Original Article

Plasma Free Fatty Acids and Peroxisome Proliferator–Activated Receptor α in the Control of Myocardial Uncoupling Protein Levels

Andrew J. Murray,[1] Marcello Panagia,[1] David Hauton,[2] Geoffrey F. Gibbons,[2] and Kieran Clarke[1]

Diabetic patients have abnormal cardiac energy metabolism associated with high plasma free fatty acid (FFA) concentrations. We investigated whether high plasma FFAs increase mitochondrial uncoupling protein (UCP) levels in the mouse heart by activating the nuclear transcription factor peroxisome proliferator–activated receptor (PPAR)α. We used Western blotting to measure UCP protein levels in isolated cardiac mitochondria from PPARα$^{-/-}$ and diabetic mice. Cardiac UCP2 and UCP3 were significantly lower in the PPARα$^{-/-}$ mouse than in the wild type. Treatment with the PPARα-specific agonist, WY-14,643, increased cardiac UCP2 and UCP3 levels in wild-type mice but did not alter UCP levels in PPARα$^{-/-}$ mice. Inhibition of β-oxidation with etomoxir increased cardiac UCP2 and UCP3 levels in wild-type mice and UCP2 levels in PPARα$^{-/-}$ mice but did not alter UCP3 levels in PPARα$^{-/-}$ mice. Streptozotocin treatment, which increased circulating FFAs by 91%, did not alter cardiac UCP2 levels in wild-type or PPARα$^{-/-}$ mice but increased UCP3 levels in wild-type, and not in PPARα$^{-/-}$, mice. The diabetic *db/db* mouse had 50% higher plasma FFA concentrations and elevated cardiac UCP2 and UCP3 protein levels. We conclude that high plasma FFAs activated PPARα to increase cardiac UCP3 levels, but cardiac UCP2 levels changed via PPARα-dependent and -independent mechanisms. *Diabetes* 54:3496–3502, 2005

The phosphocreatine (PCr)-to-ATP ratio, an index of myocardial energetic status, has been shown to correlate negatively with plasma free fatty acid (FFA) concentrations in patients with type 2 diabetes (1), but the cellular link between energy metabolism and circulating FFA concentrations has yet to be defined. Conditions that increase plasma FFA levels, such as high-fat feeding, fasting, and streptozotocin (STZ)-induced diabetes, increase cardiac and skeletal muscle mitochondrial uncoupling protein (UCP)3 levels in the rat (2–5). A positive correlation between circulating FFA concentrations and both UCP2 and UCP3 protein levels occurs in human heart (6), suggesting that plasma FFAs may control UCP levels in the heart. The UCPs are believed to dissipate the proton electrochemical gradient by allowing protons to reenter the mitochondrial matrix without the concomitant synthesis of ATP (7).

Long-chain FFAs are natural ligands for the peroxisome proliferator–activated receptors (PPARs) (8–10), and the UCP genes have PPAR response elements in their promoter regions (11,12). Thus, plasma FFAs control cardiac UCP levels via PPAR activation. The PPARα link has been shown by decreased UCP3 mRNA levels in the PPARα$^{-/-}$ mouse heart and increased UCP3 mRNA levels in rat heart after treatment with the specific PPARα agonist, WY-14,643 (5). Most studies of changes in cardiac UCPs, associated with alterations in circulating metabolite concentrations, have reported UCP mRNA levels (5,10,13,14), yet UCP mRNA and protein do not necessarily change in parallel (15,16).

Studies in brown adipose tissue (15,16) and gastrocnemius muscle (16) have demonstrated that dramatic changes in levels of UCP2 or UCP3 transcripts were translated to much smaller changes, or no significant changes, at the protein level. Dietary conjugated linoleic acid was found to increase skeletal muscle UCP3 protein in mice, despite unchanged UCP3 mRNA levels (17). These findings indicate complex posttranslational control of UCP levels and expose a limitation in the conclusions drawn from studies that have demonstrated changes only in UCP mRNA. Furthermore, changes in mRNA are not necessarily concordant between studies; for example, treatment with WY-14,643 increased UCP2 mRNA in rat cardiomyocytes (9) but not in rat heart (5). To draw conclusions about UCP function, it is important to use protein level measurements.

In this study, we have investigated the links between plasma FFA concentrations, PPARα, and UCPs by measuring cardiac UCP2 and UCP3 protein levels in mutant PPARα$^{-/-}$ mice and two mouse models of diabetes known to have high plasma FFA levels, the STZ-treated mouse and the diabetic (*db/db*) mouse. Thus, we have determined how plasma FFAs, which have been associated with changes in UCP mRNA levels, affect cardiac UCP2 and UCP3 protein levels in vivo, thereby highlighting posttranslational control of UCP levels. Part of this work has been published in abstract form (18).

From the [1]University Laboratory of Physiology, University of Oxford, Oxford, U.K.; and the [2]Metabolic Research Laboratory, Oxford Centre for Diabetes, Endocrinology and Metabolism, Nuffield Department of Clinical Medicine, University of Oxford, Churchill Hospital, Oxford, U.K.

Address correspondence and reprint requests to Professor Kieran Clarke, University Laboratory of Physiology, University of Oxford, Parks Road, Oxford, OX1 3PT, U.K. E-mail: kieran.clarke@physiol.ox.ac.uk.

Received for publication 25 April 2005 and accepted in revised form 9 September 2005.

FFA, free fatty acid; PCr, phosphocreatine; PPAR, peroxisome proliferator–activated receptor; STZ, streptozotocin; UCP, uncoupling protein.

TABLE 1
Fasting plasma concentrations of FFAs, triacylglycerol, cholesterol, and insulin in wild-type and PPARα$^{-/-}$ mice fed on a chow diet or treated with WY-14,643, etomoxir, or WY-14,643 and etomoxir

	Wild-type			
	Chow	WY-14,643	Etomoxir	WY and etomoxir
FFAs (mmol/l)	0.38 ± 0.01	0.43 ± 0.01	0.51 ± 0.07*	0.43 ± 0.03
Triacylglycerol (mmol/l)	1.9 ± 0.1	1.3 ± 0.2*	2.4 ± 0.2*	1.1 ± 0.1†
Cholesterol (mmol/l)	2.1 ± 0.2	2.0 ± 0.1	2.4 ± 0.2	2.3 ± 0.1
Insulin (μg/l)	1.2 ± 0.4	0.4 ± 0.1*	1.2 ± 0.3	0.4 ± 0.1*
	PPARα$^{-/-}$			
	Chow	WY-14,643	Etomoxir	WY and etomoxir
FFAs (mmol/l)	0.44 ± 0.11	0.38 ± 0.02	0.34 ± 0.02	0.42 ± 0.05
Triacylglycerol (mmol/l)	1.4 ± 0.2	1.7 ± 0.1	1.2 ± 0.1	1.4 ± 0.1
Cholesterol (mmol/l)	2.6 ± 0.1	2.4 ± 0.1	2.4 ± 0.2	2.5 ± 0.2
Insulin (μg/l)	1.7 ± 0.7	0.8 ± 0.4	0.7 ± 0.1	1.3 ± 0.7

Data are means ± SE. $n = 4$ for all groups. *$P < 0.05$, †$P < 0.01$ compared with untreated mice of the same strain.

RESEARCH DESIGN AND METHODS

This investigation conformed with U.K. Home Office and National Institutes of Health guidelines on the care and use of laboratory animals. All mice were studied at 6–7 months of age.

PPARα$^{-/-}$ mice. The PPARα$^{-/-}$ mouse was generated a little over 10 years ago by disruption of the ligand-binding domain of PPARα using homologous recombination (19) and has since become a much-used and invaluable tool for studying the role of the receptor in fatty acid metabolism (5,20,21). Mice homozygous for the mutation do not express PPARα protein and fail to display the peroxisome proliferator pleiotropic response when challenged with WY-14,643 (19). Here we used male wild-type SVEV129 and PPARα$^{-/-}$ mice, which were a gift from Frank Gonzalez (National Cancer Institute, Bethesda, MD).

WY-14,643 and etomoxir treatment. Wild-type and PPARα$^{-/-}$ mice were treated with the PPARα-specific agonist, WY-14,643, and/or etomoxir, an inhibitor of β-oxidation that raises intracellular levels of intermediary fatty acid metabolites (22). Direct activation of PPARα with WY-14,643, or indirect stimulation by high intracellular fatty acid concentrations resulting from etomoxir treatment, were expected to increase UCP levels in wild-type, but not in PPARα$^{-/-}$, mouse hearts. Hence, PPARα$^{-/-}$ mice and SVEV129 wild-type mice were fed for 7 days on one of four diets: *1*) standard chow diet alone, *2*) standard chow diet supplemented with WY-14,643 (0.1%), *3*) etomoxir (0.02%), or *4*) both WY-14,643 (0.1%) and etomoxir (0.02%).

Streptozotocin injection. When injected into rodents, STZ destroys pancreatic β-cells leading to a hypoinsulinemic state and high levels of circulating FFAs (23). Male wild-type SVEV129 and PPARα$^{-/-}$ mice were given 210 mg/kg STZ (Sigma, St. Louis, MO) dissolved in 50 mmol/l citrate, pH 4.5. The STZ was administered in three daily intraperitoneal injections of 85 mg/kg, 70 mg/kg, and 55 mg/kg (24). Wild-type animals were injected with vehicle. All STZ-injected animals were assessed for the development of diabetes as described previously (25) and were killed 6 weeks after the initial injection.

***db/db* mice.** Although diabetes in humans rarely occurs as a result of a defect in the leptin receptor, the *db/db* mouse, which has a single leptin receptor gene mutation, is a well-characterized model of diabetic cardiomyopathy, with metabolic changes similar to those in human type 2 diabetes (26). The *db/db* mouse has peripheral insulin resistance and increased pancreatic β-cell insulin secretion. Hyperglycemia eventually develops when enhanced insulin secretion can no longer compensate for peripheral and hepatic insulin resistance. Body weights of *db/db* mice increase progressively and plateau at 40–50 g at about 2 months of age, almost double the weight of wild-type mice (26), with greater lipolysis of endogenous adipose stores leading to increased levels of circulating FFAs (27). As diabetic patients have abnormally high levels of plasma FFAs (1,28), we measured plasma metabolites and cardiac UCP2 and UCP3 levels in the *db/db* mouse. Male C57BL/KsJ-lepr^{db} *db/db* mice and their nondiabetic (*db/+*) controls were obtained from Harlan U.K. (Bicester, U.K.).

Isolation of mitochondria from mouse heart. For each experiment, whole mouse hearts (~100 mg) were isolated, minced, washed, and suspended in 5 ml of isolation medium (0.3 mol/l sucrose, 10 mmol/l Na-HEPES, and 0.2 mmol/l EDTA, pH 7.2). The tissue was subjected to mild trypsin digestion (0.7 mg trypsin, 15 min, 4°C) and diluted with 5 ml of isolation medium containing 1 mg/ml BSA and 3.25 mg trypsin inhibitor. The suspension was stirred and the supernatant discarded. The partially digested tissue was resuspended in 10 ml isolation medium/BSA and homogenized with a handheld Teflon-glass homogenizer. The homogenate was centrifuged (10 min, 600*g*, 4°C). The supernatant solution was decanted and centrifuged (15 min, 8,000*g*, 4°C). The supernatant solution was discarded, and the pellet was twice resuspended in 10 ml isolation medium/BSA and centrifuged (15 min, 8,000*g*, 4°C), each time discarding the supernatant solution. The final, washed mitochondrial pellet was suspended in 1 ml of isolation medium/BSA. The mitochondrial solution was stored at −80°C for the preparation of protein lysates for UCP2 and UCP3 immunoblotting (29).

Immunoblotting for UCP2 and UCP3. Levels of cardiac UCP2 and UCP3 were measured in mitochondrial preparations by immunoblotting, as described previously (29), using polyclonal rabbit anti-UCP2 and anti-UCP3 antibodies (Alpha Diagnostic, San Antonio, TX) and an anti-rabbit IgG peroxidase conjugate polyclonal antibody (Autogen Bioclear, Wiltshire, U.K.). Membranes were covered in enhanced chemiluminescence detection solution (Amersham, U.K.) and exposed to X-ray film for visualization of protein bands. Developed films were digitized using a scanner and quantified using Quantiscan 32 (Biosoft, U.K.).

Blood plasma analyses. Blood samples, taken from mice immediately after killing, were centrifuged and the supernatant frozen at −80°C for determination of glucose, cholesterol, triacylglycerol, β-hydroxybutyrate, and insulin levels or frozen with a final concentration of 30 μg/ml lipoprotein lipase inhibitor (tetrahydrolipstatin Xenical; Roche) for determination of FFA concentrations. Plasma FFAs were measured using the spectrophotometric NEFA C kit (Wako Chemicals, Neuss, Germany), and insulin levels were determined using an enzyme-linked immunosorbent assay kit (Mercodia, Uppsala, Sweden). All other plasma metabolites were quantified using a spectrophotometric plasma analysis system (Monarch, Maumee, OH).

Statistics. Data are presented as means ± SE. Statistical significance was assessed using two-way ANOVA followed by a modified *t* test (Instat v 3.05; GraphPad Software, San Diego, CA). Differences were considered significant at $P < 0.05$.

RESULTS

PPARα$^{-/-}$ mice. There were no significant differences in the fasting plasma lipid or insulin concentrations between chow-fed wild-type and PPARα$^{-/-}$ mice (Table 1). Levels of myocardial UCP2 and UCP3 were 44 and 33% lower, respectively, in PPARα$^{-/-}$ than in wild-type mice ($P < 0.05$) (Fig. 1).

WY-14,643 and etomoxir feeding. In wild-type mice, feeding with the PPARα activator, WY-14,643, decreased plasma triacylglycerol levels by 32% ($P < 0.05$) and plasma insulin levels by 66% ($P < 0.05$) with no changes in plasma FFA levels (Table 1). Feeding the β-oxidation inhibitor etomoxir increased plasma FFA levels by 34% ($P < 0.05$)

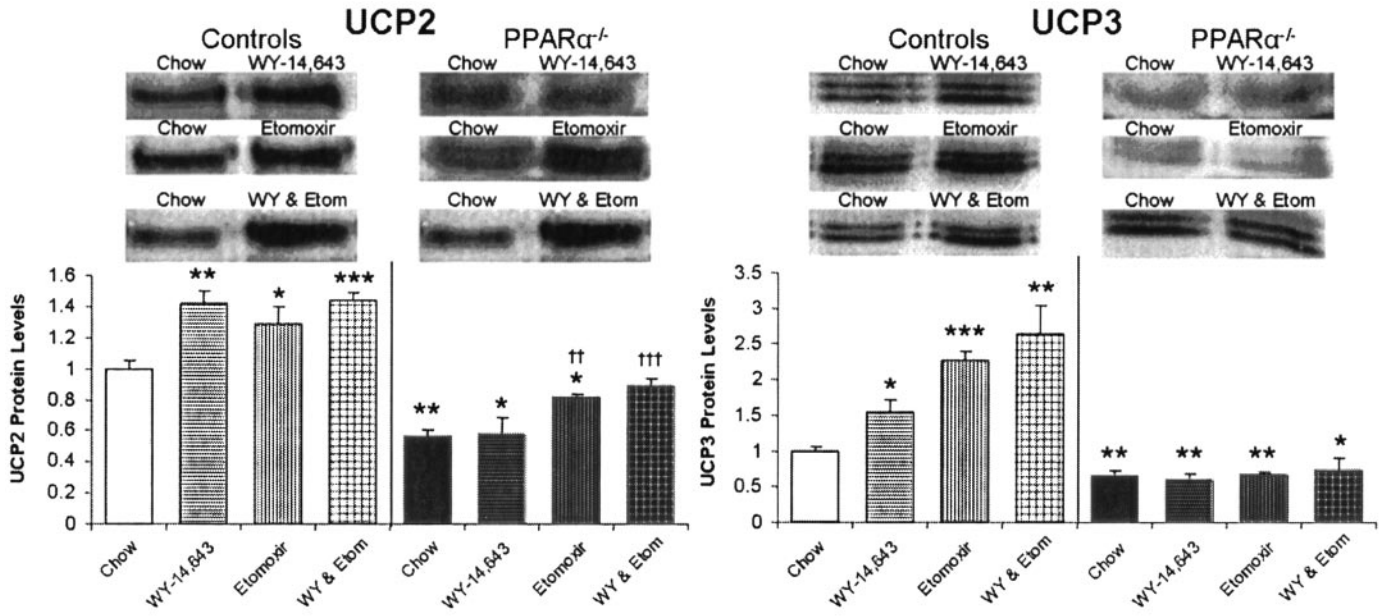

FIG. 1. Protein levels of UCP2 and UCP3 (relative to chow-fed wild-type mice) in cardiac mitochondria from PPARα$^{-/-}$ (■) and wild-type (□) mice fed WY-14,643 (▤), etomoxir (▥), or both WY-14,643 and etomoxir (▦). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with untreated wild-type mice; ††$P < 0.01$, †††$P < 0.001$ compared with untreated PPARα$^{-/-}$ mice. All groups $n = 4$.

and triacylglycerol levels by 26% ($P < 0.05$). Treatment with both WY-14,643 and etomoxir decreased triacylglycerol levels by 42% ($P < 0.01$) and plasma insulin levels by 66% ($P < 0.05$). Levels of cholesterol were the same for all wild-type mice (Table 1). In the PPARα$^{-/-}$ mice, treatment with WY-14,643, etomoxir, or WY-14,643 plus etomoxir resulted in no significant changes in levels of plasma FFAs, triacylglycerol, cholesterol, or insulin (Table 1).

Levels of myocardial UCP2 in wild-type mice increased by 42% with WY-14,643 treatment ($P < 0.01$), by 29% with etomoxir treatment ($P < 0.05$), and by 44% with both WY-14,643 and etomoxir ($P < 0.001$) (Fig. 1). Feeding PPARα$^{-/-}$ mice with WY-14,643 did not alter cardiac UCP2 levels, although etomoxir increased UCP2 levels by 46% ($P < 0.01$), and the combination of WY-14,643 plus etomoxir increased UCP2 levels by 58% ($P < 0.001$). UCP2 levels in PPARα$^{-/-}$ mouse hearts always remained lower than in untreated wild-type mice (Fig. 1).

Myocardial levels of UCP3 in wild-type mice increased by 54% with WY-14,643 ($P < 0.05$), by 126% with etomoxir ($P < 0.001$), and by 164% with the combined WY-14,643 plus etomoxir treatment ($P < 0.01$) (Fig. 1). UCP3 levels were not altered in PPARα$^{-/-}$ mouse hearts by any treatment (Fig. 1).

Streptozotocin treatment. Six weeks after STZ injections, plasma FFAs were increased by 91% in wild-type mice ($P < 0.01$) and by 196% in PPARα$^{-/-}$ mice ($P < 0.001$), and plasma triacylglycerol levels were increased by 61% in the wild-type mice ($P < 0.05$) and by 71% in the PPARα$^{-/-}$ mice ($P < 0.05$). Cholesterol levels were increased by 27% in both wild-type mice ($P < 0.001$) and the PPARα$^{-/-}$ mice ($P < 0.01$). Levels of plasma glucose were increased by 99% in the wild-type mice ($P < 0.01$) and by 143% in the PPARα$^{-/-}$ mice ($P < 0.01$). Insulin levels were 50% lower in the wild-type mice ($P < 0.01$) and 45% lower in the PPARα$^{-/-}$ mice ($P < 0.05$) (Table 2). Cardiac UCP2 levels were not altered by STZ treatment in wild-type or in PPARα$^{-/-}$ mice, but UCP3 levels increased by 50% in wild-type mouse hearts with no increase in UCP3 levels in the PPARα$^{-/-}$ mice (Fig. 2). Cardiac levels of UCP3 correlated positively with plasma FFA levels in control and STZ-treated mice ($r = 0.84$, $P < 0.01$) (Fig. 4).

***db/db* mice.** The *db/db* mice were 90% heavier than *db/+* mice ($P < 0.01$), with no significant increase in heart weight (Table 3). The *db/db* mice had 58% higher plasma FFA, 59% higher triacylglycerol, 31% higher cholesterol, and 248% higher glucose concentrations than the *db/+* mice. Plasma insulin levels were 310% higher in the *db/db*

TABLE 2
Body weights, heart weights, and fasting plasma concentrations of FFAs, triacylglycerol, glucose, cholesterol, and insulin in wild-type and PPARα$^{-/-}$ mice, with and without STZ treatment

	Wild-type		PPARα$^{-/-}$	
	−STZ	+STZ	−STZ	+STZ
Body weight (g)	34 ± 2	25 ± 1	29 ± 1	27 ± 2
Heart weight (mg)	284 ± 19	226 ± 12	244 ± 24	232 ± 14
FFAs (mmol/l)	0.35 ± 0.03	0.67 ± 0.07†	0.27 ± 0.05	0.80 ± 0.06‡
Triacylglycerol (mmol/l)	0.71 ± 0.08	1.14 ± 0.09*	0.59 ± 0.05	1.01 ± 0.13*
Cholesterol (mmol/l)	2.50 ± 0.04	3.17 ± 0.09‡	2.51 ± 0.05	3.19 ± 0.15†
Glucose (mmol/l)	11.0 ± 0.8	21.9 ± 2.0†	10.3 ± 1.4	25.0 ± 3.0†
Insulin (μg/l)	1.4 ± 0.2	0.7 ± 0.1†	1.1 ± 0.1	0.6 ± 0.2*

Data are means ± SE. $n = 4$ for all groups.* $P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ compared with untreated mice of the same strain.

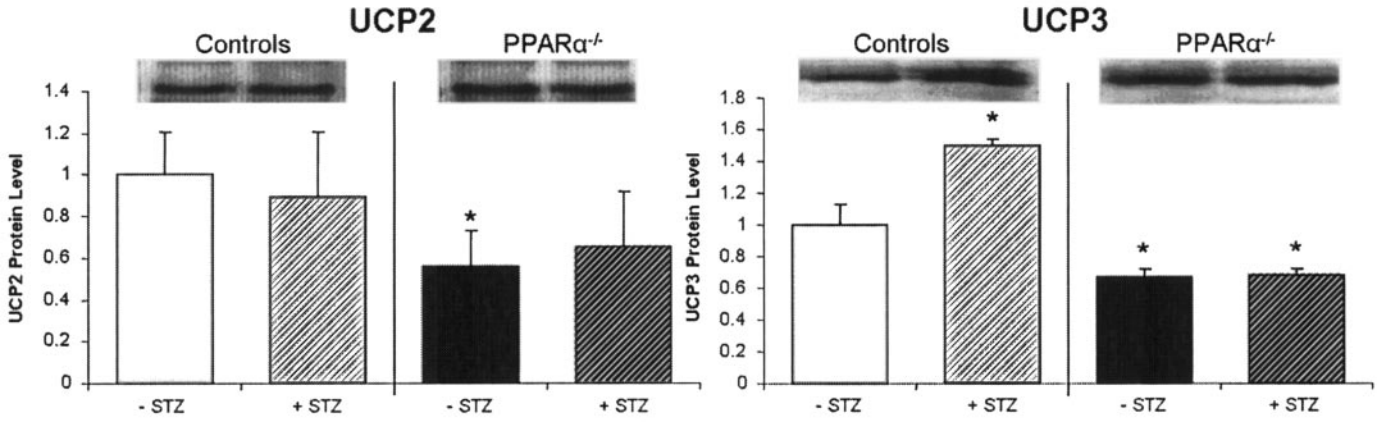

FIG. 2. Levels of UCP2 and UCP3 protein (relative to untreated wild-type mice) in cardiac mitochondria from PPARα$^{-/-}$ (■) and wild-type (□) mice. ▨, mice treated with STZ. *$P < 0.05$ compared with untreated wild-type mice. All groups $n = 4$.

mice ($P < 0.05$) (Table 3). Cardiac levels of UCP2 and UCP3 were, respectively, 37 ($P < 0.01$) and 38% ($P < 0.01$) higher in *db/db* mice than in *db/+* mice (Fig. 3). Cardiac UCP3 levels correlated positively with plasma FFA levels in *db/db* and *db/+* mice ($r = 0.66$; $P < 0.05$) (Fig. 4).

DISCUSSION

This study represents a comprehensive analysis of changes in cardiac UCP protein levels occurring with abnormal metabolic conditions, specifically those associated with high plasma FFA concentrations. A number of studies have measured changes in UCP mRNA expression in the heart (5,13,14) and other tissues (2,3,30,31) in response to circulating metabolite changes. Often, however, changes in UCP mRNA are not accompanied by similar changes at the protein level (15–17). The aim of the current study was to demonstrate how cardiac UCPs are regulated at the protein level by changes in FFA concentrations through PPARα activation. This is an extensive investigation into control of cardiac UCP protein levels and its importance to our understanding of the physiological role of these intriguing proteins.

In human heart, levels of cardiac UCP2 and UCP3 protein correlated positively with levels of plasma FFAs (6), the natural ligands of PPARα. A link between FFAs, PPARα activation, and UCP mRNA levels has been suggested in mouse heart. Reduced dietary fatty acid availability decreased cardiac expression of both UCP2 and UCP3 (5), and cardiac overexpression of PPARα increased UCP2 and UCP3 mRNA levels twofold and fivefold, respectively (32).

TABLE 3
Fasting plasma concentrations of FFAs, triacylglycerol, glucose, cholesterol, and insulin in nondiabetic (*db/+*) and diabetic *db/db* mice

	db/+ mice	*db/db* mice
Body weight (g)	28 ± 1	52 ± 2†
Heart weight (mg)	180 ± 6	169 ± 7
FFAs (mmol/l)	0.19 ± 0.02	0.30 ± 0.02†
Triacylglycerol (mmol/l)	1.02 ± 0.09	1.62 ± 0.16*
Cholesterol (mmol/l)	2.26 ± 0.14	2.95 ± 0.16*
Glucose (mmol/l)	12.2 ± 0.3	42.4 ± 3.1‡
Insulin (μg/l)	1.2 ± 0.1	4.3 ± 1.3*

Data are means ± SE. $n = 5$ for both groups. *$P < 0.05$, †$P < 0.01$, ‡$P < 0.0001$ compared with control mice.

In the PPARα$^{-/-}$ mouse, hepatic fatty acid oxidation is inhibited due to decreased β-oxidation enzyme activity (21). Cardiac expression of β-oxidation enzymes, including medium-chain acyl-CoA dehydrogenase and carnitine palmitoyltransferase-2, are lower in PPARα$^{-/-}$ mice (33). Levels of UCP3 mRNA were lower in the PPARα$^{-/-}$ mouse heart, whereas UCP2 mRNA levels were unchanged (5). In contrast, we found that both UCP2 and UCP3 protein concentrations were lower, by 44 and 33%, respectively, in the PPARα$^{-/-}$ mouse heart compared with the wild-type mouse. Thus, changes in mRNA and protein levels may be discordant, although PPARα exercises some control over protein levels of both UCPs in heart.

WY-14,643, a highly specific agonist for PPARα, promotes binding of PPARα and 9-*cis*-retinoic acid receptor heterodimers to PPAR response elements on DNA, upstream of target genes (8). Incubation of rat cardiomyocytes with fatty acids or WY-14,643 increased only UCP2 mRNA levels (9); however, feeding WY-14,643 increased only UCP3 mRNA levels in rat heart (5) and WY-14,643 incubation increased UCP3 mRNA levels in rat preadipocytes, with no change in UCP2 mRNA levels (30). Here, WY-14,643 feeding, alone or with etomoxir, increased both cardiac UCP2 and UCP3 protein levels in wild-type but not PPARα$^{-/-}$ mice.

Etomoxir, an irreversible inhibitor of carnitine palmitoyltransferase-1 originally developed for treating type 2 diabetes (34), has no acute cardiovascular effects in rats,

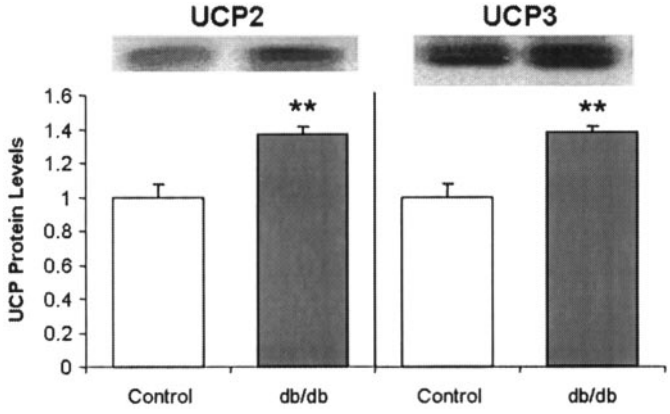

FIG. 3. Levels of UCP2 and UCP3 protein (relative to *db/+* mice) in cardiac mitochondria from *db/db* (■) and *db/+* (□) mice. **$P < 0.01$. All groups $n = 5$.

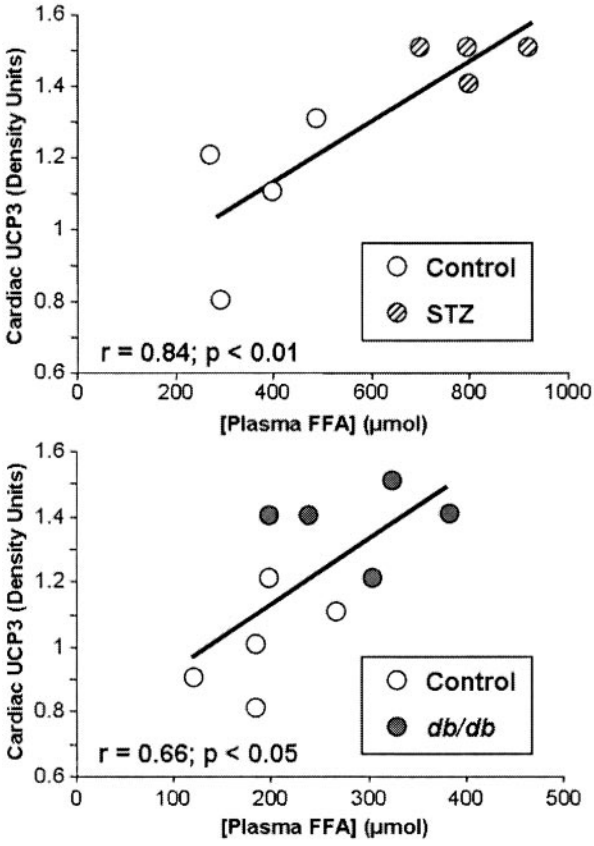

FIG. 4. Positive correlations between plasma FFA levels and cardiac levels of UCP3 protein in STZ-injected mice and their controls (r = 0.84; $P < 0.01$) and *db/db* mice and their controls (r = 0.66; $P < 0.05$). Trendlines are shown to guide the eye.

as shown by unaltered heart rate and blood pressure (35). Etomoxir feeding increases cardiac lipid droplet number (36) and, due to systemic inhibition of β-oxidation, increases plasma FFA levels (22,37). Etomoxir increased UCP3 protein levels in mouse and human skeletal muscle (4,38), and was found to increase UCP3 mRNA levels in rat preadipocytes, with no change in UCP2 mRNA (39). We found that etomoxir feeding, with or without WY-14,643, increased protein levels of UCP2 and UCP3 in mouse heart, suggesting that increased circulating FFA concentrations can increase protein levels of both UCPs in wild-type mouse heart.

Furthermore, it would appear from this finding that β-oxidation is not required for UCP levels to increase. Should the UCPs uncouple electron transport from ATP synthesis in cardiac mitochondria, then the increase in UCPs with etomoxir treatment suggests that partial inhibition of fatty acid oxidation, using such inhibitors as etomoxir, trimetazidine, perhexiline, and ranolazine, may have negative effects if used to treat heart failure (40,41). Activation of PPARα, due to the build up of fatty acid intermediary metabolites, may increase cardiac UCP levels, potentially increasing the energy deficit associated with higher mortality in heart failure patients (42).

In PPARα$^{-/-}$ mouse heart, neither WY-14,643 nor etomoxir feeding increased UCP3 levels, suggesting that fatty acid control of UCP3 occurred solely via PPARα. WY-14,643 did not alter, but etomoxir increased, cardiac UCP2 levels, which suggests a PPARα-independent mechanism, possibly triggered by high intracellular fatty acid levels. Increased hepatic UCP2 mRNA levels have been reported in PPARα$^{-/-}$ mice treated with the modified fatty acid tetradecylthioacetic acid, further supporting the case for a fatty acid–dependent, PPARα-independent regulation of UCP2 (43). As PPARβ/δ may partially compensate for a lack of PPARα (20), and as PPARβ/δ is present in the heart (44), it is possible that cardiac UCP2 protein can be increased in PPARα$^{-/-}$ mice via fatty acid activation of PPARβ/δ.

STZ-induced diabetes has been associated with increased UCP2 and UCP3 mRNA levels in rat skeletal muscle (3) and increased UCP3 mRNA in the rat heart (5) (13). Consequently, we measured UCP2 and UCP3 in the hearts of STZ-treated wild-type and PPARα$^{-/-}$ mice to establish whether such changes occur at the protein level and whether they depend on PPARα.

Whereas cardiac UCP2 protein levels did not change after STZ treatment in wild-type or PPARα$^{-/-}$ mice, cardiac UCP3 levels increased by 50% in wild-type but not in the PPARα$^{-/-}$ mice, again suggesting that UCP3 is under the control of PPARα. These findings, though consistent with previous studies, are hard to reconcile with our hypothesis that high plasma FFAs can increase myocardial UCP2 levels, especially given the clear increase in UCP3 levels in the same mice. The explanation for a lack of an increase in UCP2 in hearts of STZ-treated mice is unknown. In contrast, STZ-induced diabetes was associated with increased mRNA levels of both UCP2 and UCP3 in rat skeletal muscle (3).

Our results suggest that UCP3 is more sensitive than UCP2 to PPARα control in heart (5). In contrast to the STZ-treated animals, however, diabetic *db/db* mice, which had higher plasma levels of FFAs, triacylglycerol, glucose, cholesterol, and insulin, had higher levels of both cardiac UCP2 and UCP3 levels than the *db/+* mice. Against the background of a completely altered metabolic state, it is impossible to attribute the UCP changes to a single factor; however, other work suggests that increased levels of circulating FFAs increase UCP mRNA (14) and protein (6) levels in the heart.

In summary, our results are remarkably consistent in that high plasma FFA concentrations increased UCP3 levels through PPARα activation. This appears to be a very sensitive control mechanism, as we found significant positive correlations between cardiac UCP3 levels and plasma FFA concentrations, in both types of diabetic animals and their controls (Fig. 4). The mechanism for control of cardiac UCP2 levels appears to be far less straightforward with an alternative, PPARα-independent mechanism for upregulating cardiac UCP2 levels, and the link between plasma FFA levels and UCP2 expression being less sensitive than for UCP3, as exemplified by the STZ-treated mouse heart.

The precise role of the UCPs in the heart remains to be resolved, and the effect of the changes that we report here is, as yet, unknown. There is emerging evidence that UCP3 may function as a mitochondrial fatty acid anion transporter because, when upregulated, it is associated with a greater capacity for lipid utilization (31). There are conflicting reports on whether high UCP levels impair the mitochondrial proton electrochemical potential, with UCP2 (43) but perhaps not UCP3 (2,30,45) eliciting such an effect. Although an effect of increased UCP levels on cardiac energetics remains to be demonstrated, high plasma FFA concentrations are associated with low cardiac PCr-to-ATP ratios in diabetic patients (1), a decreased

myocardial PCr-to-ATP ratio being a strong predictor of mortality in patients with dilated cardiomyopathy (42). The hyperthyroid rat heart, which has a decreased PCr-to-ATP ratio (46), has increased myocardial UCP2 and UCP3 levels, increased mitochondrial uncoupling, and reduced cardiac efficiency (29). Furthermore, PPARγ agonist treatment, which lowers plasma FFA levels, has a beneficial effect on cardiac energy metabolism in diabetic rodents (47,48).

It remains to be established whether changes in cardiac levels of UCP2 and/or UCP3, such as those reported here, would lead to altered mitochondrial respiration, or indeed to changes in cardiac function. Further studies correlating changes in UCP levels with functional effects will define the role of mitochondrial uncoupling in the heart. Similarly, whereas a strength of this study over previous investigations is the measurement of cardiac UCP protein levels, the study is somewhat limited by the lack of data on UCP mRNA. Such data may have permitted clarification as to whether the discrepancies between changes in UCP mRNA in other studies and protein in our own are due to posttranscriptional control mechanisms. The small amount of ventricular tissue available from a single mouse was sufficient only for preparation of mitochondrial protein for the Western blots. A larger animal model, in which UCP mRNA and protein levels could both be measured, alongside mitochondrial respiration, may allow links between UCP gene expression and cardiac energy metabolism to be determined.

Our results show that UCP2 and UCP3 protein levels are regulated in heart by different mechanisms. Levels of both proteins are sensitive to plasma fatty acid concentrations and appear to be controlled by PPARα. UCP2, however, may also be controlled by another, PPARα-independent mechanism, possibly via PPARβ/δ. We suggest that there are discrete roles for UCP2 and UCP3 in the heart, with more work required to fully define their physiological importance.

ACKNOWLEDGMENTS

This study was supported by grants from the British Heart Foundation and the Medical Research Council.

We thank Frank Gonzalez (National Institutes of Health) for the gift of PPARα$^{-/-}$ mice and Gillian Watson for plasma insulin and metabolite assays. A.J.M. thanks the British Heart Foundation, and M.P. thanks the Rhodes Trust for their DPhil studentships.

REFERENCES

1. Scheuermann-Freestone M, Madsen P, Manners D, Blamire A, Buckingham R, Styles P, Radda G, Neubauer S, Clarke K: Abnormal cardiac and skeletal muscle energy metabolism in patients with type 2 diabetes. *Circulation* 107:3040–3046, 2003
2. Chou CJ, Cha MC, Jung DW, Boozer CN, Hashim SA, Pi-Sunyer FX: High-fat diet feeding elevates skeletal muscle uncoupling protein 3 levels but not its activity in rats. *Obes Res* 9:313–319, 2001
3. Kageyama H, Suga A, Kashiba M, Oka J, Osaka T, Kashiwa T, Hirano T, Nemoto K, Namba Y, Ricquier D, Giacobino J-P, Inoue S: Increased uncoupling protein-2 and-3 gene expression in skeletal muscle of STZ-induced diabetic rats. *FEBS Lett* 440:450–453, 1998
4. Schrauwen P, Hoeks J, Schaart G, Kornips E, Binas B, van der Vusse G, van Bilsen M, Luiken J, Coort S, Glatz J, Saris W, Hesselink M: Uncoupling protein 3 as a mitochondrial fatty acid transporter. *FASEB J* 17:2272–2274, 2003
5. Young M, Patil S, Ying J, Depre C, Ahuja H, Shipley G, Stepkoeski S, Davies P, Taegtmeyer H: Uncoupling protein 3 transcription is regulated by peroxisome proliferator-activated receptor a in the adult rodent heart. *FASEB J* 15:833–845, 2001
6. Murray A, Anderson R, Watson G, Radda G, Clarke K: Uncoupling proteins in human heart. *Lancet* 364:1786–1788, 2004
7. Boss O, Hagen T, Lowell B: Uncoupling proteins 2 and 3: potential regulators of mitochondrial energy metabolism. *Diabetes* 49:143–156, 2000
8. Forman B, Chen J, Evans R: Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. *Proc Natl Acad Sci U S A* 94:4312–4317, 1997
9. Gilde A, van der Lee K, Willemsen P, Chinetti G, van der Leij F, van der Vusse G, Staels B, van Bilsen M: Peroxisome proliferator-activated receptor (PPAR) alpha and PPAR beta/delta, but not PPAR gamma, modulate the expression of genes involved in cardiac lipid metabolism. *Circ Res* 92:518–524, 2003
10. van Bilsen M, van der Vusse G, Gilde A, Lindhout M, van der Lee K: Peroxisome prolilferator-activated receptors: lipid binding proteins controlling gene expression. *Mol Cell Biochem* 239:131–138, 2002
11. Tu N, Chen H, Winnikes U, Reinert I, Marmann G, Pirke KM, Lentes KU: Molecular cloning and functional characterization of the promoter region of the human uncoupling protein-2 gene. *Biochem Biophys Res Commun* 265:326–334, 1999
12. Acin A, Rodriguez M, Rique H, Canet E, Boutin JA, Galizzi JP: Cloning and characterization of the 5′ flanking region of the human uncoupling protein 3 (UCP3) gene. *Biochem Biophys Res Commun* 258:278–283, 1999
13. Hidaka S, Kakuma T, Yoshimatsu H, Sakino H, Fukuchi S, Sakata T: Streptozotocin treatment upregulates uncoupling protein 3 expression in the rat heart. *Diabetes* 48:430–435, 1999
14. Vettor R, Fabris R, Serra R, Lombardi A, Tonello C, Granzotto M, Marzolo M, Carruba M, Federspil G, Nisoli E: Changes in FAT/CD36, UCP2, UCP3 and GLUT4 gene expression during lipid infusion in rat skeletal and heart muscle. *Int J Obes Relat Metab Disord* 26:838–847, 2002
15. Sivitz W, Fink B, Donohoue P: Fasting and leptin modulate adipose and muscle uncoupling protein: divergent effects between messenger ribonucleic acid and protein expression. *Endocrinology* 140:1511–1519, 1999
16. Rodriguez VM, Portillo MP, Pico C, Macarulla MT, Palou A: Olive oil feeding up-regulates uncoupling protein genes in rat brown adipose tissue and skeletal muscle. *Am J Clin Nutr* 75:213–220, 2002
17. Ealey KN, El-Sohemy A, Archer MC: Effects of dietary conjugated linoleic acid on the expression of uncoupling proteins in mice and rats. *Lipids* 37:853–861, 2002
18. Murray A, Panagia M, Hauton D, Gibbons G, Clarke K: PPARa, free fatty acids and mitochondrial UCP3 in mouse heart. *J Mol Cell Cardiol* 37: 282, 2004
19. Lee SS, Pineau T, Drago J, Lee EJ, Owens JW, Kroetz DL, Fernandez-Salguero PM, Westphal H, Gonzalez FJ: Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. *Mol Cell Biol* 15:3012–3022, 1995
20. Muoio D, MacLean P, Lang D, Li S, Houmard J, Way J, Winegar D, Corton J, Dohm G, Kraus W: Fatty acid homeostasis and induction of lipid regulatory genes in skeletal muscles of peroxisome proliferator-activated receptor (PPAR) alpha knock-out mice. *J Biol Chem* 277:26089–26097, 2002
21. Kersten S, Seydoux J, Peters J, Gonzalez F, Desvergne B, Wahli W: Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J Clin Invest* 103:1489–1498, 1999
22. Koundakjian P, Turnbull D, Bone A, Rogers M, Younan S, Sherratt H: Metabolic changes in fed rats caused by chronic administration of ethyl 2[5(4-chlorophenyl)pentyl] oxirane-2-carboxylate, a new hypoglycaemic compound. *Biochem Pharmacol* 33:465–473, 1984
23. Ito M, Kondo Y, Nakatani A, Naruse A: New model of progressive non-insulin-dependent diabetes mellitus in mice induced by streptozotocin. *Biol Pharm Bull* 22:988–989, 1999
24. Panagia M, Gibbons GF, Radda GK, Clarke K: PPAR-alpha activation required for decreased glucose uptake and increased susceptibility to injury during ischemia. *Am J Physiol Heart Circ Physiol* 288:H2677–H2683, 2005
25. King LM, Sidell RJ, Wilding JR, Radda GK, Clarke K: Free fatty acids, but not ketone bodies, protect diabetic rat hearts during low-flow ischemia. *Am J Physiol Heart Circ Physiol* 280:H1173–H1181, 2001
26. Aasum E, Hafstad A, Severson D, Larsen T: Age-dependent changes in metabolism, contractile function and ischemic sensitivity in hearts from *db/db* mice. *Diabetes* 52:434–441, 2003
27. Aasum E, Belke DD, Severson DL, Riemersma RA, Cooper M, Andreassen M, Larsen TS: Cardiac function and metabolism in type 2 diabetic mice after treatment with BM 17.0744, a novel PPAR-alpha activator. *Am J Physiol Heart Circ Physiol* 283:H949–957, 2002
28. Taegtmeyer H, McNulty P, Young ME: Adaptation and maladaptation of the heart in diabetes: Part 1 general concepts. *Circulation* 105:1727–1733, 2002

29. Boehm E, Jones B, Radda G, Veech R, Clarke K: Increased uncoupling proteins and decreased cardiac efficiency in the palmitate-perfused hyperthyroid rat heart. *Am J Physiol* 280:H977–H983, 2001
30. Cabrero A, Alegret M, Sanchez R, Adzet T, Laguna JC, Vazquez M: Peroxisome proliferator-activated receptor alpha (PPARalpha) activators, bezafibrate and Wy-14,643, increase uncoupling protein-3 mRNA levels without modifying the mitochondrial membrane potential in primary culture of rat preadipocytes. *Arch Biochem Biophys* 380:353–359, 2000
31. Schrauwen P, Hoppeler H, Billeter R, Bakker AH, Pendergast DR: Fiber type dependent upregulation of human skeletal muscle UCP2 and UCP3 mRNA expression by high-fat diet. *Int J Obes Relat Metab Disord* 25:449–456, 2001
32. Finck BN, Lehman JJ, Leone TC, Welch MJ, Bennett MJ, Kovacs A, Han X, Gross RW, Kozak R, Lopaschuk GD, Kelly DP: The cardiac phenotype induced by PPARalpha overexpression mimics that caused by diabetes mellitus. *J Clin Invest* 109:121–130, 2002
33. Watanabe K, Fujii H, Takahashi T, Kodama M, Aizawa Y, Ohta Y, Ono T, Hasegawa G, Naito M, Nakajima T, Kamijo Y, Gonzalez F, Aoyama T: Constitutive regulation of cardiac fatty acid metabolism through peroxisome proliferator-activated receptor alpha associated with age-dependent cardiac toxicity. *J Biol Chem* 275:22293–22299, 2000
34. Hubinger A, Knode O, Susanto F, Reinauer H, Gries F: Effects of the carnitine-acyltransferase inhibitor etomoxir on insulin sensitvity, energy expenditure and substrate oxidation in NIDDM. *Horm Metab Res* 29:436–439, 1997
35. Rupp H, Zarain-Herzberg A, Maisch B: The use of partial fatty acid oxidation inhibitors for metabolic therapy of angina pectoris and heart failure. *Herz* 27:621–636, 2002
36. Rupp H, Schulze W, Vetter R: Dietary medium-chain triglycerides can prevent changes in myosin and SR due to CPT-1 inhibition by etomoxir. *Am J Physiol* 269:R630–R640, 1995
37. Reaven GM, Chang H, Hoffman BB: Additive hypoglycemic effects of drugs that modify free-fatty acid metabolism by different mechanisms in rats with streptozotocin-induced diabetes. *Diabetes* 37:28–32, 1988
38. Schrauwen P, Hinderling V, Hesselink MK, Schaart G, Kornips E, Saris WH, Westerterp-Plantenga M, Langhans W: Etomoxir-induced increase in UCP3 supports a role of uncoupling protein 3 as a mitochondrial fatty acid anion exporter. *FASEB J* 16:1688–1690, 2002
39. Cabrero A, Alegret M, Sanchez R, Adzet T, Laguna J, Vazquez M: Etomoxir, Sodium 2-[6-(4-Chlorophenoxy)hexyl]oxirane-2-carboxylate, up-regulates uncoupling protein-3 mRNA levels in primary culture of rat preadipocytes. *Biochem Biophys Res Commun* 263:87–93, 1999
40. Lee L, Horowitz J, Frenneaux M: Metabolic manipulation in ischaemic heart disease, a novel approach to treatment. *Eur Heart J* 25:634–641, 2004
41. Stanley WC, Chandler MP: Energy metabolism in the normal and failing heart: potential for therapeutic interventions. *Heart Fail Rev* 7:115–130, 2002
42. Neubauer S, Horn M, Cramer M, Harre K, Newell J, Peters W, Pabst T, Ertl G, Hahn D, Ingwall J, Kochsiek K: Myocardial phosphocreatine-to-ATP ratio is a predictor of mortality in patients with dilated cardiomyopathy. *Circulation* 96:2190–2196, 1997
43. Grav HJ, Tronstad KJ, Gudbrandsen OA, Berge K, Fladmark KE, Martinsen TC, Waldum H, Wergedahl H, Berge RK: Changed energy state and increased mitochondrial beta-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-alpha independent induction of UCP-2 expression. *J Biol Chem* 278:30525–30533, 2003
44. Gilde A, van Bilsen M: Peroxisome proliferator-activated receptors (PPARS): regulators of gene expression in heart and skeletal muscle. *Acta Physiol Scand* 178:425–434, 2003
45. Essop MF, Razeghi P, McLeod C, Young ME, Taegtmeyer H, Sack MN: Hypoxia-induced decrease of UCP3 gene expression in rat heart parallels metabolic gene switching but fails to affect mitochondrial respiratory coupling. *Biochem Biophys Res Commun* 314:561–564, 2004
46. Clarke K, Sunn N, Willis R: 31P NMR spectroscopy of hypertrophied rat heart: effect of graded global ischemia. *J Mol Cell Cardiol* 21:1315–1325, 1989
47. Sidell R, Cole M, Draper N, Desrois M, Buckingham R, Clarke K: Thiazolidinedione treatment normalizes insulin resistance and ischemic injury in the Zucker fatty rat heart. *Diabetes* 51:1110–1117, 2002
48. Carley AN, Semeniuk LM, Shimoni Y, Aasum E, Larsen TS, Berger JP, Severson DL: Treatment of type 2 diabetic db/db mice with a novel PPARgamma agonist improves cardiac metabolism but not contractile function. *Am J Physiol Endocrinol Metab* 286:E449–E455, 2004