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Mitochondrial regulators of fatty acid metabolism reflect metabolic dysfunction in type 2 diabetes mellitus

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ABSTRACT

The delicate homeostatic balance between glucose and fatty acid metabolism in relation to whole-body energy regulation is influenced by mitochondrial function. We determined expression and regulation of mitochondrial enzymes including pyruvate dehydrogenase kinase (PDK) 4, PDK2, carnitine palmitoyltransferase 1b, and malonyl–coenzyme A decarboxylase in skeletal muscle from people with normal glucose tolerance (NGT) or type 2 diabetes mellitus (T2DM). Vastus lateralis biopsies were obtained from NGT (n = 79) or T2DM (n = 33) men and women matched for age and body mass index. A subset of participants participated in a 4-month lifestyle intervention program consisting of an unsupervised walking exercise. Muscle biopsies were analyzed for expression and DNA methylation status. Primary myotubes were derived from biopsies obtained from NGT individuals for metabolic studies. Cultured skeletal muscle was exposed to agents mimicking exercise activation for messenger RNA (mRNA) expression analysis. The mRNA expression of PDK4, PDK2, and malonyl–coenzyme A decarboxylase was increased in skeletal muscle from T2DM patients. Methylation of the PDK4 promoter was reduced in T2DM and inversely correlated with PDK4 expression. Moreover, PDK4 expression was positively correlated with body mass index, blood glucose, insulin, C peptide, and hemoglobin A_{1c}. A lifestyle intervention program resulted in increased PDK4 mRNA expression in NGT individuals, but not in those with T2DM. Exposure to caffeine or palmitate increased PDK4 mRNA in a cultured skeletal muscle system. Our findings reveal that skeletal muscle expression of PDK4 and related genes regulating mitochondrial function reflects alterations in substrate utilization and clinical features associated with T2DM. Furthermore, hypomethylation of the PDK4 promoter in T2DM coincided with an impaired response of PDK4 mRNA after exercise.

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1. Introduction

Skeletal muscle substrate metabolism plays a fundamental role in whole-body nutrient utilization and homeostasis. Metabolic flexibility, defined by the ability to switch between lipid and glucose oxidation in response to a meal or after insulin stimulation, is impaired in skeletal muscle from insulin-resistant (type 2 diabetes mellitus [T2DM]) patients [1-3]. A number of key mitochondrial genes determine the balance between glucose and fatty acid metabolism [4], including pyruvate dehydrogenase kinases (PDKs), carnitine palmitoyltransferase (CPT1b, also known as *CPT1 muscle, CPT I, CPT1-M, CPTI-M*), and malonyl–coenzyme A (CoA) decarboxylase (MCD). The PDK enzymes phosphorylate the pyruvate dehydrogenase complex, which leads to inhibition of the complex and reduced carbohydrate oxidation. Thus, pyruvate dehydrogenase complex inhibition is recognized as a flux point at which fuel selection in skeletal muscle can be shifted toward fat oxidation [5]. Four isoforms of PDK have been identified in the human genome [6,7], and the primary isoforms expressed in skeletal muscle are PDK2 and PDK4 [8]. Malonyl CoA decarboxylase is a key enzyme regulating cellular malonyl CoA levels [9], and malonyl-CoA is an allosteric inhibitor of CPT1b [10]. Carnitine palmitoyltransferase 1b is located at the outer mitochondrial membrane and controls the transfer of long-chain fatty acid–CoA molecules from the cytosol to the mitochondria where they are oxidized [10]. Like the PDK enzymes, MCD and CPT1b also play a pivotal role in fuel selection and drive increases in fatty acid oxidation.

The role of PDK4 in skeletal muscle has been examined with various metabolic challenges. Pyruvate dehydrogenase kinase 4 expression is increased in response to short-term fasting [11] or high-fat diet [12], two circumstances that increase the supply of lipids to skeletal muscle. Pyruvate dehydrogenase kinase 4 transcription and PDK4 messenger RNA (mRNA) are markedly increased in human skeletal muscle during exercise of acute high intensity or prolonged low intensity [13]. Increased PDK activity in skeletal muscle suppresses glucose oxidation and thus may cause or exacerbate hyperglycemia. Mice with a targeted deletion of PDK4 have lower blood glucose levels and slightly improved glucose tolerance as compared with wild-type mice after an 18-week high-fat diet [14], highlighting a role of PDK4 in the development of hyperglycemia. Furthermore, insulin exposure during the euglycemic-hyperinsulinemic clamp reduces PDK4 mRNA expression in normal glucose-tolerant (NGT), but not insulin-resistant, people, indicating that elevations in PDK4 expression and activity may further increase plasma glucose levels [15-17].

Malonyl-CoA decarboxylase and CPT1b also coordinate fuel balance during exercise and states of metabolic disease. Exercise reduces skeletal muscle malonyl-CoA [18,19] and increases expression of MCD1 [19], which may contribute to the increase in lipid oxidation at the onset of exercise [18]. Increased levels of malonyl-CoA have been reported in insulin-resistant animal models [20]. However, MCD knockdown protects against the development of dietary-induced whole-body insulin resistance in mice [21] and enhances insulin-stimulated glucose uptake in cultured human muscle cells [9]. Carnitine palmitoyltransferase 1b expression and function have been linked to the regulation of insulin sensitivity. Chemical inhibition of CPT1b with the pharmacological agent etomoxir increases lipid deposition and exacerbates insulin resistance in rats fed a high-fat diet [22], whereas overexpression of CPT1b in rat hind limb muscle by electrotransfer prevents the dietary-induced insulin resistance on glucose uptake [23]. Whether MCD or CPT1b levels are altered or related to the metabolic phenotype in T2DM is unknown.

The aim of this study was to profile the expression pattern of PDK4, PDK2, MCD1, and CPT1b in human skeletal muscle from T2DM and matched NGT volunteers. We hypothesized that lifestyle modification resulting in weight loss and improved glycemic control would be accompanied by normalization of these regulators of metabolic flexibility.

2. Materials and methods

2.1. Subjects

Type 2 diabetes mellitus (n = 33) patients and NGT (n = 79) male and female volunteers were matched for age (61 ± 5 years) and body mass index (BMI) (30 ± 5 kg/m^2). The ethics committee at Karolinska Institutet approved all study protocols. Patients on insulin treatment and with symptomatic coronary heart disease were excluded. The clinical characteristics of the patients are presented in Table 1. All individuals provided a muscle biopsy upon entry into the study.

2.2. Intervention program

This was a randomized controlled study. Volunteers were randomized to the exercise or control group and asked to maintain their usual eating habits. The participants in the exercise group were instructed to increase their weekly level of physical activity by 5 hours of walking with walking poles (Nordic walking) for 4 months. They received instructions for Nordic walking from a physiologist/personal trainer. Of the total number of volunteers, a select group of male participants in each category (n = 23, NGT; n = 17, T2DM) underwent an exercise intervention and provided muscle biopsies before and after exercise training.

2.3. Skeletal muscle biopsies

Skeletal muscle biopsies were obtained from subjects in the morning, following an overnight fast. Local anesthesia (lidocaine hydrochloride, 5 mg/mL) was administered, and an incision (5 mm long/10 mm deep) was made in the skin and skeletal muscle fascia. A biopsy (20-100 mg) was obtained from the vastus lateralis portion of the quadriceps femoris using a conchotome tongue. Biopsies were immediately frozen and stored in liquid nitrogen until analysis.

2.4. Human skeletal muscle cells

Skeletal muscle biopsies (rectus abdominis) were obtained during scheduled abdominal surgery with informed consent from the donors. The subjects were 61 ± 5 years of age, were of

Table 1 – Anthropometric measurements and metabolic parameters in NGT and T2DM subjects

	NGT	T2DM	Total	NGT vs T2DM
N	79	33	112	*P* value
Sex (M/F)	30/49	24/9	66/69	
Age (y)	60 ± 1	62 ± 1	61 ± 0.5	.165
Height (cm)	169.8 ± 1.0	174.2 ± 1.6	171.1 ± 0.9	.024
Waist circumference (cm)	98.4 ± 1.1	105.0 ± 1.6	100.3 ± 0.9	.001*
SBP (mm Hg)	136 ± 1.62	141.52 ± 2.26	137.63 ± 1.34	.060
DBP (mm Hg)	82.66 ± 0.89	81.82 ± 1.39	82.41 ± 0.75	.611
BMI (kg/m^2)	29.8 ± 0.4	30.7 ± 0.6	30.1 ± 0.3	.246
FBG (mmol/L)	5.54 ± 0.05	8.08 ± 0.36	6.29 ± 0.16	<.001*
2-h BG (mmol/L)	7.20 ± 0.11	15.53 ± 0.59	9.66 ± 0.41	<.001*
HbA_{1c} (%)	4.7 ± 0.0	6.1 ± 0.2	5.1 ± 0.1	<.001*
Insulin (pmol/L)	61.78 ± 4.04	69.71 ± 9.43	63.99 ± 3.91	.366
Cholesterol (mmol/L)	5.50 ± 0.09	4.71 ± 0.15	5.27 ± 0.09	<.001*
LDL (mmol/L)	1.63 ± 0.09	1.28 ± 0.14	1.52 ± 0.08	.002*
HDL (mmol/L)	3.32 ± 0.05	2.79 ± 0.06	3.17 ± 0.04	<.001*
C-peptide (nmol/L)	0.83 ± 0.03	1.05 ± 0.07	0.89 ± 0.03	<.001*
TG (mmol/L)	1.23 ± 0.06	1.38 ± 0.09	1.28 ± 0.05	.173
Apo A-I (g/L)	1.60 ± 0.03	1.38 ± 0.04	1.54 ± 0.03	<.001*
Apo B (g/L)	1.01 ± 0.02	0.93 ± 0.04	0.99 ± 0.02	.063
Vo_2 (L/min)	2.01 ± 0.07	2.07 ± .08	2.03 ± 0.06	.654
RQ	1.10 ± 0.01	1.08 ± 0.01	1.09 ± 0.01	.333

Data are presented as means ± SEM. SBP indicates systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; TG, triglycerides; Vo_2, oxygen consumption; RQ, respiratory quotient. *Significant at $P < .01$ level.

average weight (BMI, 26 ± 0.5 kg/m^2), and had no known metabolic disorders. The ethics committee at Karolinska Institutet approved the study protocols. Satellite cells were isolated from the biopsies by trypsin and collagenase digestion and grown to confluent myoblasts that were differentiated to myotubes as described [24,25]. Dulbecco modified Eagle medium, Ham F-10 medium, fetal bovine serum, penicillin, streptomycin, and Fungizone were obtained from Gibco BRL (Invitrogen, Stockholm, Sweden). Unless specified, all reagents were purchased from Sigma (Stockholm, Sweden). Radioactive reagents were purchased from Amersham.

2.5. *mRNA expression analysis in muscle biopsies*

Messenger RNA was prepared from vastus lateralis muscle biopsies for gene expression analysis. Pure mRNA was extracted from 30 mg of frozen tissue using a standard Trizol extraction method (Invitrogen). Complementary DNA was generated from 1 μg of mRNA using the High-Capacity cDNA RT kit (Applied Biosystems, Stockholm, Sweden). Gene expression analysis was carried out using a TaqMan-based multifluidic card (MFC) gene expression assay (Applied Biosystems, Foster City, CA). The following primer and probe sets from Applied Biosystems were lyophilized in the MFC well: MFC internal endogenous control 18S; Hs99999907_m1 (β2-microglobulin); Hs99999905_m1 (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]); Hs00176865_m1 (PDK2); Hs00176875_m1 (PDK4); Hs03046298_s1 (CPT1B [muscle]); Hs00201955_m1 (MCD-1, MLYCD). Samples were applied into the MCF well, and polymerase chain reaction (PCR) amplification was performed using an Applied Biosystems Prism 7900HT sequence detection system (Applied Biosystems, Stockholm, Sweden). Messenger RNA was quantified according to technical documents supplied by the manufacturer, and the relative abundance of target transcripts was calculated after normalization of the data against the housekeeping genes.

2.6. *Immunoblot analysis*

Aliquots of human skeletal muscle lysates (20 μg) were resuspended in Laemmli buffer. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Bio-Rad, Danvers, MA), blocked with 7.5% nonfat milk, washed with TBST (10 mmol/L Tris-HCL, 100 mmol/L NaCl, and 0.02% Tween 20), and incubated with primary antibodies overnight at 4°C. Membranes were washed with TBST and incubated with appropriate horseradish peroxidase–conjugated secondary antibodies (Bio-Rad, diluted 1:25 000). Proteins were visualized by enhanced chemiluminescence (GE Healthcare, Stockholm, Sweden). A rabbit monoclonal antibody against PDK2 (catalog no. ab76152) was purchased from Abcam (Cambridge, UK), and mouse monoclonal antibody against MCD (catalog no. H00023417-B01P) was purchased from Abnova (Taipei City, Taiwan).

2.7. *mRNA expression analysis in cultured cells*

Myotubes, prepared as described above, were washed 3 times with RNase-free phosphate-buffered saline and then harvested directly for RNA extraction (RNeasy Mini Kit, Qiagen, Sollentuna, Sweden). Total RNA concentration was measured and reverse transcribed with random hexamers using the high-capacity reverse transcription kit (Applied Biosystems). Reactions were performed in duplicate in a 96-well format using Prism 7000 Sequence Detector and TaqMan-based technology (Applied Biosystems). TaqMan probes were purchased from Applied Biosystems. Relative quantities of target

transcripts were calculated after normalization of the data using the standard curve method or comparative CT method.

2.8. Bisulfite sequencing

A subset of muscle biopsies derived from NGT and T2DM subjects was selected for gDNA methylation analysis (age- and BMI-matched male donors; n = 4, NGT; n = 4, T2DM). Bisulfite treatment was performed as described [26], with the following adaptations: 1 μg of genomic DNA was embedded in a 2% low–melting point agarose solution, and 10 agarose beads were formed. A freshly prepared bisulfite solution (4 mol/L sodium β-bisulfite, Sigma; 250 mmol/L hydroquinone, Sigma; pH 5.0) was added to each reaction tube containing one single bead. The reaction mixtures were incubated for 4 hours at 50°C under exclusion of light. Treatment was stopped by equilibrations against 1 mL of Tris-EDTA (4× 15 minutes) followed by desulfonation in 500 μL of 0.2 mol/L NaOH (2× 15 minutes). The reaction was neutralized, and beads were washed with 1 mL Tris-EDTA (2× 15 minutes). Before the PCR analysis, the beads were equilibrated against 1 mL of H_2O (2× 30 minutes). For the amplification of regions +160 to +446 of the PDK4 promoter, the following primers were used: sense 5′-GGT ATT TTT AAA TTT TAG TTT AGG T-3′, antisense 5′-ATC CAA TAA CTA CTT CAT AAA CAA C-3′. The PCR fragments were purified from an agarose gel using MinElute Gel Extraction Kit (Qiagen) and cloned into pDrive vector using a PCR cloning kit (Qiagen) according to the manufacturer's protocol. Individual clones were grown, and plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen). For each condition, 10 to 50 clones were sequenced using T7 promoter primer on an ABI 3730xl DNA Analyzer platform at Cogenics (Hope End, UK).

2.9. Statistical analysis

All data are presented as mean ± SEM. Baseline differences in gene expression between NGT subjects and T2DM patients were analyzed using an independent *t* test. Pearson correlations were calculated for baseline measurements of gene and clinical parameters. Regression models controlled for covariates (age, sex, and BMI) were used to examine clinical predictors of gene expression. Computation of the fold change following exercise intervention was performed using a paired *t* test. Comparisons were considered statistically significant at $P < .05$. Natural log transformation was applied when data were not normally distributed. Analyses were performed using SPSS version 17.0 (SPSS, Chicago, IL).

3. Results

3.1. PDK4, PDK2, and MCD1 mRNA expression is increased in skeletal muscle from T2DM patients

We measured skeletal muscle mRNA expression of PDK4, PDK2, CPT1b, and MCD1 in T2DM and in age- and BMI-matched NGT subjects (clinical characteristics are reported in Table 1). Skeletal muscle mRNA expression of PDK4 was increased approximately 70% ($P < .01$) in T2DM patients as compared with BMI- and age-matched NGT volunteers (Fig. 1A). Messenger RNA expression of PDK2 and MCD1 was increased approximately 50% in skeletal muscle from T2DM patients (Fig. 1B-C, $P < .05$). In contrast, mRNA expression of CPT1b was similar between cohorts. Protein expression of PDK2 and MCD was determined in a subset of subjects where material permitted (Fig. 1E). Protein levels support the observed mRNA expression patterns. Antibody detection of protein levels of PDK4 and CPT1b using commercially available antibodies was unsuccessful, with antibodies detecting several nonspecific bands.

3.2. Skeletal muscle mRNA expression of PDK4 correlates with BMI and with PDK2, CPT1, and MCD mRNA expression

Skeletal muscle PDK4 mRNA expression was positively correlated with BMI (represented graphically in Fig. 2B), waist circumference, fasting and 2-hour blood glucose, hemoglobin A_{1c} (HbA_{1c}), insulin, and circulating C peptide in the entire cohort of study participants (n = 112, Table 2). The differential clustering pattern of NGT and T2DM subjects may suggest a tighter relationship between PDK4 mRNA and BMI in the presence of maintained glucose regulation. Pyruvate dehydrogenase kinase 4 mRNA expression was negatively correlated ($P < .05$) with cholesterol and low-density lipoprotein (LDL). Pyruvate dehydrogenase kinase 2 mRNA was positively correlated with fasting and 2-hour blood glucose measurements ($P < .05$), and negatively correlated with diastolic blood pressure, high-density lipoprotein (HDL) and apolipoprotein (apo) A-I. Malonyl-CoA decarboxylase was inversely correlated with HDL and apo A-I (Table 2, $P < .05$). Messenger RNA expression of the mitochondrial genes was positively and highly correlated ($r = 0.246$-0.889, $P < .001$), with the strongest correlation observed for expression of PDK2 and MCD (Fig. 2A, $r = 0.889$, $P < .0001$).

Multiple regression analysis revealed that BMI is associated with PDK4 gene expression after adjustment for age, sex, and diabetes status (Table 3). Regression models demonstrate that the relationship between PDK4 and BMI was significant, independent of age, sex, cohort, and glucose tolerance. After correcting for age, sex, and cohort, the expression of PDK4 remained independently determined by BMI ($r = 0.348$, $P = .002$). Although the expression of the genes correlated highly, similarly strong relationships did not exist between other genes and the clinical factors after controlling for age, sex, and cohort.

3.3. PDK4 promoter methylation is reduced in skeletal muscle from T2DM patients

Using bisulfite sequencing, we determined the methylation status of cytosines in the +160 to +446 region of the PDK4 promoter in skeletal muscle from T2DM patients and NGT volunteers. The increase in PDK4 mRNA expression noted in the T2DM patients was accompanied by a parallel reduction in PDK4 promoter methylation (Fig. 3).

3.4. Effect of lifestyle modification on skeletal muscle mRNA expression

We further determined the effect of a 4-month lifestyle intervention involving approximately 4 hours of Nordic

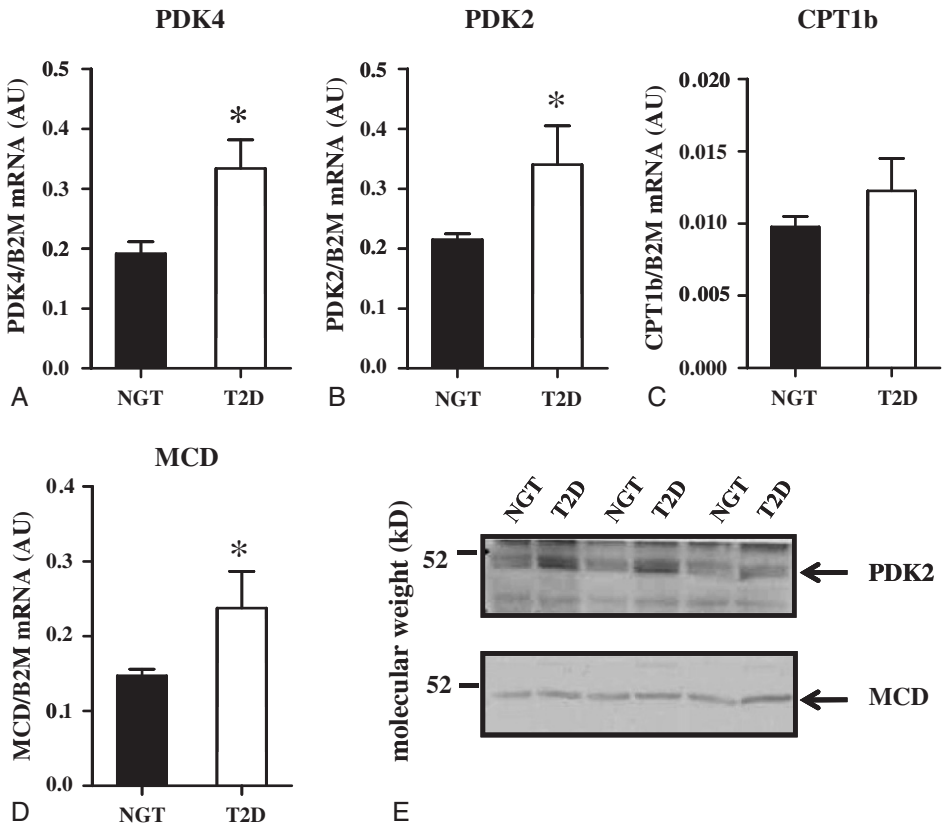

Fig. 1 – Gene expression analysis. Pyruvate dehydrogenase kinase 4 (A), PDK2 (B), CPT1b (C), and MCD (D) mRNA expressions were measured in skeletal muscle of age- and BMI-matched subjects with NGT (n = 79) and T2DM (n = 33). The mRNA expression of PDK4, PDK2, and MCD was significantly elevated in T2DM patients (*P < .05). The mRNA expression is reported as a relative expression related to ß2 microglobulin (B2M) mRNA. Values are means ± SE arbitrary units (AU) for subjects in each group. E, Representative immunoblot analysis of skeletal muscle protein expression of PDK2 and MCD.

walking a week on skeletal muscle mRNA expression in a subgroup of this population. Walking exercise was associated with modest weight loss in NGT and T2DM subjects ($P < .05$; Table 4). In the T2DM patients, weight loss was accompanied by a reduction in 2-hour plasma glucose levels, whereas in the NGT subjects, the fasting plasma insulin level was reduced. Skeletal muscle PDK4 mRNA expression was increased in response to lifestyle intervention, but only in NGT subjects (Fig. 4A). Although PDK4 expression in skeletal muscle was elevated in T2DM before the exercise intervention (Fig. 1A), no further increase was noted upon the completion of the intervention program. Skeletal muscle mRNA expression of PDK2, MCD1, or CPT1b was unaltered by the lifestyle intervention program (Fig. 4 B-D).

3.5. *Regulation of mRNA expression of PDK4, PDK2, CPT1b, and MCD1 in cultured human muscle cells*

We determined mRNA expression of PDK4, PDK2, CPT1b, and MCD1 in cultured skeletal muscle following 48 hours of incubation in the presence of 3 mmol/L caffeine, 1 mmol/L AICAR, or 200 μmol/L palmitate. These agents were selected to mimic metabolic conditions generated in skeletal muscle during exercise, such as increased Ca^{2+} (induced in cultured muscle following exposure to caffeine [27]), activation of AMPK [28], or increased lipids. Caffeine and palmitate independently increased mRNA expression of PDK4 approximately 40-fold and 15-fold, respectively (Fig. 5A). Caffeine exposure increased mRNA expression of PDK2 approximately 50% ($P < .05$; Fig. 5B). Palmitate exposure increases CPT1 mRNA, whereas MCD1 expression increased in response to both caffeine and palmitate (Fig. 5C, D).

4. Discussion

Type 2 diabetes mellitus is associated with abnormal substrate metabolism, raising the possibility that alterations in the expression of mitochondrial enzymes controlling lipid uptake and metabolism may by altered. Here we determined skeletal muscle expression of key mitochondrial genes that orchestrate the switch of substrate utilization between glucose and lipid sources. We show that mRNA expression of PDK4, PDK2, and MCD1 is upregulated in human skeletal muscle from T2DM patients. We have previously demonstrated that reduction of MCD1 enhances glucose and reduces lipid metabolism in cultured human muscle cells [9]. Thus, increased MCD1 expression may play a role in reducing skeletal muscle glucose utilization in glucose-intolerant individuals.

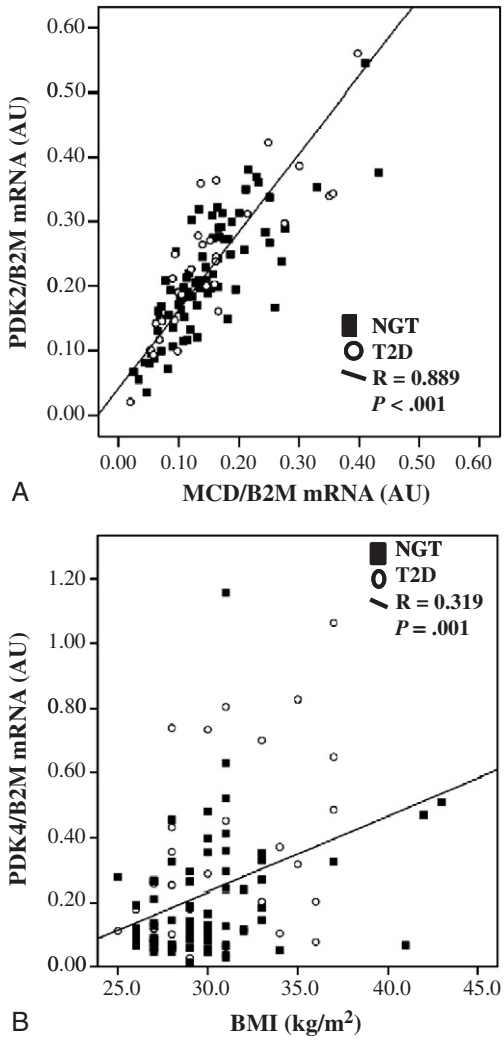

Fig. 2 – Baseline correlation analysis. A, Gene expression of PDK4, PDK2, CPT1b, and MCD is highly correlated in the entire cohort of subjects examined. Correlations between PDK2 and MCD represent the strongest correlation (n = 112, r = 0.889, P < .0001). The mRNA expression is reported as a relative expression related to B2M mRNA. B, The PDK4 gene expression correlates weakly with BMI (r = 0.319, P = .001). NGT (n = 79, black, filled squares) and T2DM (n = 33, open circles).

Elevated PDK4 gene expression corresponds with various clinical phenotypes. Circulating lipid levels have been highlighted as a key factor determining PDK4 expression [5]. In rats, a 4-week high-fat diet increases PDK4 mRNA expression [29]. In our study, plasma triglyceride levels were unaltered between NGT volunteers and T2DM patients; and thus, PDK4 mRNA expression was unrelated to triglyceride levels. A subset of the T2DM patients in the present study regularly used cholesterol-lowering medications or other pharmacological interventions, which may mask such relationships. Messenger RNA expression of PDK4 is also acutely regulated by insulin [17,30]; and thus, the increased PDK4 mRNA in the T2DM patients may be a consequence of hyperinsulinemia and insulin resistance at the level of the enzyme, such that expression is not appropriately suppressed. Reductions in skeletal muscle glycogen is known to increase PDK4 mRNA [31]. Although muscle glycogen content was not determined, PDK4 was positively correlated with fasting glucose levels. However, a significant correlation between PDK4 mRNA expression and BMI highlights the relationship between the expression of mitochondrial genes related to lipid metabolism and body mass. Examination of gene-gene correlations revealed positive relationships between PDK4, PDK2, CPT1b, and MCD in the entire study cohort (Table 2), further supporting that this cluster of genes is similarly coordinated and regulated in healthy and diseased individuals. The inverse correlation noted between PDK4 methylation and PDK4 expression in skeletal muscle suggests the possibility that epigenetic modification orchestrates the regulation of mitochondrial genes involved in substrate switching. Although the verification of PDK4 regulation at the protein level would further strengthen this finding, we were unable to reliably detect PDK4 protein using currently available antibodies. Our analysis of PDK2 and MCD protein reflects the increase noted in mRNA levels. Because the elevation of these genes at the mRNA level correlates highly (Fig. 2A), this suggests that an increase in PDK4 transcript may consequently result in an end-point increase in protein expression also, as has been reported for the majority of mammalian genes [32]. Collectively, our findings provide evidence that the level of PDK4 gene expression reflects glucose/lipid metabolism, which is possibly potentiated by epigenetic control.

A 4-month lifestyle modification program increased skeletal muscle PDK4 mRNA expression in NGT subjects, but was without effect on PDK4 expression in T2DM patients. This differential response could be due to a higher compliance with the exercise program in the NGT subjects or to an inability of the T2DM patients to appropriately respond to the exercise training program. Pyruvate dehydrogenase kinase 2, CPT1b, or MCD gene expression was unaltered following exercise intervention in our study, indicating a set level of stability and/or a resistance of fluctuation in response to this particular exercise stimulation. However, the understanding of the overall metabolic regulation of PDK4 is complicated by seemingly conflicting results, as an elevation is observed both in T2DM and following exercise intervention. The apparent paradox of skeletal muscle lipid metabolism in states of insulin resistance vs exercise training has been discussed previously [33].

Conditions of obesity and insulin resistance are strongly associated with impaired rates of fatty acid oxidation in skeletal muscle [34]. Further indication of high fatty acid availability is a buildup of intramyocellular lipids (IMCL), which reflects an elevated fatty acid flux into the muscle [35,36]. Thus, our finding that PDK4 mRNA is elevated in T2DM and correlates with BMI further supports that an elevated lipid flux in an insulin-resistant state requires an upregulation of gene pathways involved in the switch from carbohydrate to lipid oxidation. However, the molecular pathways involved in lipid uptake and storage are not entirely transparent, as the muscle of highly trained athletes also contains excess IMCL [33]. The observation that PDK4 is elevated after exercise, concomitant with an observed decrease in BMI, may appear

Table 2 – Correlation analysis between skeletal muscle mRNA expression and clinical parameters in NGT and T2DM subjects

	PDK4 mRNA		PDK2 mRNA		CPT1b mRNA		MCD mRNA	
	r	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value
PDK4 mRNA	–	–	0.278†	.003	0.246†	.009	0.374†	<.001
PDK2 mRNA	0.278†	.003	–	–	0.637†	<.001	0.889†	<.001
CPT1b mRNA	0.246†	.009	0.637†	<.001	–	–	0.689†	<.001
MCD mRNA	0.374†	<.001	0.889†	<.001	0.689†	<.001	–	-
Height (cm)	–0.066	.489	0.061	.520	–0.117	.221	–0.046	.631
Waist circumference (cm)	0.328†	<.001	–0.033	.730	–0.141	.140	–0.065	.496
SBP (mm Hg)	0.057	.550	0.005	.956	0.009	.925	0.025	.792
DBP (mm Hg)	–0.045	.641	–0.203*	.032	–0.087	.364	–0.192*	.042
BMI (kg/m^2)	0.319†	.001	–0.010	.919	0.006	.950	0.030	.752
FBG (mmol/L)	0.248†	.008	0.198*	.037	0.149	.120	0.185	.050
2-h BG (mmol/L)	0.284†	.002	0.227*	.016	0.149	.120	0.198*	.036
HbA_{1c} (%)	0.251†	.008	0.165	.083	0.089	.352	0.147	.122
Insulin (pmol/L)	0.263*	.011	0.107	.306	0.085	.422	0.190	.068
Cholesterol (mmol/L)	–0.241	.022	–0.105	.270	–0.030	.757	–0.057	.548
LDL (mmol/L)	–0.186*	.050	–0.029	.761	0.045	.641	0.007	.939
HDL (mmol/L)	–0.155	.103	–0.224*	.018	–0.216*	.022	–0.156	.102
C-peptide (nmol/L)	0.328†	<.001	0.053	.580	0.077	.420	0.081	.395
Apo A-I (g/L)	–0.104	.274	–0.230*	.014	–0.243*	.010	–0.185	.051
Apo B (g/L)	–0.067	.482	–0.069	.472	0.055	.563	–0.012	.898
Vo_2 (L/min)	–0.048	.622	0.080	.410	0.156	.107	0.052	.588
RQ	–0.076	.435	–0.183	.056	0.033	.737	–0.115	.232

* Significant at $P < .05$ level.
† Significant at $P < .01$ level.

contradictory given the positive correlation between PDK4 and BMI observed at baseline. However, reports examining the effects of exercise on skeletal muscle fatty acid transport and oxidation, particularly low-intensity exercise, demonstrate a high reliance of the muscle on lipid sources of fuel [37,38]. The initiation of exercise mobilizes FFAs from adipose tissue as a fuel source [39], and subsequent oxidation in muscle mitochondria requires increased participation of relevant players involved in the metabolic switch [40]. Thus, as with states of insulin resistance, exercise intervention elicits an increase in PDK4 expression in NGT subjects, reflecting a metabolic adjustment to prioritize lipid fuel handling. We did not observe a PDK4 elevation in T2DM following exercise, which

Table 3 – Multiple regression analysis between age, sex, BMI, and PDK4 mRNA expression in NGT and T2DM subjects

	PDK4	
	β-Coefficient	*P* value
Model 1		
Age	0.023	.132
Sex	–0.065	0.685
Cohort	0.208	.020*
BMI	2.296	.002†
Model 2		
Age	0.022	.154
Sex	–0.066	.683
Cohort	0.143	.443
BMI	2.285	.002†
2-h BG	0.176	.689
Model 3		
Age	0.018	.305
Sex	0.006	.972
Cohort	0.227	.016*
BMI	1.731	.029*
Insulin	0.342	.026*

* Significant at $P < .05$ level.
† Significant at $P < .01$ level.

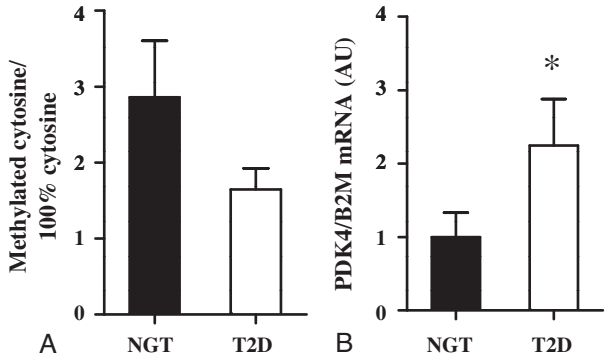

Fig. 3 – Methylation analysis. A, Methylation analysis of the PDK4 promoter by bisulfite sequencing. The PDK4 promoter methylation is expressed as a percentage of cytosine methylation per 100% of total cytosine in subjects with NGT (n = 4) and T2DM (n = 4). Results are mean ± SEM. B, Skeletal muscle PDK4 mRNA expression in the same set of subjects. Expression is reported as a relative expression related to B2M mRNA. Values are means ± SE AU for subjects in each group (*P < .05).

Table 4 – Anthropometric measurements and metabolic parameters in NGT and T2DM men before and after exercise training

		Pre ± SEM	Post ± SEM	Δ ± SEM	P value		
					Pre vs post	Pre, NGT vs T2DM	Δ, NGT vs T2DM
N	NGT	23					
	T2DM	17					
Age	NGT	61 ± 1					
	T2DM	62 ± 1				.253	
Weight (kg)	NGT	89.9 ± 2.04	88.5 ± 2.16	−1.39 ± 0.43	.003†		
	T2DM	94.1 ± 2.74	92.5 ± 2.71	−1.54 ± 0.59	.018*	.220	.830
BMI (kg/m^2)	NGT	28.7 ± 0.43	28.1 ± 0.46	−0.57 ± 0.15	.001†		
	T2DM	29.9 ± 0.81	29.5 ± 0.78	−0.44 ± 0.20	.042*	.184	.628
Waist circumference (cm)	NGT	101.57 ± 1.63	98.00 ± 1.64	−3.57 ± 0.54	<.0001†		
	T2DM	105.11 ± 2.05	103.06 ± 2.07	−2.06 ± 0.63	.005†	.178	.076
FBG (mmol/L)	NGT	5.57 ± 0.11	5.48 ± 0.11	−0.09 ± 0.11	.434		
	T2DM	8.32 ± 0.52	8.01 ± 0.52	−0.32 ± 0.24	.207	<.0001†	.407
2-h BG (mmol/L)	NGT	7.07 ± 0.22	6.84 ± 0.27	−0.23 ± 0.26	.392		
	T2DM	15.11 ± 0.82	13.37 ± 1.00	−1.73 ± 0.55	.006†	<.0001†	.021*
Insulin (pmol/L)	NGT	65.82 ± 6.91	44.76 ± 4.01	−17.33 ± 5.02	.003†		
	T2DM	88.19 ± 12.93	87.07 ± 13.35	10.06 ± 12.91	.917	.139	.066
V_{O_2} (L/min)	NGT	2.46 ± 0.14	2.61 ± 0.12	0.15 ± 0.12	.249		
	T2DM	2.13 ± 0.12	2.22 ± 0.12	0.03 ± 0.15	.872	.102	.540
Nordic walking (h/wk)	NGT		4.895 ± 0.34				
	T2DM		4.061 ± 0.38			.110	

* Significant at $P < .05$ level.
† Significant at $P < .01$ level.

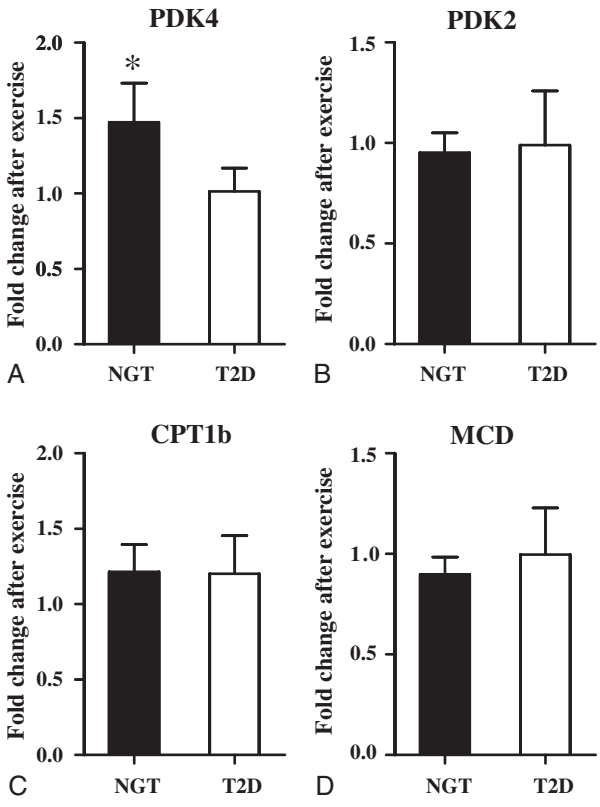

Fig. 4 – Effects of exercise on gene expression. The relative change in skeletal muscle mRNA expression of PDK4 (A), PDK2 (B), CPT1b (C), and MCD (D) mRNA in age- and BMI-matched NGT and T2DM subjects following a 4-month lifestyle intervention program. Values are means ± SEM (*P < .05).

may signify that this exercise intervention was not of sufficient intensity to elicit a response in insulin-resistant participants. An examination of IMCL was not performed in this study.

The expression of the FAT/CD36 transporter in skeletal muscle reflects a similar pattern of regulation as we report for PDK4. Increases in FAT/CD36 mRNA and/or protein have been observed in human obesity, in T2DM [41], and after a high-fat diet [42,43]. Elevation in FAT/CD36 transporter levels in skeletal muscle is also observed following exercise training, through a proposed metabolic adaptation of greater fuel utilization [44]. Thus, an increase in expression of genes involved in the switch of fuel sources could be expected in two contrary circumstances, both of which require similar molecular orchestration to balance an elevated fatty acid influx.

DNA methylation alters gene transcription by regulating the accessibility of the transcription factors and transcription machinery to the DNA strand. Whether the differential exercise-dependent mRNA change in PDK4 is related to the difference in PDK4 promoter methylation noted between NGT and T2DM patients remains to be investigated. Increased promoter methylation of peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α), another key regulator of mitochondrial activity, has been reported in skeletal muscle from people with impaired glucose tolerance or T2DM [45] and in subjects with low birth weight [46]. Furthermore, exposure of cultured muscle cells to fatty acids [45] or to a high-fat diet in low-birthweight men [46] increases PGC1α promoter methylation. Whether PDK4 promoter methylation is similarly regulated remains to be determined. However, here we provide evidence that reduced PDK4 promoter methylation is associated with metabolic disease, whereas our previous study established that PGC1α promoter methylation is

increased [45]. This suggests that altered methylation patterns associated with metabolic disease may be promoter specific.

Weight loss is accompanied by a marked reduction in skeletal muscle PDK4 expression following surgery-induced weight reduction [47,48], and the depression in PDK4 expression has been correlated to improvements in insulin sensitivity [47]. Here we note that BMI is associated with PDK4 expression independent of age, sex, or state of glucose tolerance. A diet-mediated weight reduction program, resulting in a stabilized 10% weight loss, was associated with a nonsignificant tendency for reduced skeletal muscle PDK4 expression [49]. The weight loss achieved following lifestyle intervention in the present study was relatively modest (1.5 kg), yet PDK4 mRNA was elevated in NGT subjects after exercise training. This finding perhaps reflects a modulation of fuel selection. Although PDK4 has been highlighted as a point of dysregulation in the context of T2DM and obesity [17,50], the overall impact of a loss of PDK4 is surprisingly slight. Mice lacking PDK4 have a modest reduction in fasting glucose and are slightly more glucose tolerant than wild-type mice after 16 weeks on a high-fat diet [14]. Clearly, PDK4 is only recognized in certain circumstances as a key regulator of glucose vs lipid utilization. For example, we have recently reported that in response to exposure to glucocorticoids, skeletal muscle cultures increase lipid oxidation and reduce glucose metabolism; and a key mediator of this response is induction of PDK4 expression [51].

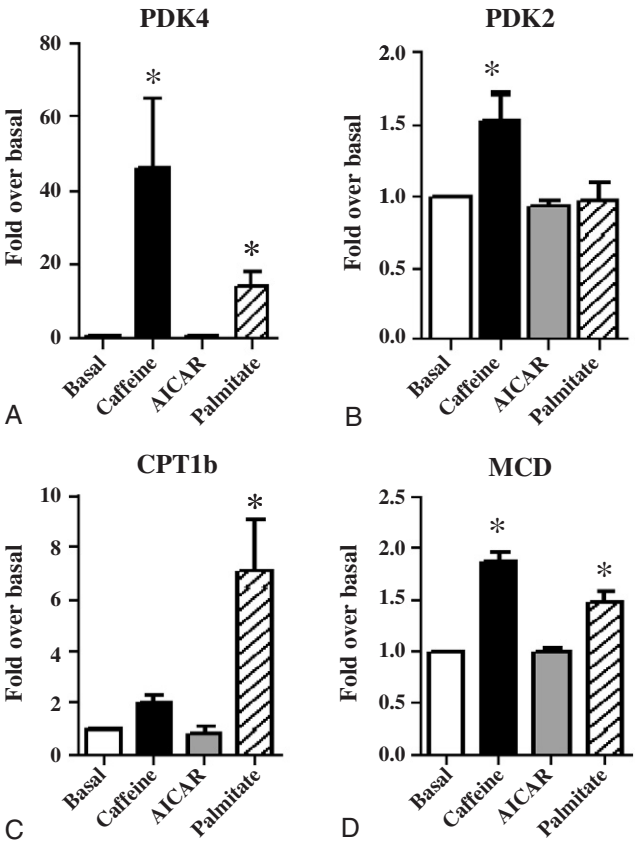

Fig. 5 – Effect of exercise mimetics on gene expression. Effect of caffeine, AICAR, and palmitate treatment on mRNA expression of PDK4, PDK2, CPT1b, and MCD (A, B, C, and D, respectively) in cultured human skeletal myotubes. Differentiated myotubes were incubated in the absence or presence of caffeine (3 mmol/L, 48 hours), AICAR (1 mmol/L, 18 hours), or palmitate (200 μmol/L, 48 hours) before the extraction of total RNA. Results are reported as a relative expression related to B2M mRNA. Values are means ± SEM (*P < .05).

The role of mitochondria in metabolic disease, and whether increasing fatty acid flux into muscle mitochondria reduces or exacerbates insulin sensitivity, is the subject of debate [52-55]. One crucial role in muscle mitochondrial function may be the ability to adapt to or show a preference for different fuel substrates, that is, the flexibility of the muscle to adapt to altered environments [1]. Because the same lifestyle modification program in NGT subjects increased skeletal muscle PDK4 expression, the final PDK4 expression noted in T2DM patients may be appropriate for the level of substrate demand placed on the muscle as a result of the increased exercise performed. To dissect the relative impact of different exercise-associated factors, we exposed cells in culture to agents that increase intracellular Ca^{2+} (caffeine [27]), activate AMPK [28], or change the lipid supply to mimic exercise-dependent increase in circulating fatty acids. Activation of AMPK (in response to AICAR) was insufficient to induce alterations in mRNA expression of these genes. In fact, no single agent resulted in a coordinated increase in expression of the genes under study; however, caffeine exposure led to a profound increase in PDK4. Indeed, caffeine exposure resulted in increased expression of PDK4, PDK2 and MCD, which were also increased in skeletal muscle from the T2DM patients. Whether intracellular Ca^{2+} signaling is enhanced in T2DM is unknown. However, because caffeine exposure in cultured muscle resulted in a profound (40-fold) increase in PDK4 mRNA, the lack of exercise-dependent induction of PDK4 in diabetic muscle would argue against enhanced Ca^{2+} signaling in diabetic skeletal muscle.

In conclusion, we provide evidence that expression of key enzymes regulating mitochondrial function in skeletal muscle is altered in T2DM patients. Increased expression of PDK4 mRNA was coincident with decreased PDK4 promoter methylation, indicative of epigenetic regulation. In addition, a lifestyle intervention involving low-intensity exercise resulted in increased expression of PDK4 mRNA in NGT volunteers but not T2DM patients, possibly implicating resistance to the appropriate adaptive responses to exercise and changes in metabolic demands in skeletal muscle associated with insulin resistance.

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Conflict of interest

The authors declare that they have no conflict of interest as pertains to the data presented in this paper.

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