

Decreased glycolytic and tricarboxylic acid cycle intermediates coincide with peripheral nervous system oxidative stress in a murine model of type 2 diabetes

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Abstract

Diabetic neuropathy (DN) is the most common complication of diabetes and is characterized by distal-to-proximal loss of peripheral nerve axons. The idea of tissue-specific pathological alterations in energy metabolism in diabetic complications-prone tissues is emerging. Altered nerve metabolism in type 1 diabetes models is observed; however, therapeutic strategies based on these models offer limited efficacy to type 2 diabetic patients with DN. Therefore, understanding how peripheral nerves metabolically adapt to the unique type 2 diabetic environment is critical to develop disease-modifying treatments. In the current study, we utilized targeted liquid chromatography–tandem mass spectrometry (LC/MS/MS) to characterize the glycolytic and tricarboxylic acid (TCA) cycle metabolomes in sural nerve, sciatic nerve, and dorsal root ganglia (DRG) from male type 2 diabetic mice (BKS.Cg-m +/+ Lepr^{db}; *db/db*) and controls (*db/+*). We report depletion of glycolytic intermediates in diabetic sural nerve and sciatic nerve (glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate (sural nerve only), 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate, and lactate), with no significant changes in DRG. Citrate and isocitrate TCA cycle intermediates were decreased in sural nerve, sciatic nerve, and DRG from diabetic mice. Utilizing LC/electrospray ionization/MS/MS and HPLC methods, we also observed increased protein and lipid oxidation (nitrotyrosine; hydroxyoctadecadienoic acids) in *db/db* tissue, with a proximal-to-distal increase in oxidative stress, with associated decreased aconitase enzyme activity. We propose a preliminary model, whereby the greater change in metabolomic profile, increase in oxidative stress, and decrease in TCA cycle enzyme activity may cause distal peripheral nerves to rely on truncated TCA cycle metabolism in the type 2 diabetes environment.

Key Words

- ▶ diabetes
- ▶ neuropathy
- ▶ metabolomics
- ▶ sural nerve
- ▶ sciatic nerve
- ▶ dorsal root ganglia

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Introduction

According to statistics published by the Centers for Disease Control (2011), ~8% of the US population has diabetes mellitus, with 1.9 million new cases diagnosed in 2010. Although both type 1 diabetes and type 2 diabetes are characterized by impaired insulin signaling and hyperglycemia, the two disease forms are distinct. Type 2 diabetes, accounting for 95% of all cases (CDCP 2011), is a metabolic disease characterized by high pancreatic insulin production and insulin resistance in muscle, fat, and liver. The remaining 5% of individuals have type 1 diabetes (CDCP 2011), which is characterized by auto-immune destruction of the pancreas and loss of insulin production.

Diabetic neuropathy (DN) is the most common complication of diabetes and over time affects ~60% of all patients with the disease (Vincent & Feldman 2004). DN is characterized by progressive, length-dependent loss of peripheral nerve axons in a stocking and glove (distal-to-proximal) pattern (Said 2007), resulting in pain, decreased sensation, and eventually complete loss of sensation. In the USA, DN is the leading cause of diabetes-related hospital admissions and non-traumatic amputations (Edwards *et al.* 2008, Feldman 2008). Despite the morbidity associated with DN, there is currently no treatment available to patients suffering from DN other than glycemic control, largely due to our incomplete understanding of disease mechanisms. In order to gain insight into DN pathogenesis and develop disease-modifying treatments, we have recently started to investigate the role of aberrant energy metabolism in the peripheral nervous system. We hypothesize that DN arises from tissue-specific metabolic reprogramming, resulting in alterations in fuel utilization that lead to dysfunction of the tissue.

Glucose is the major substrate for peripheral nerves, although they do not rely on insulin for its uptake (Greene & Winegrad 1979). In rodent models of type 1 diabetes, peripheral nerve glucose content is elevated (Thurston *et al.* 1995, Kishi *et al.* 1999, Obrosova *et al.* 2005b) and oxidative stress is increased within the sciatic nerve and the dorsal root ganglia (DRG) containing the sensory neuron cell bodies (Vincent *et al.* 2004). It is also established that all mechanisms known to be contributory to the onset and progression of DN are in some way related to oxidative stress (Vincent *et al.* 2011). Based on the findings primarily in cultured endothelial cells, oxidative stress was thought to be related to hyperglycemia-induced overproduction of superoxide ($O_2^{\cdot-}$) by the mitochondrial electron transport chain (Brownlee 2001). Thus, it was

proposed that increased glycolytic flux leads to increased hyperglycemia-derived electron donors from the tricarboxylic acid (TCA) cycle (FADH₂ and NADH), which generates a high mitochondrial membrane potential, facilitating the reduction of O_2 to $O_2^{\cdot-}$ (Brownlee 2001, Tomlinson & Gardiner 2008). Indeed, a study by Thurston *et al.* (1995) in the mid 1990s reported an increase in nerve glycolytic intermediates in long-term type 1 diabetes in rats. Recent reports, however, suggest downregulation of glycolytic intermediates in complication-prone tissues in type 1 diabetes, including peripheral nerves (Gardiner *et al.* 2007), retina (Ola *et al.* 2006), and lens (Obrosova & Stevens 1999). Recent metabolomic studies in models of type 1 diabetes reported downregulation of key TCA cycle and mitochondrial proteins (Akude *et al.* 2011) and enzyme activities (Chowdhury *et al.* 2010) in DRG, with decreased mitochondrial $O_2^{\cdot-}$ production (Akude *et al.* 2011). Conversely, Schwann cells grown under hyperglycemic conditions demonstrated upregulation of the TCA cycle and mitochondrial proteins (Zhang *et al.* 2010). Critically, these data were produced in models of type 1 diabetes.

While the mechanisms underlying type 1 diabetes and type 2 diabetes are distinct, it is generally held that DN is due to hyperglycemic damage, regardless of the type of diabetes (Callaghan *et al.* 2012). However, DN is more common in patients with type 2 diabetes (Young *et al.* 1993), and DN in type 2 diabetic patients is less amenable to tight glycemic control, indicating differing underlying pathogenic mechanisms (Callaghan *et al.* 2012). Despite the significant progress in our understanding of altered peripheral nerve metabolism in models of type 1 diabetes, fundamental differences between type 1 diabetes and type 2 diabetes confer a critical need to understand how peripheral nerves adapt to the unique type 2 diabetic environment (hyperglycemia, hyperinsulinemia, and hyperlipidemia). We hypothesize that measures of oxidative stress are increased in a proximal-to-distal gradient in peripheral nerves in type 2 diabetes, consistent with DN pathogenesis. This will be reflected in a decrease in the glycolytic and TCA cycle metabolomes and related enzymes moving proximally to distally from the DRG to the sciatic nerve and its terminal branches, including the sural nerve.

In the current study, we utilized liquid chromatography–tandem mass spectrometry (LC/MS/MS) and LC–electrospray ionization (ESI)–MS/MS to explore glycolytic and TCA cycle metabolomic changes within sural nerve, sciatic nerve, and DRG of the BKS.Cg-m +/+Lepr^{db}

(db/db) mouse model of type 2 diabetes. We present steady-state metabolomics data demonstrating decreased glycolytic intermediates in the sural and sciatic nerves and a decrease in TCA cycle intermediates in DRG, sciatic nerve, and sural nerve, complementing the published data in peripheral nerves in type 1 diabetes. We confirm that these changes occur concurrently with decreased aconitase enzyme activity as well as increased protein and lipid oxidation in the sciatic nerve and DRG. We present the first evidence that oxidative stress is more pronounced distally in peripheral nerves from a mouse model of type 2 DN. Finally, we propose a preliminary model whereby these changes may cause the sciatic nerve to rely on truncated TCA cycle metabolism in the type 2 diabetes environment.

Materials and methods

Diabetic mice

Male type 2 diabetic (BKS.Cg-m +/+Lepr^{db}; db/db) and control (db/+) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). A mutation in the leptin receptor of the db/db mouse results in hyperphagia, severe obesity, hyperinsulinemia, and hyperglycemia beginning at ~4 weeks of age (Jackson Laboratories, http://jaxmice.jax.org/jaxmicedb/html/model_66.shtml). At 24 weeks of age (20 weeks of diabetes), db/db mice exhibit insensitivity to mechanical and thermal stimuli, along with slowed nerve conduction velocities and reduced intra-epidermal nerve fiber (IENF) density (Sullivan *et al.* 2007, Cheng *et al.* 2009). Animals were maintained at the University of Michigan in a pathogen-free environment and cared for following the University of Michigan Committee on the Care and Use of Animals guidelines. Mice were given continuous access to food (Purina 5001, Purina Mills LLC, St Louis, MO, USA) and water.

Blood glucose (6-h fasting) and body weight were measured every 4 weeks to document the onset and duration of diabetes. One drop of tail blood was analyzed using a standard glucometer (One Touch Profile, LIFESCAN, Inc., Milpitas, CA, USA). At the end of the experimental period, glycated hemoglobin (GHb) was measured using the Helena Laboratories Test Kit (Glyco-Tek Affinity Column Method).

Tissue harvest

Mice were killed by sodium pentobarbital overdose at 24 weeks of age, followed by transcardial PBS perfusion

to remove residual contaminating blood. This method was chosen over CO₂/cervical dislocation (recommended by Stevens *et al.* (2000)) as CO₂/cervical dislocation is not compatible with transcardial perfusion, a step that was necessary to remove blood artifact and for collaborative use of the other tissues. The left sural and sciatic nerves and lumbar DRG were dissected, briefly rinsed in double-distilled water, frozen by immersion in liquid nitrogen, and stored at -80 °C until metabolomic analysis was conducted (eight db/+ and db/db DRG; nine db/+ and db/db sural nerve and sciatic nerve). The right sciatic nerve and lumbar DRG were dissected and immediately submerged in ice-cold antioxidant buffer, rapidly frozen by immersion in liquid nitrogen, and stored at -80 °C for quantification of end products of oxidative damage (six db/+ and db/db DRG; 12 db/+ and db/db sciatic nerve). The right sciatic nerve from a separate cohort of mice was dissected, cut in thirds, and the proximal and distal thirds prepared as earlier for quantification of oxidative damage (six db/+ and six db/db). The left sciatic nerve and lumbar DRG were used for determination of aconitase enzyme activity as per manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI, USA). Although the method of killing confers a greater time from death to tissue harvest than CO₂/cervical dislocation, the timings were standardized across all mice (sural and sciatic nerves 8 min, DRG 15 min), allowing comparative results within this study.

Targeted metabolomic analysis by LC/MS/MS

Frozen tissue samples were extracted with 150 µl of chilled 8:1:1 methanol:chloroform:water containing ¹³C-labeled glycolysis and TCA cycle standards. Samples were sonicated on ice for 20 s (20% duty cycle, 20% maximum power), held at 4 °C for a further 5 min, and centrifuged at 15 000 g for 5 min at 4 °C. Supernatant was transferred to autosampler vials and directly analyzed by LC/MS.

Chromatographic separation of 18 targeted glycolytic and TCA intermediates was performed based on the methods of Lorenz *et al.* (2011). Briefly, hydrophilic interaction liquid chromatography was performed using a Phenomenex Luna NH₂ column (3 µm, 150 × 1 mm i.d.) on an Agilent 1200 RRLC coupled to an Agilent 6410 triple quadrupole mass spectrometer. Mobile phase A was 5 mM ammonium acetate in water, adjusted to pH 9.9 with ammonium hydroxide. Mobile phase B was acetonitrile. The initial gradient was a 10-min linear ramp from 20 to 100% B, followed by a 3-min hold at 100% B. The mobile phase was returned to 20% B and held for 17 min for re-equilibration before the next run.

The instrument was operated in multiple reaction monitoring (MRM) mode using MS/MS transitions previously optimized by analysis of authentic standards. The following parameters were implemented: flow rate was 0.07 ml/min, column temperature 25 °C, injection volume 20 µl, spray voltage 4.0 kV in negative ion mode, desolvation gas flow rate 10 l/min, desolvation gas temperature 325 °C, and nebulizer pressure 40 psi.

The ratio of each metabolite peak area to that of the closest-matching ¹³C-labeled standard was calculated. Metabolite concentration was determined using calibration curves generated from known concentrations of authentic standards and equal concentrations of ¹³C-labeled compounds as were present in the samples. Concentrations were normalized to total protein content of the tissue sample, determined by the Bradford–Lowry method using the reagents and protocol supplied by Bio-Rad Laboratories. Final values are expressed as picomole/microgram tissue. Intermediates of the pathways not included in the LC/MS/MS output were due to lack of commercial availability of standard, insufficient concentration to reach detection limits, or degradation issues preventing accurate analysis by the current method.

Oxidative stress measures

DRG and sciatic nerve samples were analyzed for nitrated protein (3-nitrotyrosine) and oxidized lipids (hydroxy-octadecadienoic acids (HODEs)) as described previously (Wiggin *et al.* 2008, Vivekanandan-Giri *et al.* 2011). Tissue was homogenized at 4 °C in antioxidant buffer (100 µM diethylenetriaminepentaacetic acid, 50 µM butylated hydroxytoluene, 1% (v/v) ethanol, 10 mM 3-amino-1,2,4-triazole, and 50 mM sodium phosphate buffer (pH 7.4)) to prevent *ex vivo* oxidation, frozen, and thawed. Protein was precipitated with ice-cold trichloroacetic acid (10% v/v), collected by centrifugation, washed with 10% trichloroacetic acid, and delipidated twice with water/methanol/water-washed diethyl ether (1:3:7 v/v). Isotopically labeled internal standards were added and samples were hydrolyzed in 4 M methane sulfonic acid at 110 °C for 24 h under argon as described previously (Pennathur *et al.* 2004). HODEs were quantified by reverse-phase C-18 HPLC analysis of triphenylphosphine-reduced lipid extracts after base hydrolysis. The protein content of tissue pellets was determined by a modified Lowry protein assay using BSA as a standard (Pennathur *et al.* 2005). Amino acids were isolated from the acid hydrolysate using a solid-phase C-18 column (Supelco; Pennathur *et al.* 2004, 2005) and quantified

by MRM using isotope dilution LC/ESI/MS/MS as described previously (Vivekanandan-Giri *et al.* 2011). Results were normalized to tyrosine content, the precursor of 3-nitrotyrosine.

Statistical analysis

Data analysis was performed using GraphPad Prism 5.0 (GraphPad Software). Comparisons between groups were performed using either a one-way ANOVA with Tukey posttest for multiple comparisons or an unpaired *t*-test, as applicable. Assumptions about the Gaussian distribution of data and rules for transformation of nonnormative data were made as described previously (Russell *et al.* 1999). Significance was assigned when *P* < 0.05.

Results

Metabolomic intermediates are decreased in sural nerve, sciatic nerve, and DRG tissue in db/db mice

Diabetes was confirmed in db/db mice by significant elevations in body weight, blood glucose, and GHb compared with their age-matched db/+ controls (*P* < 0.001; Table 1). To begin to characterize the changes in glycolytic and TCA cycle intermediates within peripheral nervous system tissue of the db/db mouse, we performed targeted LC/MS/MS metabolomic analysis on sural nerve, sciatic nerve, and DRG extracts (Table 2). At 24 weeks of age (20 weeks of diabetes), there was a significant decrease in four of the five measured glycolytic intermediates within both diabetic sural nerve and sciatic nerve compared with db/+ controls (glucose-6-phosphate/fructose-6-phosphate (G6P/F6P), 62, 45%; 3-phosphoglycerate/2-phosphoglycerate (3PG/2PG), 75, 63%; phosphoenolpyruvate (PEP), 75, 75%; and lactate (LAC), 46, 56% respectively), with additional diabetic decrease in fructose-1,6-bisphosphate (FBP) content of sural nerve (63%; Table 2). By contrast, diabetes had no effect on measured glycolytic intermediates in DRG

Table 1 Body weight, fasting blood glucose, and GHb for db/+ and db/db mice at 24 weeks of age. Data are mean ± S.E.M.

Genotype	Weight (g)	Blood glucose (mg/dl)	GHb (%)
24 weeks db/+ (9)	32.0 ± 1.0	203 ± 9	6.6 ± 0.2
24 weeks db/db (9)	52.6 ± 4.8*	672 ± 67*	13.7 ± 0.4*

**P* < 0.001 vs db/+. Nine db/+ and db/db.

Table 2 Quantified LC/MS/MS metabolomic data from db/+ and db/db mouse sural nerve, sciatic nerve, and DRG. Data are mean picomole/microgram protein for each group \pm s.e.m.

Metabolite	24 weeks db/+ sural nerve	24 weeks db/db sural nerve	24 weeks db/+ sciatic nerve	24 weeks db/db sciatic nerve	24 weeks db/+ DRG	24 weeks db/db DRG
G6P/F6P	16.3 \pm 4.9	6.2 \pm 3.1*	10.9 \pm 1.9	6.0 \pm 1.1*	5.5 \pm 1.0	6.8 \pm 1.2
FBP	3.8 \pm 0.6	1.4 \pm 0.3 [†]	9.7 \pm 1.6	6.2 \pm 1.3	7.1 \pm 0.9	6.3 \pm 0.9
3PG/2PG	0.4 \pm 0.1	0.1 \pm 0.03*	2.2 \pm 0.4	0.8 \pm 0.2 [†]	0.1 \pm 0.03	0.1 \pm 0.02
PEP	0.4 \pm 0.1	0.1 \pm 0.02 [†]	0.4 \pm 0.1	0.1 \pm 0.02 [†]	0.1 \pm 0.03	0.1 \pm 0.03
LAC	136.8 \pm 19.0	73.3 \pm 19.7*	220.7 \pm 45.8	96.1 \pm 14.4*	149.8 \pm 14.7	162.4 \pm 14.1
CIT	8.3 \pm 1.0	4.3 \pm 0.6 [†]	49.7 \pm 6.9	22.2 \pm 4.4 [†]	8.5 \pm 0.8	5.6 \pm 0.4 [†]
ICIT	0.2 \pm 0.03	0.1 \pm 0.03*	0.6 \pm 0.1	0.3 \pm 0.1*	0.2 \pm 0.02	0.1 \pm 0.01*
SUC	1.4 \pm 0.05	1.1 \pm 0.2	5.9 \pm 0.9	4.7 \pm 0.8	0.6 \pm 0.04	0.9 \pm 0.2
FUM	4.4 \pm 0.4	3.6 \pm 0.8	6.0 \pm 1.1	3.6 \pm 0.6	4.9 \pm 0.6	3.6 \pm 0.1
MAL	8.0 \pm 0.9	6.2 \pm 1.0	16.9 \pm 2.5	11.5 \pm 2.0	10.9 \pm 0.9	10.6 \pm 0.7

* $P < 0.05$; [†] $P < 0.01$ vs db/+. Eight db/+ and db/db DRG; nine db/+ and db/db sciatic nerve and sural nerve; all data from male mice. G6P/F6P, glucose-6-phosphate/fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; 3PG/2PG, 3-phosphoglycerate/2-phosphoglycerate; PEP, phosphoenolpyruvate; LAC, lactate; CIT, citrate; ICIT, isocitrate; SUC, succinate; FUM, fumarate; MAL, malate.

(Table 2). Among the TCA cycle metabolites, citrate (CIT) and isocitrate (ICIT) were significantly lower in all three tissues from db/db mice compared with db/+ controls, with a greater decrease seen in sural nerve and sciatic nerve (48, 50% sural nerve; 55, 50% sciatic nerve; and 34, 50% DRG). Sural nerve, sciatic nerve, and DRG content of the remaining measured TCA cycle intermediates were not significantly altered with diabetes (Table 2). These observations indicate that type 2 diabetes affects the TCA cycle metabolome of sural nerve, sciatic nerve, and DRG, as well as the glycolytic metabolome of the sural nerve and sciatic nerve, and suggest that the effect is greater in the peripheral nerve than the DRG.

Protein oxidation and lipid peroxidation markers are elevated in sciatic nerve and DRG tissue in db/db mice

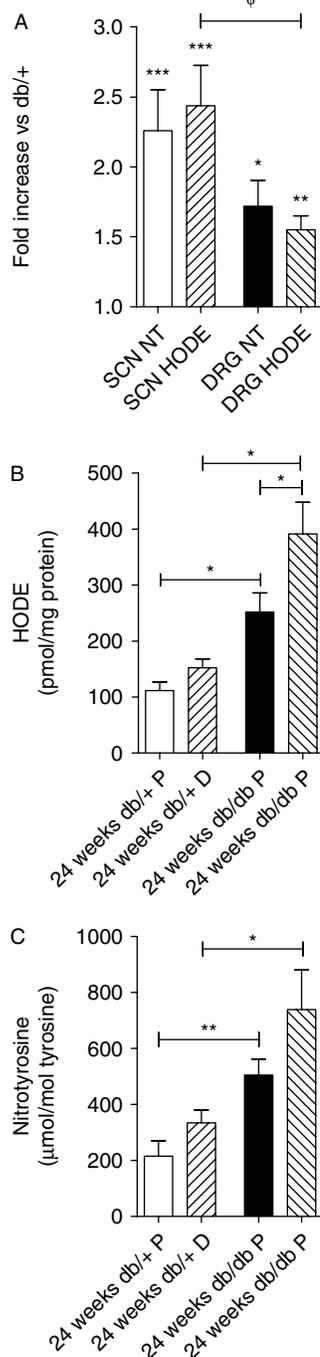
Oxidative stress is a major mechanism of hyperglycemia-induced DN in humans and rodents, particularly through the oxidation of proteins and lipids (Vincent & Feldman 2004, Vincent *et al.* 2004, 2009b). Hydrogen peroxide (H₂O₂) and O₂^{•-} react with nitrite, derived from NO, to produce peroxynitrite in peripheral nerves in experimental diabetes (Obrosova *et al.* 2005a). 3-Nitrotyrosine accumulates when peroxynitrite oxidizes tyrosine (Corbett *et al.* 1992, van der Veen & Roberts 1999, Du *et al.* 2002). To confirm whether protein oxidation was elevated in the db/db peripheral nervous system *in vivo*, we used isotope dilution LC/ESI/MS/MS to quantify levels of protein-bound 3-nitrotyrosine in sciatic nerve and DRG tissue from the db/+ and db/db mice (Fig. 1A). Nitrotyrosine in tissues of the peripheral nervous system was significantly increased by diabetes (sciatic nerve nitrotyrosine 2.3-fold

increase, $P < 0.001$; DRG nitrotyrosine 1.7-fold increase, $P < 0.05$). To determine whether lipid peroxidation was also elevated, we used HPLC to quantify HODEs in lipid extracts from the sciatic nerve and DRG of db/+ and db/db mice (Fig. 1A). The sciatic nerve and DRG samples from the db/db mice contained significantly more HODEs than those from the db/+ mice (sciatic nerve HODEs 2.4-fold increase, $P < 0.001$; DRG HODEs 1.6-fold increase, $P < 0.01$). This increase in oxidized lipids was greater in sciatic nerve than in DRG ($P < 0.05$).

The data presented in Fig. 1A are those from whole sciatic nerve. Owing to the greater observed effect of diabetes on the metabolome of sural nerve and sciatic nerve than DRG (Table 2), and the peripheral presentation of signs of DN in rodents (Sullivan *et al.* 2007) and in humans (Edwards *et al.* 2008), we explored whether oxidative stress is greater distally in the sciatic nerve by repeating the analyses for 3-nitrotyrosine and HODEs on proximal and distal segments of the sciatic nerve from db/+ and db/db mice (Fig. 1B and C). There were no differences in oxidative stress measures between proximal and distal segments of db/+ control nerves. Oxidized lipids (HODEs) were 2.3- and 2.6-fold greater in proximal and distal db/db sciatic nerve respectively compared with those of db/+ mice ($P < 0.05$). Furthermore, diabetic sciatic nerve HODEs were 1.6-fold greater distally than proximally ($P < 0.05$; proximal db/+ 112 \pm 15, proximal db/db 252 \pm 35, distal db/+ 152 \pm 16, distal db/db 391 \pm 56 pmol/mg protein; Fig. 1B). Nitrated protein was 2.3- and 2.2-fold greater in proximal and distal db/db sciatic nerve respectively compared with those of db/+ mice (proximal db/+ 216 \pm 54, proximal db/db 505 \pm 57 μ mol/mol tyrosine, $P < 0.01$; distal db/+ 335 \pm 45,

distal db/db 739 ± 142 $\mu\text{mol/mol}$ tyrosine, $P < 0.05$). Nitrated protein content was not significantly altered proximally to distally in diabetic sciatic nerve (Fig. 1C).

Together, these observations indicate that type 2 diabetes increases both protein and lipid oxidation in db/db mouse sciatic nerve and DRG. The diabetic increase in lipid peroxidation is greater in sciatic nerve than in DRG, and the extent of this oxidation is greater distally in sciatic nerve.



Aconitase enzyme activity is decreased in sciatic nerve and DRG tissue in db/db mice

Aconitase catalyzes the conversion of CIT to ICIT in the TCA cycle and is the most sensitive TCA cycle enzyme to reactive oxygen species (ROS) inhibition (Tretter & Adam-Vizi 2000). Work by Tretter & Adam-Vizi (2000) established that when aconitase is inhibited, a segment of the TCA cycle between α -ketoglutarate (α -KG) and oxaloacetate still functions via anaplerosis of glutamate in to the TCA cycle. To begin to understand the lack of change in TCA cycle intermediates other than CIT and ICIT, we measured aconitase enzyme activity in sciatic nerve and DRG from the db/+ and db/db mice (Fig. 2). Aconitase activity was 1.6-fold greater in DRG than sciatic nerve in control (db/+) mice (DRG 1.50 ± 0.06 ; sciatic nerve 0.96 ± 0.10 μM NADPH/mg protein per min, $P < 0.01$), with this tissue difference maintained in the db/db mice (DRG 0.91 ± 0.12 ; sciatic nerve 0.46 ± 0.06 μM NADPH/mg protein per min $P < 0.05$). Type 2 diabetes was associated with a decrease in aconitase activity, with a greater effect in sciatic nerve than DRG (DRG 0.39-fold decrease, $P < 0.01$; sciatic nerve 0.51-fold decrease, $P < 0.01$).

Discussion

Recent metabolomic studies in models of type 1 diabetes reported downregulation of key TCA cycle and mitochondrial proteins (Akude *et al.* 2011) and enzyme activities (Chowdhury *et al.* 2010) in DRG and upregulation of TCA cycle and mitochondrial proteins in Schwann

Figure 1

Increased oxidative stress in diabetic sciatic nerve and DRG. (A) Fold changes in oxidative stress measures from whole sciatic nerve and DRG were calculated from the ratio of db/db to db/+. The nitrated protein content and HODEs were increased in whole sciatic nerve and DRG from diabetic mice compared with those from db/+ controls ($*P < 0.05$; $**P < 0.01$; and $***P < 0.001$ vs db/+). The diabetic fold increase in oxidized lipids was greater in sciatic nerve than in DRG ($^{\phi}P < 0.05$). Data expressed as mean fold-change \pm S.E.M.; six db/+ and db/db DRG; 12 db/+ and db/db sciatic nerve. There were no changes in oxidative stress measures between proximal and distal segments of db/+ control sciatic nerves (B and C). (B) Oxidized lipids (HODEs) were elevated in proximal and distal db/db sciatic nerve respectively compared with those of db/+ mice. Diabetic sciatic nerve HODEs were greater distally than proximally. (C) The nitrotyrosine-to-tyrosine ratio was elevated in proximal and distal db/db sciatic nerve respectively compared with those of db/+ mice. Data expressed as mean \pm S.E.M.; six db/+ and db/db sciatic nerve. $*P < 0.05$ and $**P < 0.01$. SCN, sciatic nerve; DRG, dorsal root ganglia; P, proximal; D, distal; NT, 3-nitrotyrosine. All data from male mice.

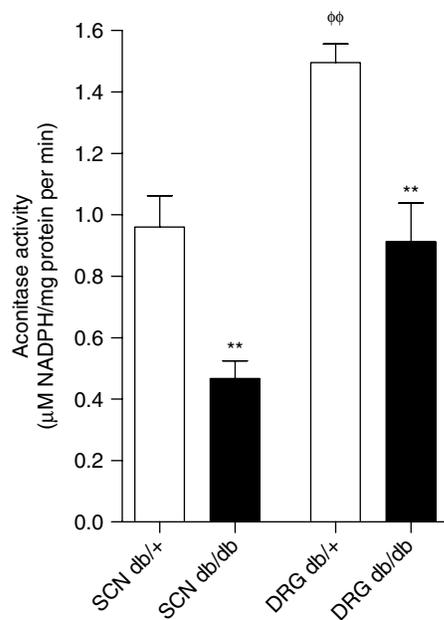


Figure 2

Decrease in aconitase enzyme activity in diabetic sciatic nerve and DRG. Aconitase activity was 1.6-fold greater in DRG than in sciatic nerve in control (db/+) mice ($\phi\phi P < 0.01$), with this tissue difference maintained in the db/db mice. Type 2 diabetes was associated with a decrease in aconitase activity in both tissues ($**P < 0.01$ compared with db/+). Data expressed as mean \pm s.e.m.; six db/+ and db/db. SCN, sciatic nerve; DRG, dorsal root ganglia. All data from male mice.

cells (Zhang *et al.* 2010). Differences in metabolic profiles, prevalence of DN, and efficacy of glycemic control on DN in type 1 diabetes and type 2 diabetes suggest that therapeutic strategies based on models of type 1 diabetes may offer limited efficacy to the type 2 diabetic patient with DN (Callaghan *et al.* 2012). There is therefore a critical need to understand peripheral nerve-specific metabolomic changes in type 2 diabetes, and their role in nerve injury, to develop disease-modifying treatments. To this end, we evaluated changes in the glycolytic and TCA cycle metabolomes of key components of the peripheral nervous system in the db/db mouse model of type 2 diabetes. We performed targeted LC/MS/MS metabolomic analysis on sural nerve, sciatic nerve, and DRG from db/db mice. To confirm that ROS is elevated in the db/db peripheral nervous system, we used both LC/ESI/MS/MS and HPLC to measure nitrated protein and peroxidated lipid content respectively. We observed decreased glycolytic and TCA cycle intermediates in the db/db tissue, with concurrent increases in protein and lipid oxidation. Additionally, our data suggest a greater metabolic impact of diabetes distally, a proximal–distal increase in the severity of oxidative

stress, and a proximal–distal decrease in aconitase enzyme activity in the db/db nerve.

Oxidative stress is strongly implicated in DN pathogenesis, particularly through the oxidation of proteins and lipids (Vincent & Feldman 2004, Vincent *et al.* 2004, 2009b). To confirm whether protein and lipid oxidation are elevated in the db/db peripheral nervous system *in vivo*, we quantified levels of protein-bound 3-nitrotyrosine in tissue extracts, and HODEs in lipid extracts respectively from the sciatic nerve and DRG of db/+ and db/db mice. Nitrotyrosine and HODEs were significantly elevated in the db/db peripheral nerve and DRG (Fig. 1), consistent with previous work showing that diabetes increases neuronal oxidized protein (Vincent *et al.* 2004, 2005, Obrosova *et al.* 2005a, Pennathur *et al.* 2005, Wiggin *et al.* 2008) and lipids (Vincent *et al.* 2004, 2005, Wiggin *et al.* 2008). Elevated oxidized proteins and oxidized lipids in distal diabetic sciatic nerve compared with distal control sciatic nerve indicate greater oxidative stress at peripheral nerve extremities in diabetes (Fig. 1B and C), paralleling the dying-back axonopathy observed in animal models (Sullivan *et al.* 2007) and human patients (Edwards *et al.* 2008). The diabetic elevation in lipid peroxidation (HODEs) was greater in the sciatic nerve than in the DRG and increased moving distally along the db/db sciatic nerve (Fig. 1). There are at least two possible explanations for these findings. First, the increased HODEs as a measure of oxidative stress could simply represent the greater lipid content in sciatic nerve compared with DRG (data not shown) or, alternatively, the increase is a true reflection of impaired axonal function with secondary impairment of axon/Schwann cell interactions leading to Schwann cell injury. To our knowledge, these are novel data that oxidative stress increases proximally-to-distally in the neuropathic diabetic peripheral nerve. DN predominantly occurs in a distal symmetrical pattern, with skin denervation (reduced IENF density; Said 2007, Sullivan *et al.* 2007) increasing with diabetes duration (Shun *et al.* 2004), and proximal-to-distal graded loss of myelinated fiber density observed in diabetic patients (Sullivan *et al.* 2003). The current data suggest that there may be a link between this proximal-to-distal graded loss of myelinated fiber density and a graded increase in oxidative stress. In addition to its role in nitration of proteins (Corbett *et al.* 1992, van der Veen & Roberts 1999, Du *et al.* 2002, Obrosova *et al.* 2005a), peroxyxynitrite formation is required for myelin–lipid peroxidation in *in vitro* myelin suspensions (van der Veen & Roberts 1999); the greater fold increases observed in sciatic nerve than in DRG lipid peroxidation measures (Fig. 1A) may reflect oxidation of myelin in the peripheral nerve.

Aconitase catalyzes the conversion of CIT to ICIT in the TCA cycle and is the most sensitive TCA cycle enzyme to ROS inhibition (Tretter & Adam-Vizi 2000). To begin to understand the specificity of the change in CIT and ICIT TCA cycle intermediates, we measured aconitase enzyme activity in sciatic nerve and DRG from the db/+ and db/db mice. The diabetes-related depletion of aconitase activity (Fig. 2) is consistent with our previous report in cultured DRG neurons (Vincent *et al.* 2005). The greater diabetic impairment observed in sciatic nerve than DRG is likely related to greater oxidative stress-mediated aconitase inhibition (Gardner & Fridovich 1992, Gardner *et al.* 1995, Tretter & Adam-Vizi 2000) in the sciatic nerve. This is contradictory to our previous *in vitro* observation that DRG neurons are more susceptible to oxidative stress than non-myelinating Schwann cells (Vincent *et al.* 2009c). It is important to note that our 2009 study investigated the effects of mild, acute oxidative stress induced by high glucose and H₂O₂ in cells derived from immature rat embryos and pups. We did not assess the effects of myelin-lipid peroxidation, or high free fatty acids and elevated ox-LDL, both of which are associated with type 2 diabetes and linked to oxidative stress-mediated injury in these cells (Vincent *et al.* 2009a, Suzuki *et al.* 2011).

LAC production via the reversible LAC dehydrogenase-catalyzed reaction from pyruvate, the last metabolic intermediate of glycolysis, is intimately linked to the NAD⁺/NADH cellular redox status. Further flux experiments are required, but the pattern of glycolytic and TCA cycle intermediates in the sural nerve and sciatic nerve in diabetic mice compared with controls suggests that the observed significant depletion of sural nerve and sciatic nerve LAC (Table 2) is related to inhibition of glycolysis. These data are in contrast with findings of elevated LAC in sciatic nerve from type 1 diabetic streptozotocin (STZ)-treated rats (Obrosova *et al.* 1999, Stevens *et al.* 2000). Our finding that LAC levels were unchanged in db/db DRG is in agreement with reports in STZ-diabetic lens (Obrosova *et al.* 1998, Obrosova & Stevens 1999). Work by Stevens *et al.* (2000) highlighted concerns over anesthetic-induced elevations in LAC levels in control peripheral nerves when compared with the CO₂/cervical dislocation method of killing. Absolute values stated in Table 2 may therefore be an underestimate of true tissue LAC content, but relative comparisons between db/+ and db/db mice may be made. Interestingly, the observed depletions in db/db sural and sciatic nerves are in contrast with elevations reported in STZ rats killed via the CO₂/cervical dislocation method (Obrosova *et al.* 1999, Stevens *et al.* 2000). The significant decrease in LAC content we observe argues against the

potential for an anesthesia-driven LAC elevation to introduce artifact into the data. In addition, it should be noted that the above studies by Obrosova and Stevens were performed in a model of type 1 diabetes in rats and over shorter diabetes duration (3–6 weeks).

Our finding that G6P and F6P were decreased in sciatic nerve, with additional decrease in FBP in sural nerve, in db/db mice at 24 weeks of age (Table 2), is in contrast to findings in STZ diabetic lens (Obrosova *et al.* 1998, Obrosova & Stevens 1999). As G6P and F6P are derived from the same pool in the current MS approach, we cannot determine the contribution from each intermediate to the observed measurements. Whether there is a true decrease in both F6P and upstream G6P or a decrease in F6P due to redirection of G6P into the polyol (or other) pathways remains unclear.

Hexokinase saturation and maximal glycolytic flux is one proposed mechanism underlying the accumulation of nerve glucose and its direction into the polyol pathway in diabetes (Tomlinson & Gardiner 2008). Gardiner *et al.* (2007) observed diminished hexokinase activity in DRG from STZ diabetic rats. Studies on excised rat retinas (Ola *et al.* 2006) concluded that glycolysis and glucose metabolism downstream of hexokinase are not elevated by hyperglycemia or type 1 diabetes but that intermediates of alternative glucose metabolism, such as those of the polyol pathway, are increased. This is in agreement with conclusions of studies by the Greene group in STZ diabetic rat sciatic nerve (Obrosova *et al.* 1999, Stevens *et al.* 2000). Thus, it is possible that glucose is being preferentially directed into the polyol pathway in the db/db sural nerve and sciatic nerve, away from energy-producing glycolysis.

Decreased steady-state concentrations of metabolites in the lower segment of glycolysis (3PG/2PG; PEP) in db/db sural and sciatic nerves compared with controls agrees with previously published reports in the STZ diabetic lens (Obrosova *et al.* 1998, Obrosova & Stevens 1999; levels of these intermediates were similar between control and diabetic DRG in the current study), which concluded that sites of regulation are at glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reaction and downstream (e.g. enolase and pyruvate kinase). This conclusion is supported in the current model by our recent report of downregulation of the gene encoding enolase (Eno2) in db/db sciatic nerve at 24 weeks of age (Pande *et al.* 2011), the fact that GAPDH is vulnerable to oxidative damage (Du *et al.* 2000), and greater markers of lipid and protein oxidative damage in distal sciatic nerve compared with DRG (Fig. 1). In the more distal sural nerve, data suggest additional upstream sites of glycolysis inhibition (e.g. hexokinase,

phosphoglucose isomerase, and phosphofructokinase), with a greater diabetic decrease in metabolic intermediates in sural nerve compared with sciatic nerve (Table 2). In addition, slow axonal transport, responsible for the transport of glycolytic enzymes, is impaired in db/db diabetes at 20–24 weeks of age (Vitadello *et al.* 1983, 1985). Decreased peripheral enzyme availability coupled with increased distal oxidative stress may be related to the greater distal metabolomic changes observed in the current study.

Our observed decreases in CIT and ICIT (Table 2) are consistent with work by Akude *et al.* reporting down-regulation of key TCA cycle proteins, including CIT synthase, in DRG from 22-week STZ diabetic rats (Akude *et al.* 2011). One question arising from the TCA cycle metabolomic data is the lack of change in additional intermediates other than CIT and ICIT. Aconitase, catalyzing the CIT to ICIT reaction in the TCA cycle (Fig. 3), is inhibited by ROS, including $O_2^{\cdot -}$ (Gardner & Fridovich 1992, Gardner *et al.* 1995) and H_2O_2 (Tretter & Adam-Vizi 2005). When aconitase is fully inhibited by H_2O_2 in nerve terminals, α -KG dehydrogenase (α -KGDH;

catalyzing the α -KG to succinyl-CoA reaction) remains functional and a segment of the TCA cycle (α -KG to oxaloacetate) is maintained by glutamate, which is converted to α -KG via transamination (see Tretter & Adam-Vizi (2005)) for comprehensive explanation). ROS-mediated inhibition of aconitase (Fig. 2) may activate this truncated TCA in diabetic sural nerve, sciatic nerve, and DRG (Fig. 3), explaining the maintenance of levels of succinate, fumarate, and malate in the face of decreased CIT and ICIT observed in this study. This truncated segment of the TCA cycle may function in the absence of glucose (Yudkoff *et al.* 1994, Erecinska *et al.* 1996). In this respect, a state of oxidative stress when aconitase is completely inhibited but α -KGDH remains active is similar to a glucose-free state. Additionally, α -KGDH is itself a source of ROS production (see Gibson *et al.* (2010) for review of α -KGDH in neurodegeneration), the level of which increases when α -KG is utilized as a fuel source over glucose in isolated brain synaptosomes (Tretter & Adam-Vizi 2004). The greater decrease in CIT and ICIT observed in db/db sural and sciatic nerves (compared with DRG) may be due to reduced anaplerosis of intermediates from upstream glycolysis to the TCA cycle. This may be interpreted as a glucose-deficient state, activating the α -KG-utilizing truncated TCA cycle pathway, further contributing to ROS production, and potentially contributing to the greater degree of oxidative stress observed in diabetic sciatic nerve than in DRG (Fig. 1A). Contribution to these pathways from an altered diabetic lipid metabolome should be considered but has not been explored in the current study.

Collectively, our data and those of the Fernyhough, LaNoue, and Obrosova groups support the emerging idea of tissue-specific alterations in energy metabolism in diabetic complications-prone tissues such as the peripheral nerve (Gardiner *et al.* 2007, Chowdhury *et al.* 2010, Akude *et al.* 2011), retina (Ola *et al.* 2006), and lens (Obrosova *et al.* 1998, Obrosova & Stevens 1999). For the first time, we expand this knowledge to neuropathy in a type 2 model of diabetes, additionally demonstrating different metabolomic profiles within specific tissues of the peripheral nervous system. The greater metabolomic changes in sural nerve and sciatic nerve than DRG may reflect increased oxidative stress and subsequent inhibition of key metabolic enzymes, including aconitase. However, our static measurements of metabolites are not direct indicators of flux through a pathway: these data are the beginnings of a full characterization of bioenergetic alterations in diabetic peripheral nerve and are necessary to develop effective disease-modifying treatments for DN.

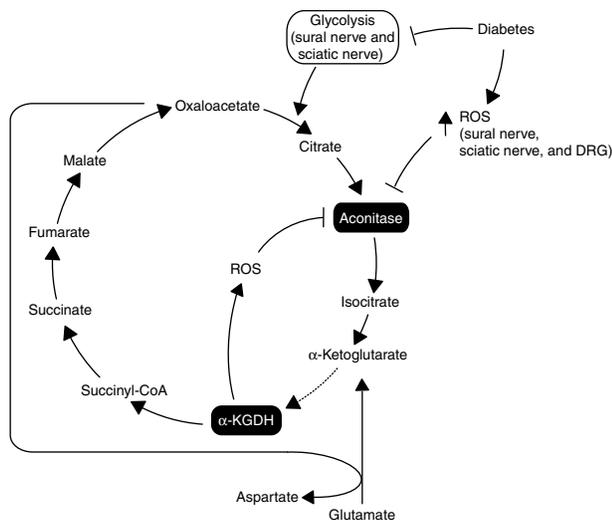


Figure 3

Proposed relationship between diabetes, ROS, and the TCA cycle. ROS-mediated inhibition of aconitase may truncate the TCA cycle in diabetic sural nerve, sciatic nerve, and DRG (bold arrows). This would result in the observed maintenance of succinate, fumarate, and malate levels in the face of decreased citrate and isocitrate. Reduced glycolytic intermediates feeding into the TCA cycle in sural and sciatic nerves may contribute to the observed decreases in citrate and isocitrate and be interpreted as a glucose-deficient state. Activation of the α -KG-utilizing truncated TCA cycle pathway in the sural and sciatic nerves would further contribute to ROS production and to the greater oxidative stress observed in diabetic sciatic nerve than DRG. α -KGDH, α -ketoglutarate dehydrogenase; DRG, dorsal root ganglia; TCA, tricarboxylic acid; ROS, reactive oxygen species.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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