14C]palmitoyl-CoA, and [1-14C]-palmitate were purchased from Perkin-Elmer, Boston, MA. Unlabeled palmitoyl-carnitine, palmitoyl-CoA, myristoyl-CoA, acetyl-CoA, palmitate, primuline, atractylate, and all other chemicals were of analytical grade.

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grade and purchased from Sigma Chemical, St. Louis, MO. Silica gel plates with flexible polyester backing were purchased from Whatman, Clifton, PA.

Generation and glycemic profile of STZ-diabetic rats. Six-week-old male Wistar rats were made diabetic with a bolus injection of STZ (65 mg/kg) in a tail vein and characterized as described previously (10, 16, 40). Both normal and STZ-injected mice were maintained on sterilized Harlan Teklad LM-485 mouse/rat chow and water ad libitum. Six-week-old diabetic and age-matched control rats were used in the study because at 6 wk diabetic rats exhibit changes greater than earlier time periods and also exhibit cardiomyopathy (10, 16, 40). The animal use protocol for the investigation was approved by the Institutional Animal Care and Use Committee.

Isolation and measurement of respiration of mitochondria. Mitochondria from ventricular myocardium were isolated and rates of state 3 (presence of ADP) and state 4 respiration (absence of ADP) were measured at 37°C as described previously (10), in the presence of 20 μM palmitoyl-carnitine + 50 μM malate, to generate oxaloacetic acid for utilization of acetyl-CoA in TCA cycle. This concentration of malate did not increase respiration appreciably without palmitoyl-carnitine. The assay medium (1.5 ml) contained 120 mM KCl, 0.1 mM EGTA, 2 mM potassium phosphate buffer (pH 7.2), 20 mM HEPES buffer (pH 7.2), 0.66 mg/ml BSA and 1 mg mitochondrial proteins.

Quantitative immunoblotting of MTE-1 and UCPs. SDS-PAGE and Western blotting were performed as described previously (40). The antisera used were diluted as follows: MTE-1, 1:4,000; UCP-3, 1:500; UCP-2, 1:250; and UCP-1, 1:100. Four different amounts of mitochondrial proteins from a pair of control and diabetic rat hearts were loaded on to the same SDS-PAGE gel. Protein levels of MTE-1 and UCPs were determined by linear regression analysis of the straight lines of number of pixels vs. amounts (μg) of mitochondrial proteins of control and diabetic rats. The slope of the linear regression line of the control heart mitochondria was taken as 100% to determine the change in the diabetic mitochondria. The data represent means ± SE of at least four pairs of control and diabetic rat heart mitochondrial preparations.

Measurements of free fatty acid generation and export from mitochondria. Synthesis of [1-14C]palmitic acid was determined by providing isolated mitochondria with [1-14C]palmitoyl-carnitine to be transported via the carnitine/acylcarnitine transporter into the matrix, where it would be converted to [1-14C]palmitoyl-CoA by carnitine palmitoyltransferase II and then to CoA-SH + [1-14C]palmitate by matrix thioesterases. The procedure for separation and quantification of [1-14C]palmitate generated in intact mitochondria is as follows: Isolated mitochondria (1.0 mg protein) were incubated at 37°C in 1.5 ml of a medium containing 120 mM KCl, 20 mM HEPES buffer (pH 7.2), 2 mM potassium phosphate buffer (pH 7.2), 0.1 mM EGTA, and 0.66 mg/ml BSA. Unless otherwise stated, the reaction was initiated by adding 20 μM [1-14C]palmitoyl-carnitine (sp. act. 8,300 cpm/nmol), prepared by mixing [1-14C]palmitoyl-carnitine with unlabeled palmitoyl-carnitine. The suspension was then incubated at 37°C for 3 to 6 min. The incubation was terminated by removing half of the suspension and mixing it with an equal volume of the assay medium without [1-14C]palmitoyl-carnitine and 3 vol of chloroform/methanol mixture (2:1 vol/vol). The remaining suspension was filtered through nitrocellulose Millipore filters (0.45 μm), presoaked in assay buffer containing BSA (0.66 mg/ml) and unlabeled palmitate (40 nmol/ml). The filter was washed with a half volume of the assay medium and mixed with 3 vol of chloroform/methanol mixture. Lipids were extracted from the mitochondrial suspension, and supernatant obtained by filtration separately, according to an established procedure (11). The samples were mixed in a vortex mixer and extraction continued for 30 min. The samples were then centrifuged at 3,000 rpm for 20 min at 4°C, in a swing-out rotor attached to a bench-top refrigerated centrifuge, to distinctly separate aqueous and organic layers. The organic solvent fraction was dried under a nitrogen gas stream and resuspended in 40 μl chloroform containing 3.6 nmol of nonradioactive palmitate to aid visualization of the spot in the TLC plate. The entire sample was applied onto a spot in a 250-μm-thick (60 Å) silica gel plate with flexible polyester backing. The chromatogram was developed in hexane/diethyl ether/formic acid (50:50:1 vol/vol/vol). Nonpolar lipids and lipid esters did not migrate with the solvent, but palmitate migrated with an Rf of ~0.8. The TLC plate was sprayed with a solution containing 80% acetone, 20% water, and 0.05% (wt/vol) primuline and visualized under UV light. The spot of [1-14C]palmitate was identified as a dark spot in the same vertical position as a known [1-14C]palmitic acid standard in a parallel lane in the TLC plate. The identified palmitate spot was cut out and soaked in scintillation fluid before counting for radioactivity in a scintillation counter. The recovery of [1-14C]palmitoyl-carnitine and [1-14C]palmitic acid in the organic solvent was ~97%. There was no detectable [1-14C]palmitate generated in heat-inactivated mitochondria (90°C for 5 min).

Thioesterase and citrate synthase activities. Thioesterase and citrate synthase activities were determined in intact mitochondria under identical conditions as described above under “Measurements of free fatty acid generation and export from mitochondria,” except that nonradioactive palmitoyl-carnitine was used during incubation. These activities were also determined in these mitochondria after breaking them by 3 cycles of freezing at −20°C for 10 min and thawing at 4°C, followed by sonication of 3-s duration, 3 times. The enzyme assays were carried out according to established methods (2, 33).

Exogenous NADH oxidation. NADH oxidation in intact and broken mitochondria was determined spectrophotometrically at 340 nm using 0.67 mM NADH in the mitochondria samples, as used for the assays of citrate synthase and thioesterase.

RNA isolation, DNA microarray hybridization and scanning. The rats were killed, the hearts were excised, and the ventricles were freeze-clamped in liquid N2 within 30 s. Total RNA from ventricles was isolated and purified with TRizol reagent according to the manufacturer’s recommendation. Gene expression profile analysis was performed with Affymetrix chips. The Affymetrix rat genome microarray chip U34A was used, which contained 8,799 cDNAs and ESTs, including a large number of FAO genes. The University of Cincinnati/Children’s Hospital Affymetrix GeneChip Microarray Core (cincinnatichildren.org/research/cores/affymetrix) determined the final integrity of the RNA preparations, biotinylated them using the Affymetrix protocol, hybridized them to the chips, and scanned and quantified fluorescence signals with GENECHIP MAS v5.0 (Affymetrix.com).

Microarray data analysis. Expression ratios were calculated using GeneSpring software (Silicon Genetics). The 50th percentile of all measurements was used as a positive control for each sample; each measurement for each gene was divided by this synthetic positive control, assuming that this was at least 10. The bottom 10th percentile was used as a test for correct background subtraction. This was never less than the negative of the synthetic positive control. The measurement for each gene in each sample was divided by the average of the corresponding control values, assuming that it was at least 0.01. The data were then independently subjected to three filters and those genes passing all filters were taken as significantly altered in diabetes. First, those genes that have all samples marked as absent or marginal by Affymetrix software were eliminated from the original 8,799 genes, identifying 5,149 genes. Second, genes were eliminated if the diabetic samples from each age group did not have at least 3 of 4 samples with ratios greater than 1.5 fold or less than 0.667 fold and this identified 2,672 genes. Third, statistical analysis for significant changes was performed using the Excel add-in Significance Analysis of Microarrays (SAM), developed at Stanford University Labs (34), which identified 3,210 genes. Finally, a total of 1,359 genes passed all three filters. Expression pattern clusters were defined by subjecting the
log_{2}-transformed differential data set to hierarchical tree clustering algorithms, as implemented in the GeneSpring program (Silicon Genetics).

To more thoroughly characterize sets of functionally related genes differentially expressed between control and diabetic rat heart, OntoExpress (6) was used to classify six high-level clusters of genes according to the following GeneOntology categories: biological process, cellular role, and molecular function. The numbers of genes corresponding to each GeneOntology category among the differentially expressed genes was tallied and compared with the number of genes expected for each GeneOntology category based on their representation on the Affymetrix U34A array GeneChip. Significant differences from the expected were calculated with a hypergeometric distribution. GeneOntology classes, the significant changes in each class (with Bonferroni-corrected P < 0.001), and the number of altered genes (minimum 3) in each class corresponding to each GeneOntology functional classes were identified and presented here.

Statistical analysis. All other data were expressed as the means ± SE of at least four experiments. The data were analyzed by ANOVA and significant differences were determined by Student’s t-test. A change in diabetic rats compared with control rats was considered significant when P < 0.05.

RESULTS

Measurement of rates of generation and export of palmitate from mitochondria. The data presented in Fig. 1 demonstrate location of [1-14C]palmitoyl-carnitine (A), [1-14C]palmitoyl-CoA (B), and [1-14C]palmitate (C) standards in the TLC plate. [1-14C]palmitate is widely separated from [1-14C]palmitoyl-CoA and [1-14C]palmitoyl-carnitine. Control rat heart mitochondria incubated with [1-14C]palmitoyl-carnitine generated [1-14C]palmitate as indicated by its appearance exactly at the location (Fig. 1D) as the [1-14C]palmitate standard (Fig. 1C). The total amount of palmitate generated was determined after stopping the reaction by adding an equal volume of the organic solvent to an aliquot of mitochondrial suspension, immediately mixing, and extracting palmitate. The amount of palmitate exported from mitochondria was determined after rapid Millipore filtration of an equal aliquot of mitochondrial suspension and extraction of palmitate from the filtrate. The rates of generation and export of palmitate using 20 μM [1-14C]palmitoyl-carnitine were linear for at least up to 6 min (Fig. 1E). The rate of palmitate export was saturated at 20 μM palmitoyl-carnitine, while the rate of generation appears to near saturation at 40 μM palmitoyl-carnitine (Fig. 1F). Concentrations of palmitoyl-carnitine above 40 μM appeared turbid and were not used in the experiment because of potential damaging effects on mitochondrial membranes and insolubility in assay medium. However, for uniform comparison and also to avoid membrane damage at higher palmitoyl-carnitine concentrations, 20 μM palmitoyl-carnitine was used in the rest of the experiments.

To determine that no increase in inner membrane permeability occurred in intact mitochondria incubated with 20 μM palmitoyl-carnitine, oxidation of exogenous NADH during incubation and leakage of thioesterase and citrate synthase from matrix were determined. Very little oxidation of NADH (Fig. 1G) and release of thioesterase and citrate synthase activities from mitochondria were observed before the addition of 20 μM palmitoyl-carnitine and no increase was observed after the incubation (Fig. 1H). These data demonstrate that the isolated mitochondria used in the study were intact and no membrane leak occurred during incubation with 20 μM palmitoyl-carnitine. Thus it was concluded that free palmitate was generated and exported from the matrix of intact mitochondria. Free fatty acid transport by UCPs reconstituted in liposomes has been shown to be inhibited by ATP, GTP, and GDP (18, 19). We have examined the effects of ATP and GDP on palmitate generation and export from heart mitochondria. ATP (10 mM) in the presence of oligomycin (1 mM to prevent ATP hydrolysis) produced no effect on the rate of generation, but it inhibited the rate of export by 48% (94 ± 2 in untreated vs. 46 ± 8 nmol min⁻¹ mg⁻¹ in ATP treated). GDP (10 mM) produced no effect on the rate of generation or export of palmitate.

Measurements of rates of generation and export of free fatty acid from STZ-diabetic rat heart mitochondria. To determine whether generation and/or export of palmitate is increased or decreased in a condition in which FAO is enhanced, the rates of these processes were determined in STZ-diabetic rat heart mitochondria. Employing the method of measurement described above, the rates of generation and export of palmitate from control and diabetic rat heart mitochondria were determined (Fig. 2). In control mitochondria, the rates of generation and export of palmitate were moderate and equal (Fig. 2, open bars). These rates were strikingly enhanced and were also equal in diabetic rat heart mitochondria (Fig. 2, closed bars). These data demonstrate that the long-chain fatty acid generation and export system is upregulated in STZ-diabetic rat hearts.

Long-chain FAO in isolated mitochondria. To determine whether activation of palmitate generation and export is associated with an increase or decrease in long-chain FAO, the rate of respiration of isolated mitochondria in the presence of 20 μM palmitoyl-carnitine was examined. The data demonstrate increases in both state 3 and state 4 rates of respiration in diabetic rat heart mitochondria (Fig. 3). The rate of state 3 respiration was increased by ~50% while the rate of state 4 respiration was increased by ~70%. The increase in long-chain FAO was not due to an overall increase in respiratory capacity of mitochondria of STZ-diabetic rat hearts, since the rates of respiration in the presence of pyruvate + malate and α-ketoglutarate were decreased and the rate of respiration with succinate was unchanged in these mitochondria under identical conditions (data not shown here, see similar data in Ref 9). These data demonstrate that increases in the rates of generation and export of palmitate observed in STZ-diabetic rat heart mitochondria accompany an increase in the rate of respiration with palmitoyl-carnitine. The data indicate that the activation of the free fatty acid export system does not prevent enhancement of FAO in diabetic rat heart mitochondria.

It has been reported that atractylate, an inhibitor of adenine nucleotide translocase (ANT), inhibits increased state 4 respiration induced by palmitate (1). To examine the premise that increased state 4 respiration in diabetic rat heart mitochondria could be due to export of free palmitate via ANT, the effect of its inhibitor atractylate was determined. Atractylate (10 μM) inhibited 85–90% of the state 3 respiration (in the presence of 380 nmol ADP) of control and diabetic rat heart mitochondria (data not shown). However, 10 μM atractylloside produced no effect on the rate of state 4 respiration of control or diabetic rat heart mitochondria (Fig. 4).
Measurement of protein levels MTE-1 and UCP-3 in mitochondria. Since MTE-1 catalyzes breakdown of long-chain and very long-chain fatty acyl-CoA to free fatty acid (2, 16) and UCP-3, reconstituted in liposomes, transports long-chain free fatty acids (16, 17), we examined whether these two proteins were increased in mitochondria of STZ-diabetic rat hearts. Quantitative immunoblotting was carried out to determine the levels of MTE-1 and UCP-3 in isolated mitochondria. Immunoblots of MTE-1 demonstrate a single band at 45 kDa (Fig. 5A). The absence of any other bands near this region of the gel indicated that the mitochondrial preparations used in the study were not contaminated with cytosolic and peroxisomal thioesterase-1 that the antibody could recognize. The data presented in bar graphs show that MTE-1 protein was increased in diabetic rat heart mitochondria.
mitochondria (Fig. 5C). There was no detectable UCP-1 in control or diabetic rat heart mitochondria (data not shown).

These data demonstrate that MTE-1 and UCP-3 protein are indeed elevated, accompanying increases in palmitate generation and export in STZ-diabetic rat heart mitochondria.

**Thioesterase activity in mitochondria.** To determine that the increased MTE-1 protein observed in STZ-diabetic rat heart mitochondria was correlated with an increased rate of generation of free fatty acid, thioesterase activity was measured in broken mitochondria (for access to substrate) using myristoyl-CoA (2). The activity was increased in mitochondria of STZ-diabetic rat hearts (Fig. 5D). A similar magnitude of increase in thioesterase activity was observed with palmitoyl-CoA and oleoyl-CoA (data not shown). However, no significant change in citrate synthase activity was observed in diabetic rat heart mitochondria (data not shown). Lack of an increased citrate synthase activity indicates that the change in thioesterase activity is not due to a general increase in matrix enzymes in STZ-diabetic hearts. The increased thioesterase activity and increased MTE-1 protein is consistent with the hypothesis that the increase in the rate of generation of palmitate may be carried out by MTE-1 in STZ-diabetic rat heart mitochondria.

**Gene expression in STZ-diabetic rat hearts.** To determine whether the increased respiration and palmitate generation and export observed in isolated mitochondria accompanied increased expression of critical genes of mitochondrial long-chain fatty acid transport and oxidation, the expression profile of normal and diabetic hearts at 3, 28, and 42 days of diabetes was examined. First, data for the expression of each gene was normalized to the average level shown in corresponding control animals. Genes were selected using statistical and fold difference criteria as described in the MATERIALS AND METHODS. The top-ranked 1,359 probe sets from the microarray data were then subjected to cluster analysis, and the top-level 6 clusters were analyzed for functional associations. The most significant alterations occurred in genes of mitochondrial proteins and protein biosynthesis (Fig. 6). Although the increases in expression of long-chain fatty acid transport and oxidation genes are initiated at 3 days of diabetes, more uniform and greater changes occurred in 6-wk diabetic rat hearts. The data on changes in individual genes of mitochondrial fatty acid transport and oxidation in 6-wk diabetic rat hearts are presented in Table 1. The data indicate increased expression of both long-chain saturated and polyunsaturated fatty acid transport and oxidation genes, and decreased expression of odd- and short-chain fatty acid oxidation genes in STZ-diabetic rat hearts. The increase in long-chain fatty acid transport and oxidation accompanies overexpression of MTE-1 and UCP-3 genes. The data demonstrate that the increases in palmitate generation and export, MTE-1, UCP-3, and respiration with palmitoyl-carnitine observed in isolated mitochondria of STZ-diabetic rat hearts accompany increased expression of critical genes for increases in long-chain fatty acid transport. The results also provide the evidence that increased expression of long-chain fatty acid transport and oxidation genes underlies the increased FAO observed in isolated heart mitochondria (see Fig. 3) and intact heart preparations of this model (27). The gene array data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) and are accessible through GEO Series accession number GSE4745.

**DISCUSSION**

In this report, we provide the first direct evidence of the existence of a long-chain free fatty acid generation and export system in mitochondria. It appears to be operating at a relatively low rate in normal rat heart mitochondria. The rates of generation and export are matched and very little palmitate accumulates in the matrix at the 20 μM palmitoyl-carnitine used in the study. The activation of the system in STZ-diabetic rat heart mitochondria indicates that it is inducible. In diabetic rat heart mitochondria the rates of generation and export are also matched and very little palmitate accumulates in the matrix. The activation of the system in STZ-diabetic rats is accompanied by enhanced FAO in isolated mitochondria. This observation and the reported increase in FAO observed in isolated heart preparations (26) indicate that activation of the system is not

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**Fig. 2.** Rates of generation and export of palmitate from mitochondria of streptozotocin (STZ)-diabetic rat hearts. The bars represent the means ± SE of 4 separate mitochondrial preparations, in pairs of control and STZ-diabetic hearts. *P < 0.05 vs. control.

**Fig. 3.** Respiration of isolated heart mitochondria with palmitoyl-carnitine as substrate. The data are presented as the ratio of the rate of respiration of diabetic over control rat heart mitochondria. The bars represent the mean ± SE of 4 separate mitochondrial preparations. The rates of state 3 and state 4 respiration of control rat heart mitochondria were 128 ± 31 and 43 ± 7 nmol of oxygen·min⁻¹·mg protein⁻¹, respectively. The rates of state 3 and state 4 respiration of diabetic mitochondria were 191 ± 22 and 74 ± 10, respectively. *P < 0.05 vs. control; #P < 0.05 vs. state 3.
preventing the enhancement of FAO in STZ-diabetic rat hearts. Since the heart in diabetes is almost completely dependent on fatty acids as energy substrates, activation of free fatty acid generation and the export system in STZ-diabetic rat heart mitochondria may be involved in facilitating enhanced FAO. There may be important benefits for its activation. Enhanced FAO may drastically decrease the level of matrix CoA-SH required for supporting the enhanced β-oxidation, by converting most of it to acyl-CoA (7, 38). Accumulation of intermediary metabolites and the end-product acetyl-CoA in the matrix may cause feedback inhibition of β-oxidation (7, 38). The benefits of augmentation of the long-chain free fatty acid generation and export system in STZ-diabetic rat heart mitochondria during heightened long-chain FAO would be threefold: 1) rapid regeneration of free CoA-SH essential for β-oxidation, 2) prevention of excessive accumulation long-chain fatty acid metabolites, and 3) prevention of feedback inhibition of β-oxidation by its products. Another benefit of activation of the free fatty acid generation and export system would be to prevent excessive production of acetyl-CoA and accumulation of ketone bodies, such as acetone, harmful for mito-

Fig. 4. Effect of atractylate on respiration of isolated heart mitochondria. Representative oxygraph traces of respiration of mitochondria from diabetic rat heart (A) and control rat heart (B), in the absence of ADP, under the conditions of the experiments described in Fig. 3, are presented. The substrates were 20 μM palmitoyl-carnitine and 100 μM malate. The numbers along the traces are means ± SE of the rate of respiration (nmol of oxygen· min⁻¹·mg protein⁻¹) calculated from 3 experiments. Arrows indicate addition of oligomycin (4 μg in 1 μl ethanol) and atractylate (10 μM, final concentration).

A MTE-1 Protein Expression
B UCP-3 Protein Expression
C UCP-2 Protein Expression
D Thioesterase Activity

Fig. 5. MTE-1, uncoupling protein-3 (UCP-3), and UCP-2 protein levels, and thioesterase activity in isolated mitochondria. Total mitochondrial protein (in μg as shown) was used in the Western blot probed with antibody against MTE-1. Thioesterase activity was determined in permeabilized mitochondria, as described under MATERIALS AND METHODS. The bars represent the means ± SE of 4 separate mitochondrial preparations. *P < 0.05 vs. control.
Chondria. The long-chain fatty acid generation and export system may also play a similar beneficial role in fasting and prolonged physical exercise when FAO is increased.

Potential pathological role of the system. In contrast to its potential beneficial role, increase in free fatty acid generation without concomitant increase in export would lead to accumulation of free fatty acids and their metabolites in the matrix and inhibition of β-oxidation. Failure to upregulate the system when long-chain fatty acyl-carnitine supply and transport into mitochondria are enhanced may also inhibit β-oxidation. This may happen due to inhibition of the proteins that carry out generation and export of free fatty acid from mitochondria or suppression or mutation of their encoding genes. Mutation of the genes of the proteins involved in generation and export of free fatty acid from mitochondria may predispose certain obese and/or diabetic individuals to development of lipotoxic cardiomyopathy.

Inhibition of fatty acid oxidation due to impairment of the fatty acid generation and export system of mitochondria may cause cellular dysfunction and pathogenesis of metabolic diseases, e.g., abnormal insulin release from pancreatic β-cells, insulin insensitivity, metabolic syndrome X, lipid storage in adipose tissue, and hepatic fatty acid metabolism. Thus impairment of the mitochondrial free fatty acid export system may also play an important role in the development of other metabolic diseases involving depressed fatty acid oxidation in mitochondria.

Involvement of proteins and proposed mechanism of regulation of the system. The apparent saturation of the rate of generation of palmitate indicates that this process may be mediated by an enzyme and saturation of the rate of export of palmitate indicates that this process may be carried out by a carrier protein. However, identification of the proteins that carry out generation and export of palmitate and the mecha-

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Fig. 6. Cluster analysis of genes significantly altered in STZ-diabetic rat hearts. Genes altered at 3, 28, or 42 days of diabetes were obtained by statistical filtering of the microarray data and subjected to hierarchical clustering. Color scale indicates relative expression ratio for each gene in diabetic relative to corresponding control hearts. Six clusters were each analyzed for over-representation of GeneOntology functional categories.
nism of activation of the system in STZ-induced diabetic rats remains to be determined. Since expression of long-chain fatty acid transport and oxidation genes is regulated by long-chain fatty acids via activation of peroxisome proliferator-activated receptor \( \alpha \) (PPAR\( \alpha \)) (8, 9), a similar mechanism may also be involved in the activation of long-chain fatty acid generation and export from mitochondria. PPAR\( \alpha \) has been shown to regulate expression of both MTE-1 and UCP-3 (17, 32). Increased expression of MTE-1 and UCP-3 genes has been shown in STZ-diabetic rat hearts (33). Increased expression of UCP-3 with unchanged UCP-2 has also been shown in STZ-diabetic rat hearts (14). Based on these and our observations in this study, we propose a long-chain free fatty acid generation and export pathway in mitochondria involving MTE-1 and UCP-3 as illustrated in Fig. 7. Although overexpression of MTE-1 and UCP-3 accompanied activation of long-chain fatty acid generation and export in mitochondria of STZ-diabetic rat hearts, there is no direct evidence of their involvement in the system.

MTE-1 in vitro hydrolyzes fatty acyl-CoA to free fatty acid and CoA-SH (17). The increase in MTE-1 and thioesterase activity accompanying the increase in palmitate generation suggests that MTE-1 is involved in the generation of free fatty acid in mitochondria. Since there is no specific inhibitor of MTE-1 available, a gene-targeted mouse model is needed to confirm its involvement in the free fatty acid generation in mitochondria.

UCP-3 has been shown to transport free fatty acid, when reconstituted in liposomes (18, 19). The increase in export of fatty acid from mitochondria to the cytoplasm via in vitro protein expression of UCP-3 suggests that UCP-3 may transport free fatty acid from mitochondria. Since there is no specific inhibitor of UCP-3 available, a gene-targeted mouse model is needed to confirm its involvement in the free fatty acid generation in mitochondria.

### Table 1. Gene transcript level of mitochondrial proteins involved in fatty acid oxidation

<table>
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<tr>
<th>Cellular Function Category</th>
<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Change, fold ± SE</th>
<th>Functional Change</th>
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<td>FA Transport and Oxidation</td>
<td>Carnitine palmitoyltransferase 1</td>
<td>NM_031559</td>
<td>2.31±0.16</td>
<td>( \uparrow ) acyl-carnitine synthesis</td>
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<td></td>
<td>Carnitine palmitoyltransferase 1B 1, 2, and 3</td>
<td>AF063302</td>
<td>1.66±0.17</td>
<td>( \uparrow ) acyl-carnitine synthesis</td>
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<td></td>
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<td>NM_059365</td>
<td>0.97±0.12</td>
<td>( \uparrow ) acyl-carnitine transport</td>
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<tr>
<td></td>
<td>(Trifunctional protein) ( \alpha )-subunit</td>
<td>NM_130868</td>
<td>1.67±0.14</td>
<td>( \uparrow ) LCFA oxidation</td>
</tr>
<tr>
<td></td>
<td>(Trifunctional protein) ( \beta )-subunit</td>
<td>NM_133616</td>
<td>1.28±0.13</td>
<td>( \uparrow ) LCFA oxidation</td>
</tr>
<tr>
<td></td>
<td>Acyl-CoA dehydrogenase, very long chain</td>
<td>NM_012891</td>
<td>1.35±0.12</td>
<td>( \uparrow ) VCF oxidation</td>
</tr>
<tr>
<td></td>
<td>Acyl-CoA dehydrogenase, medium chain</td>
<td>NM_016986</td>
<td>0.93±0.02</td>
<td>( \uparrow ) MCFA oxidation</td>
</tr>
<tr>
<td></td>
<td>2, 4-Dienoyl-CoA reductase 1</td>
<td>NM_057197</td>
<td>3.15±0.37</td>
<td>( \uparrow ) PUFA oxidation</td>
</tr>
<tr>
<td></td>
<td>Dodecenoyl-CoA ( \Delta ) isomerase</td>
<td>NM_017306</td>
<td>1.94±0.19</td>
<td>( \uparrow ) PUFA oxidation</td>
</tr>
<tr>
<td></td>
<td>( \alpha )-Propionyl-CoA carboxylase</td>
<td>M22631</td>
<td>0.60±0.06</td>
<td>( \downarrow ) OCFA oxidation</td>
</tr>
<tr>
<td></td>
<td>Enoyl-CoA hydratase, short chain 1</td>
<td>NM_078623</td>
<td>0.44±0.04</td>
<td>( \downarrow ) SCFA oxidation</td>
</tr>
<tr>
<td>Unknown Function</td>
<td>Mitochondrial acyl-CoA thioesterase 1</td>
<td>NM_138097</td>
<td>3.83±0.42</td>
<td>( \uparrow ) Thioesterase activity</td>
</tr>
<tr>
<td></td>
<td>Uncoupling protein 3</td>
<td>NM_013167</td>
<td>6.72±0.67</td>
<td>( \uparrow ) Uncoupling</td>
</tr>
</tbody>
</table>

FA, fatty acid; LCFA, long chain fatty acid; VCF, very long chain fatty acid; MCFA, medium chain fatty acid; PUFA, polyunsaturated fatty acid; OCFA, odd chain fatty acid; SCFA, short chain fatty acid. \( \uparrow \) significantly increased, \( \downarrow \) significantly decreased, and \( = \) no significant change.

Fig. 7. Schematic illustration of proposed free fatty acid generation and export pathway in mitochondria. FATP = fatty acid transport protein; FA = free fatty acid; FABP = fatty acid binding proteins; FA-CoA = fatty acyl-CoA; FA-carn = fatty acyl-carnitine.
palmitate from mitochondria accompanying the increase in UCP-3 suggests that it may be involved in the export of free fatty acid from STZ-diabetic rat heart mitochondria. The increase in state 4 respiration rate in STZ-diabetic rat heart mitochondria may be due to increase in UCP-3. Export of free fatty acid anions by UCP-3 may decrease the negative membrane potential on the matrix side of the inner membrane. The extra oxygen consumption is then needed to restore the membrane potential. The lack of inhibition of state 4 respiration by atraclylate suggests that ANT may not be involved in the increased rate of state 4 respiration, due to export of palmitate anion from diabetic rat heart mitochondria. However, it remains to be determined whether UCP-3 carries out free fatty acid export from mitochondria, and if so, whether it is exclusive of UCP-2 and other mitochondrial anion exporters. It also remains to be determined whether secondary modification and activation of UCPs or other anion exporters cause increased export of palmitate from diabetic rat heart mitochondria. Lack of inhibition of export of palmitate by GDP, may be an indication that UCPs may not be exclusively involved in the process. On the other hand, lack of a GDP effect on palmitate export could be due to differences between function of UCPs in situ, in intact mitochondria, and modification introduced when UCPs are reconstituted in liposomes. Since there are no inhibitors of UCP-3 or UCP-2 available, gene-targeted mouse models should confirm or deny their involvement in the free fatty acid export from mitochondria.

Activation of PPARγ in ZDF rats has been shown to reverse depressed glucose oxidation accompanying normalization of cardiac function (13). In contrast, activation of PPARγ in ob/ob and db/db diabetic mice improves cardiac metabolism but not contractile function (3, 21). The latter observation appears to contradict the benefits of upregulation of FAO and the free fatty acid generation and export system by PPARα proposed in this study. The reason for the disparity between ZDF rat and ob/ob or db/db mouse models could be twofold: 1) the underlying mechanisms of cardiomyopathy are different in these models, and 2) the difference in the long-chain FAO between ZDF rats in which it was attenuated (39), and ob/ob or db/db mice in which it is enhanced (21).

At this time it is not possible to determine whether the enhanced free fatty acid generation and export system of mitochondria in STZ-diabetic rats contributes to cardiomyopathy. Identification of proteins in the system and availability of their gene-targeted and transgenic mice, made diabetic with STZ, would help resolve this issue. However, gene array data presented in this study indicate FAO and long-chain free fatty acid generation and export, if constituted by MTE-1 and UCP-3, are enhanced at the early stage of diabetes before development of contractile dysfunction in STZ-diabetic rats (16, 40). We have shown a correlation between development of contractile dysfunction and alteration of sarcoplasmic reticulum (SR) proteins in STZ-diabetic rat hearts (40). Others have also shown altered SR proteins in this model (24). We have shown defective intracellular Ca2+ signaling associated with altered SR proteins in STZ-induced diabetic rat hearts (5). Complete restoration of cardiac contractile function with transgenic expression of SERCA2a in STZ-diabetic mice also supports the role of SR dysfunction as the major cause of cardiomyopathy in STZ-induced type 1 diabetes models (35). Thus altered SR proteins and their functions alone may be responsible for cardiomyopathy in STZ-diabetic rats. However, it is possible that early increased FAO or lipid metabolites or decreased glucose metabolites may trigger alteration of SR protein expression and function that develops over several weeks.

Summary. We report here the existence and activation of a novel fatty acid export system in mitochondria that may exert regulatory influence on cardiac fatty acid metabolism. Although the activity of the system is correlated with MTE-1 and UCP-3 levels, their involvement remains to be confirmed. It appears that the physiological role of the system is to prevent fatty acid metabolite accumulation to toxic levels and thus facilitate β-oxidation during heightened long-chain fatty acids transport and oxidation in mitochondria. Importantly, it also appears that impairment of the system may cause, and activation may prevent and cure lipotoxic cardiomyopathy in obesity and type 2 diabetes.

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