Diminished Superoxide Generation is Associated with Respiratory Chain Dysfunction and Changes in the Mitochondrial Proteome of Sensory Neurons from Diabetic Rats

Running Title - Diabetes alters the DRG mitochondrial proteome and function.

Eli Akude, 1,2,* Elena Zherebitskaya, 1,* Subir K. Roy Chowdhury, 1 Darrell R. Smith, 1,2 Rick T. Dobrowsky, 3,** and Paul Fernyhough 1,2,**

1St Boniface Hospital Research Centre, Winnipeg, MB, Canada , 2Department of Pharmacology & Therapeutics, University of Manitoba, Winnipeg, MB, Canada and 3Department of Pharmacology and Toxicology, University of Kansas, Lawrence, KS, USA.

* E. Akude and E. Zherebitskaya contributed equally
**R. T. Dobrowsky and P. Fernyhough contributed equally

Correspondence should be addressed to:
Paul Fernyhough,
E-mail: paulfernyhough@yahoo.com;

Submitted 10 June 2010 and accepted 20 September 2010.

Additional information for this article can be found in an online appendix at
http://diabetes.diabetesjournals.org

This is an uncopyedited electronic version of an article accepted for publication in Diabetes. The American Diabetes Association, publisher of Diabetes, is not responsible for any errors or omissions in this version of the manuscript or any version derived from it by third parties. The definitive publisher-authenticated version will be available in a future issue of Diabetes in print and online at http://diabetes.diabetesjournals.org.
Diabetes alters the DRG mitochondrial proteome and function.

Objective. Impairments in mitochondrial function have been proposed to play a role in the etiology of diabetic sensory neuropathy. We tested the hypothesis that mitochondrial dysfunction in axons of sensory neurons in type 1 diabetes is due to abnormal activity of the respiratory chain and an altered mitochondrial proteome.

Research design and methods. Proteomic analysis using stable isotope labeling with amino acids in cell culture (SILAC) determined expression of proteins in mitochondria from dorsal root ganglia (DRG) of control, 22 week streptozotocin (STZ)-diabetic rats and diabetic rats treated with insulin. Rates of oxygen consumption and complex activities in mitochondria from DRG were measured. Fluorescence imaging of axons of cultured sensory neurons determined effect of diabetes on mitochondrial polarization status, oxidative stress and mitochondrial matrix-specific ROS.

Results. Proteins associated with mitochondrial dysfunction, oxidative phosphorylation, ubiquinone biosynthesis and the citric acid cycle were down-regulated in diabetic samples. For example, cytochrome c oxidase subunit 4 (COX IV; a complex IV protein) and NADH dehydrogenase Fe-S protein 3 (NDUFS3; a complex 1 protein) were reduced by 29% and 36% (P<0.05), respectively, in diabetes and confirmed previous Western blotting studies. Respiration and mitochondrial complex activity was significantly decreased by 15-32% compared to control. The axons of diabetic neurons exhibited oxidative stress and depolarized mitochondria, an aberrant adaption to oligomycin-induced mitochondrial membrane hyperpolarization but reduced levels of intra-mitochondrial superoxide compared with control.

Conclusions. Abnormal mitochondrial function correlated with a down-regulation of mitochondrial proteins, with components of the respiratory chain targeted in lumbar DRG in diabetes. The reduced activity of the respiratory chain was associated with diminished superoxide generation within the mitochondrial matrix and did not contribute to oxidative stress in axons of diabetic neurons. Alternative pathways involving polyol pathway activity appear to contribute to raised ROS in axons of diabetic neurons under high glucose concentration.

Enhanced oxidative stress is thought to be a central pathological feature in the etiology of diabetic peripheral neuropathy (DPN) (1). To develop more targeted therapeutics toward ameliorating oxidative stress and the development of DPN, considerable effort has focused on identifying the cellular source of ROS over the past decade. Brownlee and colleagues suggested that mitochondrial superoxide generation may be a critical feature in the onset of diabetic complications (2). In cultured endothelial cells, hyperglycemia induced excessive electron flux through the respiratory chain that promoted mitochondrial hyperpolarization and elevated ROS production (2-3). These investigators proposed that hyperglycemia increases mitochondrial NADH levels and that increased electron availability and/or saturation caused partial reduction of oxygen to superoxide in the proximal part of the respiratory
Diabetes alters the DRG mitochondrial proteome and function.

chain (2). In support of this mechanism, epineurial arterioles serving the sciatic nerve of STZ-diabetic rats show increased levels of mitochondrial superoxide that is dependent on complex I activity (4). On the other hand, studies in diabetic retina suggest that metabolism of high glucose concentrations does not operate in a fashion that supports superoxide formation by the respiratory chain (5). Similarly, in sensory neurons from STZ-diabetic rats, the mitochondrial membrane potential is depolarized and not hyperpolarized as observed in endothelial cells exposed to hyperglycemia (6-7). Further, lumbar dorsal root ganglia (DRG) from diabetic rats exhibit reduced respiratory chain activity that correlated with the down-regulation of select proteins within the electron transport chain complexes (8). These findings are aligned with studies in diabetic heart where mitochondrial respiration and enzymatic activities are reduced (9-10). In addition, activities of citrate synthase and mitochondrial respiratory chain are decreased in skeletal muscle of patients with type 2 diabetes (11-13).

Therefore, production of mitochondrial superoxide may exhibit fundamental differences in cells that are targets of diabetic complications. Indeed, the tissue specific nature of mitochondrial remodeling in diabetes is directly underscored by results from an unbiased proteomic study that identified a differential affect of diabetes on mitochondrial protein expression and oxidative capacity. For example, proteins associated with oxidative phosphorylation were more depressed and respiratory activity decreased in heart compared to liver mitochondria from diabetic Akita mice (14). Since DRG sensory neurons are highly susceptible to glucotoxicity (15), we examined the effect of diabetes on mitochondrial proteome, respiratory capacity and superoxide production. Our previous work on sensory neurons in diabetes had demonstrated mitochondrial depolarization within the perikarya and lowered respiratory chain capacity (6; 8), however, our objective in the current work was to relate effects of diabetes on mitochondrial proteome expression to mitochondrial physiology and function within the axon where oxidative stress and degeneration are most clearly defined (16-18). To this end, we exploited the use of stable isotope labeling of cells in culture (SILAC) (19) to provide a set of culture-derived isotope tags (20) to serve as internal standards for a quantitative proteomic analysis. We demonstrate that in diabetes impaired respiratory chain function correlates with decreased protein expression and in mitochondria of axons these deficits are associated with membrane depolarization and reduced respiratory chain-derived ROS generation. These data support the conclusion that glucose-dependent superoxide production within the mitochondrial matrix is not a major contributor to oxidative stress in axons of DRG in long term diabetes.

**RESEARCH DESIGN AND METHODS**

**Induction, treatment and confirmation of type 1 diabetes.** Male Sprague Dawley rats were made diabetic with a single intraperitoneal injection of 75 mg/kg STZ (Sigma, St Louis, MO). Insulin implants (2 Linplant implants; Linshin Canada Inc., Toronto, ON, Canada) were placed subcutaneously into STZ-diabetic rats after 18 weeks of diabetes and remained in place for 4 weeks. Animals were killed and tissue collected after 22 weeks of diabetes. Animal procedures followed guidelines of University of
Diabetes alters the DRG mitochondrial proteome and function.

**Manitoba Animal Care Committee using Canadian Council of Animal Care rules.**

**Preparation of isolated mitochondria from DRG and isotopically labeled S16 cells.** Mitochondrial preparations from DRG were isolated as described (8; 21). Immortalized S16 Schwann cells were cultured in low glucose DMEM containing 125 mg/l $^{13}$C$_6$-lysine (K6) and 84 mg/l $^{13}$C$_6$,$^{15}$N$_4$-arginine (R10) (Cambridge Isotopes, Andover, MA), 10% dialyzed fetal calf serum (Atlas Biologicals, Fort Collins, CO) and antibiotics (22). Crude mitochondria from labeled cells were obtained by differential centrifugation and purified through a discontinuous Nycodenz gradient (23). For quantitative analysis of the DRG mitochondrial proteome, the K6R10 labeled mitochondria were used as a source of culture-derived isotope tags to serve as internal standards (20). After assessing the protein concentration of the preparations, the unlabeled mitochondrial protein (K0R0) obtained from each of the control (n=4), diabetic (n=3) and diabetic + insulin (n=4) treatments were mixed in a 2:1 ratio with K6R10 labeled mitochondria. Total protein (60-70 µg) was subjected to SDS-PAGE, the gel was stained with colloidal Coomassie blue and lanes were cut into 5 x 1 cm pieces.

**RP-HPLC/LTQ-FT MS/MS and protein identification and quantification criteria.** A detailed description of these parameters is provided in Supplemental Table 1 in Online Appendix 2 at http://diabetes.diabetesjournals.org. See Fig.1 for overview of SILAC approach.

**Adult rat DRG sensory neuron culture.** Lumbar DRG sensory neurons from adult male Sprague-Dawley rats were isolated and dissociated as described (6; 16). Rats were either age matched control or 22 week STZ-diabetic. Cells were plated onto poly-d-L-ornithine hydrobromide and laminin-coated 25 mm glass cover slips (Electron Microscopy Sciences, Hatfield, PA, German glass #1). Neurons were grown in defined Hams F-12 medium with N2 additives (no insulin), supplemented with neurotrophic factors: 0.1 ng/ml nerve growth factor, 1.0 ng/ml glial cell line-derived neurotrophic factor and 0.1 ng/ml neurotrophin-3 (all obtained from Sigma). Neurons from control rats were cultured with 10 mmol/l D-glucose and 10 nmol/l insulin and neurons from diabetic rats were plated with 25 mmol/l D-glucose and no insulin for 1 day (cultures were maintained for 3 days for immunofluorescent staining – see later).

**Determination of oxidative stress in axons.** Cultured neurons from control or diabetic rats were either (i) imaged in real time for intracellular ROS by loading with 1.2 µmol/l 5-(and-6)-chloromethyl-2$^7$-dichlorodihydrofluorescein diacetate acetyl ester (CM-H$_2$DCFDA), or (ii) fixed and stained for adducts of 4-hydroxy-2-nonenal (4-HNE) (a product of lipid peroxidation), as previously described (16). To study role of polyol pathway in ROS production, cultures were treated acutely with the specific sorbitol dehydrogenase (SDH) inhibitor, SDI-158 (24) (10 µM; a gift from Dr. Nigel Calcutt, UCSD).

**Assessment of mitochondrial membrane potential in cultured neurons.** Cultured DRG neurons were loaded with 3.0 nmol/l tetramethyl rhodamine methyl ester (TMRM; Molecular probes, Eugene, OR) for 1 hr and the fluorescence signal in the axons detected with a Carl Zeiss LSM510 confocal inverted microscope (X100 objective; excitation at 540 nm and emission > 560 nm). The TMRM was utilized in sub quench mode – where decreased fluorescence intensity indicates reduced mitochondrial inner
membrane potential (25). Antimycin A and oligomycin (Sigma) were injected into the culture media to a final concentration of 10 µmol/l and 1 µmol/l, respectively, at 1 min following baseline fluorescence measurements. All axons in each field were assessed as average of fluorescence pixel intensity per axon length using the Carl Zeiss software package (16).

**Intra-mitochondrial ROS measurement.** Intra-mitochondrial ROS generation, mainly superoxide, was detected using the fluorescent MitoSOX red dye (Molecular Probes, cat #M36008). Lumbar DRG neurons were loaded with 400 nmol/l of MitoSOX red (in 100% anhydrous DMSO; Molecular Probes) for 15 min with or without 1.0 µmol/l oligomycin at 37°C, and then washed three times with F-12 and excited at 514 nm and emission > 560 nm.

**Respiratory chain function.** Oxygen consumption was determined at 37°C using the OROBOROS Oxygraph-2K (OROBOROS Instruments GmbH, Innsbruck, Austria) (8). Mitochondria from freshly isolated and intact lumbar DRG were resuspended in KCl medium (80 mmol/l KCl, 10 mmol/l Tris-HCl, 3.0 mmol/l MgCl$_2$, 1.0 mmol/l EDTA, 5.0 mmol/l potassium phosphate, pH 7.4). Various substrates and inhibitors of the mitochondrial respiratory chain complexes were used as described (8). Enzymatic activities in lumbar DRG mitochondrial preparations were performed spectrophotometrically as previously described (8).

**Statistical analysis.** Where appropriate, data were subjected to one-way ANOVA with *post-hoc* comparison using Tukey’s test or regression analysis with a one-phase exponential decay parametric test with Fisher’s parameter (GraphPad Prism 4, GraphPad Software Inc., San Diego, CA). In all other cases two-tailed Student’s *t*-Tests were performed. To determine the threshold for statistical significance for the proteomic data proteins showing at least a 20 or 25% increase or decrease were grouped and compared to the entire dataset using a Kruskal-Wallis non-parametric ANOVA and Dunn’s multiple comparison test. This analysis indicated that a minimum difference of 25% was necessary for a value to be considered statistically different from the data set.

**RESULTS**

STZ-diabetic rats did not suffer weight loss during the study but showed reduced weight gain after 22 weeks of STZ-diabetes compared to age-matched controls (Table 1). Persistence of diabetes was indicated by elevated non-fasting blood glucose and glycated hemoglobin levels (Table 1). STZ-diabetic rats that received insulin supplementation for the final 4 weeks of a 22 week period of diabetes showed a partial, but statistically significant, recovery of body weight, blood glucose and glycated hemoglobin levels.

To quantitatively assess the effect of diabetes and insulin therapy on the mitochondrial proteome of lumbar DRG, we labeled S16 immortalized Schwann cells with isotopic forms of lysine (K6) and arginine (R10) and isolated labeled mitochondria to serve as internal standards (Fig. 1A). We examined the quantitative accuracy of this approach by mixing the K6R10:K0R0 mitochondria in ratios of 0.75:1, 1.5:1 and 3:1. The K6/K0 or R10/R0 ratios for individual peptides were obtained from MaxQuant analysis and a linear response was observed after plotting the average peptide ratio obtained from each mixture against the known mixing ratio (Supplemental Fig. 1).
Diabetes alters the DRG mitochondrial proteome and function.

in online appendix 1 at http://diabetes.diabetesjournals.org). A 25% decrease in protein expression was quantifiable. Unlabeled (K0R0) DRG mitochondria from the three treatments were then individually mixed in a 2:1 ratio with the K6R10 mitochondria prior to SDS-PAGE and LTQ-FT MS/MS analysis. From over 43,600 identified peptides, 12,785 SILAC pairs were sequenced and approximately 60% identified. After culling out contaminants (n=30), reverse-decoy hits (n=13) and proteins identified by only a single unique peptide, we identified 672 proteins of which 334 (49.6%) were quantified by at least one unique peptide identified in samples from at least two animals (Supplemental Table 1). The median number of quantified ratios for the three treatments was: control (n=5), diabetic (n=8) and diabetic + insulin (n=7). Of the total proteins identified, 206 (30%) were annotated as mitochondrial/glycolytic and 151 were quantified (73%).

To provide a global view of the effect of diabetes and insulin therapy on protein expression, the expression ratios were binned and a frequency distribution assessed (Fig. 1B). In general, diabetes had a more pronounced effect on decreasing protein expression. Insulin therapy induced a rightward shift toward normalizing expression and promoted a significant increase in protein expression. Pathway analysis found that proteins associated with mitochondrial dysfunction, oxidative phosphorylation and ubiquinone biosynthesis (primarily complex 1 proteins) were the most significantly over-represented and showed the greatest percentage of proteins that underwent significant down-regulation (Table 2). Consistent with the diabetic phenotype, proteins associated with ketone body biology were also over-represented and diabetes increased the expression of succinyl-CoA:3-ketoacid-coenzyme A transferase 1 (SCOT), which is critical in acetoacetate clearance.

To determine if diabetes and insulin therapy had a distinct effect on mitochondrial versus non-mitochondrial proteins, the expression ratios for each protein were plotted against each treatment (Fig. 1C). This analysis indicated that diabetes had little effect on the majority of mitochondrial and non-mitochondrial proteins that were quantified (region between solid and dotted lines). With rare exception, insulin therapy did not decrease protein expression but led to a significant increase in the expression of numerous non-mitochondrial proteins (green shading). Enrichment analysis of proteins in this region using the Biological Networks Gene Ontology (BiNGO) plugin of Cytoscape found that cluster frequency for proteins annotated to the biological process of translation was 36.1%, a 7-fold enrichment. We also noted a small group of proteins that were significantly increased by diabetes but whose expression was unchanged by insulin (blue shading). BiNGO analysis of this subset of proteins indicated that small G-protein signaling and protein transport were the enriched processes.

Diabetes caused a statistically relevant change in 27% of quantified mitochondrial proteins and insulin therapy had an ameliorative effect that, in general, normalized this decrease (Fig. 1C, yellow shading). Consistent with another proteomic study of heart mitochondria (14), bioinformatic analysis found that proteins associated with canonical pathways of mitochondrial dysfunction and oxidative phosphorylation were over-represented and mainly
Diabetes alters the DRG mitochondrial proteome and function.

decreased in expression (Table 3). Representative examples from a diabetic animal show a 51% decrease in the complex 1 protein, NADH dehydrogenase Fe-S protein 3 (NDUFS3) and a 29% decline of Mn superoxide dismutase (Mn-SOD) (Fig. 2A & B). However, insulin therapy improved the deficits in NDUFS3 and Mn-SOD expression (Fig. 2C) as previously characterized using Western blotting (8; 16). On the other hand, diabetes did not alter the expression of ATP synthase alpha (Supplemental Fig. 2 in online appendix 1).

Lumbar DRG from age-matched control and 22 week STZ-diabetic rats were analyzed for rates of oxygen consumption as shown in Fig. 3A. Respiratory chain activity, whether coupled or uncoupled, was significantly depressed in diabetic samples. In agreement with the proteomic data and oxygen consumption results, the enzymatic activities of rotenone-sensitive NADH-cytochrome c reductase (Complex I) and cytochrome c oxidase (COX; Complex IV) as well as the Krebs cycle enzyme, citrate synthase, were significantly decreased in STZ-diabetic rats compared to control (Supplemental Table 2).

Adult sensory neurons were cultured for 1 day from age matched control and 22 weeks STZ-diabetic rats and loaded with TMRM. This dye was used at a sub-quench concentration where a decrease in fluorescence signal intensity indicated reduced mitochondrial inner membrane potential (25). The live neurons were exposed to a combination of antimycin A (inhibitor of complex III) and oligomycin (inhibitor of ATP synthase) and the fluorescence signal in axons detected by confocal microscopy. Antimycin A blocks electron transfer leading to mitochondrial depolarization whereas oligomycin inhibits the ATPase and prevents reverse pumping of protons and associated generation of a proton gradient. Therefore, the mitochondrial membrane potential (and associated proton gradient) is completely dissipated in the presence of both these drugs. In the presence of antimycin A + oligomycin, the rate of mitochondrial depolarization was more rapid in axons of normal neurons compared with diabetic neurons (Fig 3B-D). This suggests that prior to addition of antimycin A + oligomycin, the axonal mitochondria were more highly polarized in the normal neurons compared with the diabetic neurons. Mitochondrial physiology was further investigated by treating cultured neurons from control and diabetic rats with oligomycin alone. Blockade of the ATPase results in a build-up of the transmembrane proton gradient and induces hyperpolarization of the mitochondrial inner membrane as indicated by elevated TMRM fluorescence (Fig. 4A) (26). In normal neurons a transient hyperpolarization was observed followed by a recovery due to adaption of the respiratory chain. For example, uncoupling proteins become active and dissipate the proton gradient under a high inner membrane potential (27). Diabetic neurons exhibited a significantly greater level of hyperpolarization upon oligomycin application and the adaptive response was impaired.

We determined if the respiratory chain was contributing to oxidative stress in diabetic neurons by loading cells with the mitochondrially-targeted ROS detector, MitoSOX red. A subset of diabetic neurons was pre-treated with oligomycin to hyperpolarize the inner mitochondrial membrane and maximize loading of MitoSOX red into the
Diabetes alters the DRG mitochondrial proteome and function.

mitochondrial matrix. Neurons were treated with the uncoupler, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), to dissipate the transmembrane electrochemical gradient and enhance the rate of electron transfer. Increased respiratory chain activity will lead to augmented electron leakage and associated generation of ROS, primarily superoxide. In normal neurons this was demonstrated with elevated FCCP-induced MitoSOX red fluorescence (Fig. 4F and G). Diabetic neurons, with or without prior oligomycin treatment, exhibited lower MitoSOX red fluorescence intensities indicative of reduced levels of superoxide being generated by the respiratory chain.

Parallel cultures demonstrated elevated oxidative stress in axons of diabetic neurons under 25 mmol/l glucose versus control neurons as exhibited by raised DCF fluorescence and enhanced staining for adducts of 4-HNE (Fig. 5A-F). Subsets of these cultures were treated acutely with the specific SDH inhibitor, SDI-158, to investigate the role of the polyol pathway in ROS production. Fig. 5G and H shows that blockade of high glucose-dependent sorbitol production resulted in reduced ROS generation in axons of diabetic neurons.

DISCUSSION
The results show that respiratory chain components of the mitochondrial proteome are down-regulated in DRG in diabetes and this phenotypic alteration was associated with impairment in respiratory chain activity. In addition, for the first time this study demonstrates that altered mitochondrial proteome expression was linked to altered mitochondrial physiology in axons of diabetic neurons. And finally, while oxidative stress was present in axons of diabetic neurons the results show that polyol pathway activity, not aberrant respiratory chain function, contributed to generation of ROS.

We used SILAC to provide a set of culture-derived isotope tags (20) to serve as internal standards for quantifying the effect of diabetes on the composition of the mitochondrial proteome from DRG. One advantage of culture-derived isotope tags is the quantitative accuracy that can be achieved relative to label-free approaches (28), especially for low abundant proteins. Despite our small sample size, a 25% change in expression was sufficient to reach statistical significance and the number of mitochondrial proteins that exceeded this threshold was similar to that previously reported in mitochondria isolated from heart tissue of diabetic Akita mice (14). Further, the results of our unbiased quantification were in close agreement with those obtained by targeted immunoblot analyses for COX subunit IV, NDUFS3, ATP synthase and Mn-SOD in two prior studies (8; 16).

Similar to a previous gene array study delineating alterations in mRNA expression in DRG from diabetic rats (29), the magnitude of changes observed in the mitochondrial proteins were rather modest and averaged 0.44 ± 0.16 fold in either direction. Interestingly, the gene array study reported that after 2 months of diabetes, no modification occurred in gene expression of enzymes associated with the tricarboxylic acid (TCA) cycle. We also observed no changes in pyruvate dehydrogenase and 6 of 8 of the TCA cycle enzymes at the protein level, the exception being fumarate hydratase and succinyl Co-A ligase. Similarly, with the exception of hexokinase 1, the remaining glycolytic enzymes that were
Diabetes alters the DRG mitochondrial proteome and function.

Initial exposure to high glucose concentration over 1-4 weeks in DRG in diabetic rats is linked to up-regulation of glycolytic pathway expression (mRNA) (29). Studies in endothelial or Schwann cells demonstrate that acute exposure to glucose elevates ROS and respiratory chain activity, respectively (2; 23). Therefore, in the short term hyperglycemia triggers enhanced glycolysis and associated respiratory chain activity (and possibly ROS). However, in the longer term the high intracellular glucose concentration provides an ample supply of ATP via several non-mitochondrial dependent pathways. Consequently, the metabolic phenotype of the cell adapts and functions in the absence of a dependence on the TCA cycle and oxidative phosphorylation for ATP production, possibly by initiating a process homologous to the ‘Crabtree effect’ (35). Thus, rates of electron donation to the respiratory chain are sub-optimal in neurons in long term diabetic rats and may predispose to lower rates of mitochondrial respiratory chain activity and oxidative phosphorylation. Key metabolic activity sensors and/or regulators such as AMPK and NRF-1 are putative candidates for this modulation, although it is unlikely subsequent adapted metabolism of glucose is channeled through glycolysis in isolation. In this regard, elevated glucose flux through the aldehyde and aldose reductase pathway could be critical (24; 36). For example, studies in lens (37), retina (5) and cardiac tissue (38) in medium to long term animal models of type 1 diabetes show that parts of glycolytic pathway function are depressed.

ROS production linked to enhanced electron leakage from the respiratory chain induced by uncoupling,
Diabetes alters the DRG mitochondrial proteome and function.

and theoretically comprising superoxide, was lower in the axons of neurons from diabetic rats (Fig.4B-E) confirming our mitochondrial physiology and proteomics work. This was also in spite of generally elevated ROS levels in axons (Fig.5A-F). The studies in Fig. 4 and Fig. 5G provide preliminary evidence that the sources of ROS in axons of diabetic neurons are not from aberrant respiratory chain function but, in part, from polyol pathway activity. The latter pathway has been proposed as a source of ROS through a putative sorbitol accumulation-dependent NADPH oxidase (NOX) route in previous studies (24; 36). Down-regulation of the respiratory chain machinery would be predicted to lead to depolarization of the mitochondrial membrane (Fig.3B-D), reduced rates of respiratory chain activity (8) (Fig.3A and Supplemental Table 2) and associated diminished electron leakage. The lower rate of loss of polarization status subsequent to uncoupling in Fig. 3C was not the result of resistance to uncoupling in the diabetic neurons since complementary measures of respiratory chain activity show lower rates of electron transfer in diabetic neurons (and see Figure 1 in (8)). These findings differ from those in cultured endothelial cells where high glucose concentration enhanced mitochondrial membrane potential and induced elevated ROS (2-3). Fig.4A reveals oligomycin treatment caused a greater level of mitochondrial inner membrane hyperpolarization above baseline in diabetic neurons compared with normal neurons further highlighting that adult sensory neurons with a history of diabetes and under high glucose concentration behave differently to endothelial cells. The adaption of mitochondria in normal neurons to hyperpolarization was not observed in diabetic neurons, again stressing the aberrant phenotype of mitochondrial physiology. Uncoupling proteins such as adenine nucleotide transporters (ANT1/2) contribute to the dissipation of a high mitochondrial membrane potential (27) and expression was depressed in diabetic mitochondria (Supplemental Table 1).

In conclusion, our proteomics data reveal a range of altered expression profiles in the mitochondrial proteome of DRG from diabetic rats. This modified expression pattern was associated with aberrant mitochondrial respiratory chain physiology and function. Under high glucose concentration the neuron cell body perceives that mitochondrial function can be downgraded, however, this may ignore the unique high energy requirements of the nerve ending and contribute to distal axon degeneration. For example, growth cone motility that underpins axonal plasticity and regeneration in the skin has an exceedingly high demand for ATP due to significant levels of actin treadmilling (39). Impaired respiratory chain function did not elevate ROS generation even though oxidative stress was observed in axons. In fact, the lower rates of respiratory chain activity were linked to mitochondrial membrane depolarization, improper adaption to oligomycin-induced membrane hyperpolarization, and reduced levels of superoxide derived from electron leakage during electron transport. In axons of neurons from long term diabetic rats sites of ROS production colocalize with the mitochondrial compartment (16; 40). Therefore, alternative mitochondrial-related sources of ROS should be considered. For example, NOX has been localized to the mitochondrial compartment of kidney cortex and mesangial cells and mediates elevated ROS under high glucose concentration.
Diabetes alters the DRG mitochondrial proteome and function.

Author contributions. E.A. and E.Z. contributed equally to the fluorescence imaging experiments described in Figs 3, 4 and 5; E.A. performed preparation of mitochondrial samples for proteomic analysis; S.K.R.C. performed the mitochondrial respiration and complex activity studies; D.R.S. was responsible for induction of diabetes and maintenance of all animal groups, analysis of blood glucose/glycated hemoglobin and dissection of DRG for mitochondrial measurements; R.T.D. designed and performed the proteomic analysis and contributed to writing and editing the manuscript; P.F. designed the animal and tissue culture studies and contributed to writing and editing of the manuscript.

ACKNOWLEDGEMENTS. This work was supported by grants from the Juvenile Diabetes Research Foundation (JDRF # 1-2008-280) and The National Institutes of Health (NIH; grants NS054847 and DK073594) to R.T.D. Mr. Eli Akude was supported by a grant from the National Science and Engineering Research Council (NSERC; grant # 3311686-06) to PF and subsequently a postgraduate scholarship from the Manitoba Health Research Council (MHRC). Drs. Subir Roy Chowdhury and Elena Zherebitskaya were supported by grants to P.F. from Canadian Institutes for Health Research (CIHR; grant # MOP-84214) and JDRF (grant # 1-2008-193). Darrell Smith was supported by a grant to P.F. from MHRC. This work was also funded by the St Boniface General Hospital and Research Foundation. We thank Dr. Gordon Glazner, University of Manitoba and St Boniface Hospital Research Centre, for permitting access to the Carl Zeiss LSM 510.

REFERENCES
Diabetes alters the DRG mitochondrial proteome and function.

Diabetes alters the DRG mitochondrial proteome and function.


FIGURE LEGENDS

**FIG. 1.** In (A) Schematic for use of culture-derived isotope tags for quantitative proteomics. Unlabeled (K0R0) mitochondrial fractions were prepared from the lumbar DRGs obtained from each animal in the three treatment groups. Each K0R0 mitochondrial fraction was mixed in a 2:1 ratio with K6R10 labeled mitochondria obtained from S16 cells that had been metabolically labeled with $^{13}$C$_6$ lysine (K6) or $^{13}$C$_6$,$^{15}$N$_4$ arginine (R10) for 10 days. The proteins were resolved by SDS-PAGE, digested with trypsin and analyzed by RP-HPLC/LTQ-FT MS/MS. For each protein, the ratio of K0R0 to K6R10 quantifies the endogenous protein relative to the internal standard. Dividing the protein ratios obtained in the diabetic or diabetic + insulin treatment by those obtained from control animals cancels out the K6R10 internal standard and provides the fold control value. (B) Effect of diabetes and insulin therapy on mitochondrial protein expression. The protein expression ratios from the diabetic and diabetic + insulin treatments were binned and the number of proteins per bin counted. (C) To determine the effect of diabetes and insulin therapy on mitochondrial versus non-mitochondrial proteins, the expression ratio for each protein was plotted against each treatment. Solid and dotted lines demarcate the threshold necessary for proteins to show a significant change in the diabetic and diabetic + insulin treatments, respectively. Proteins in-between dotted and solid lines did not change with either treatment. Yellow shading indicates proteins that were significantly up or down regulated by diabetes and normalized by insulin therapy. Blue shading indicates proteins that were increased by diabetes but not normalized by insulin therapy. Green shading indicates proteins not affected by diabetes but increased by insulin therapy.

**FIG. 2.** Representative peptide mass spectra showing the effect of diabetes and insulin therapy on NDUFS3 and MnSOD. (A) The upper spectrum shows the doubly charged ion of the unlabeled (m/z 743.90, blue) and R10 labeled (m/z 748.90, black) VVAEPVELAQEFR peptide of NDUFS3 from a control animal. Since the peptide is doubly charged, the mass difference is 5 atomic mass units and the other colored peaks represent the isotopic envelope of the monoisotopic peak. The lower spectrum shows the doubly charged ion of the unlabeled (m/z 720.91, blue) and K6 labeled (m/z 723.91, black) GDVTTQVALQPALK peptide of Mn-SOD from a control animal. Since the peptide is doubly charged, the mass difference is 3 atomic mass units and the other colored peaks represent the isotopic envelope of the monoisotopic peak. The R0/R10 and K0/K6 ratios for these peptides are indicated. (B) Upper and lower spectra show the same NDUFS3 and Mn-SOD peptides, but from a diabetic animal. The K0R0/K6R10 ratios for each peptide are indicated and the Diab/Control ratio were obtained after
Diabetes alters the DRG mitochondrial proteome and function.

(C) Upper and lower spectra show the same NDUFS3 and Mn-SOD peptide, but from a diabetic + insulin treated animal. The K0R0/K6R10 ratios for each peptide are indicated and the Diab/Control ratio were obtained after dividing by the control ratios from panel A. Note that the intensity of the K6 and R10 peptides are very similar between the treatments (A - C) indicating that the changes in protein expression are minimally influenced by the internal standard.

FIG. 3. The mitochondria of DRG sensory neurons exhibited lower respiratory chain activity. (A) Oxygen consumption was assessed in freshly isolated mitochondria from lumbar DRG of age-matched control and 22 week diabetic rats using an OROBOROS oxygraph 2k. Coupled respiration rates were measured in the presence of pyruvate, P (10 mmol/l); malate, M (5.0 mmol/l) and ADP (2.0 mmol/l). Addition of FCCP (0.5 µmol/l) permits a measure of uncoupled respiratory chain activity. Addition of ascorbate, Asc (5.0 mmol/l) and TMPD (0.5 mmol/l) permit an analysis of complex IV activity that was verified by specific inhibitors. Values are mean ± SEM; n = 5. *p < 0.05 versus controls; **p < 0.001 versus controls. (B) Images of fluorescence confocal microscopy using TMRM in live cultures of DRG neurons isolated from control adult rats showing effect of antimycin A and oligomycin. (C) Trace of TMRM fluorescence signal in the axons of cultured DRG neurons isolated from age matched controls and STZ-diabetic rats. (D) Shows the area under the TMRM fluorescence trace (AUC) for control (open bar) and diabetic (filled bar) neurons. The AUC was estimated from the baseline (at the point of injection) to a fluorescence level of 0.2 and between time points of 1.0 min and 6 min using sums-of-squares (shown by dotted line). Values are the means ± SEM, n = 65–80 axons; *p < 0.001 compared to control, t-Test. The TMRM trace was characterized by non-linear regression (one phase exponential decay). The rate constant of decay (K) = 0.013 ± 0.0004 (control) and 0.006 ± 0.0001 (diabetic). Half-life of decay = 54.19 s (control) and 108.7 s (diabetic). The F (Fisher parametric)-ratio = 409.5, P < 0.0001, control vs diabetic. The F-ratio compares the goodness-of-fit of the two curves. The red arrow indicates point of injection of antimycin A + oligomycin.

FIG. 4. Impaired respiratory function is associated with reduced ROS generation in the mitochondrial matrix of cultured neurons isolated from STZ-diabetic rats. (A) TMRM fluorescence trace of oligomycin-induced mitochondrial inner membrane hyperpolarization in the axons of control and diabetic neurons. Values are the mean ± SEM, n=65-85 axons. Inset shows the area under the TMRM fluorescence trace (AUC) for control (open bar) and diabetic (filled bar) neurons. The AUC was estimated from the baseline (at the point of injection, dotted line), and between time points of 1.0 min and 4 min using sums-of-squares. Values are the means ± SEM, n = 65–80 axons,* P < 0.01 compared to control, t-Test. The black arrow indicates point of injection of oligomycin. (B-E) Images of MitoSOX red fluorescence in cultures of DRG neurons showing effect of 5.0 µmol/l FCCP. (F) Quantification of real-time MitoSOX red fluorescence levels in the axons of cultured DRG neurons after 5.0 µmol/l FCCP treatment. MitoSOX trace was characterized by non-linear regression. F-ratio = 32.48, P < 0.0001 (control vs. diabetic with or without oligomycin by oneway ANOVA). Values are the mean ± SEM, n= 18-73 axons. (G) Shows area under the curve for Mitosox red fluorescence intensity levels. Values are
Diabetes alters the DRG mitochondrial proteome and function.

the mean ± SEM, n=35-73 axons; ** P < 0.001 compared to diabetic or diabetic + oligomycin treated cells by oneway ANOVA.

FIG. 5. Axons of sensory neurons from STZ-diabetic rats exhibit elevated oxidative stress that is ameliorated by blockade of SDH. Images of ROS levels in axons at 24 h in adult DRG neuron culture from (A) control and (B) STZ-diabetic rats. Cultures were stained for ROS using CM-H$_2$DCFDA dye (DCF being the fluorescent product resulting from oxidation). (E) Quantification of ROS accumulation in axons. Values are means ± SEM, n= 44-57 axons, * P < 0.05 by t-Test. Immunofluorescent images of accumulation of adducts of 4-HNE in axons in sensory neuron cultures after 3 days, (C) is control and (D) is diabetic culture. (F) Level of accumulation of puncta of adducts of 4-HNE in axons. Values are means ± SEM, n= 19-27 axons, ** P < 0.01 by t-Test. (G) Trace of DCF fluorescence signal in the axons of cultured DRG neurons isolated from age matched controls and STZ-diabetic rats and treated acutely with 10 µM SDI-158. DCF fluorescence trace was characterized by non-linear regression (one phase exponential decay). K = 0.09 ± 0.02 (control) and 0.13 ± 0.009 (diabetic). Half-life of decay = 7.5 mins (control) and 5.5 min (diabetic). The F-ratio = 50.33, P < 0.0001, control vs diabetic. The red arrow indicates point of injection of SDI-158. (H) Shows the area under the DCF fluorescence trace (AUC) for control (open bar) and diabetic (filled bar) neurons. The AUC was estimated from 0.2 to 1.6 on fluorescence axis and between time points 0 to 22 min using sums-of-squares (dotted lines show upper and lower limits). Values are means ± SEM, n = 42–51 axons; ** P < 0.01 compared to control by t-Test.

Table 1. Body weights, plasma glucose and glycated hemoglobin (HbA1c) of treatment groups.

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Blood glucose (mmol/l)</th>
<th>HbA1c (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 10-13</td>
<td>n = 10-13</td>
<td>n = 9-10</td>
</tr>
<tr>
<td>Control</td>
<td>770.9 ± 57.9*</td>
<td>8.24 ± 0.89*</td>
<td>4.39 ± 0.28*</td>
</tr>
<tr>
<td>Diabetic</td>
<td>415.8 ± 33.9**</td>
<td>30.95 ± 2.71**</td>
<td>11.69 ± 0.97**</td>
</tr>
<tr>
<td>Diabetic + insulin</td>
<td>519.1 ± 42.0</td>
<td>14.81 ± 4.95</td>
<td>8.59 ± 1.17</td>
</tr>
</tbody>
</table>

Values are means ± SD. *p<0.001 vs other groups; **p<0.001 vs diabetic + insulin (one-way ANOVA with Tukey’s test). Starting weights for the groups were 293 ± 8.8 g (mean ± SD; n=38). Non-fasting blood sugar concentration was measured using the Accu-Chek Compact Plus glucometer (Roche, Laval, QC, Canada) and blood glycated hemoglobin (HbA1c) levels by the A1cNow+ system (Bayer Healthcare, Sunnyvale, CA).

Table 2. Over-represented canonical pathways identified in the proteomic analysis.

<table>
<thead>
<tr>
<th>Canonical Pathways</th>
<th>Significance</th>
<th>Ratio, %</th>
<th>Total Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial dysfunction</td>
<td>3.16 x 10^{-11}</td>
<td>7.2</td>
<td>171</td>
</tr>
<tr>
<td>Oxidative phosphorylation</td>
<td>3.98 x10^{-7}</td>
<td>5.4</td>
<td>166</td>
</tr>
</tbody>
</table>
Diabetes alters the DRG mitochondrial proteome and function.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>p Value</th>
<th>Ratio</th>
<th>Total Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquinone biosynthesis</td>
<td>7.94 x 10^{-6}</td>
<td>5.0</td>
<td>119</td>
</tr>
<tr>
<td>Methane metabolism</td>
<td>1.25 x 10^{-4}</td>
<td>4.0</td>
<td>66</td>
</tr>
<tr>
<td>Breast cancer regulation by stathmin1</td>
<td>1.59 x 10^{-4}</td>
<td>4.6</td>
<td>199</td>
</tr>
<tr>
<td>Integrin signaling</td>
<td>1.99 x 10^{-4}</td>
<td>3.5</td>
<td>200</td>
</tr>
<tr>
<td>Butanoate metabolism</td>
<td>6.31 x 10^{-4}</td>
<td>3.0</td>
<td>132</td>
</tr>
<tr>
<td>14-3-3-mediated signaling</td>
<td>6.31 x 10^{-4}</td>
<td>4.4</td>
<td>114</td>
</tr>
<tr>
<td>Propanoate metabolism</td>
<td>7.94 x 10^{-4}</td>
<td>3.1</td>
<td>130</td>
</tr>
<tr>
<td>Citrate cycle</td>
<td>7.94 x 10^{-4}</td>
<td>5.2</td>
<td>58</td>
</tr>
<tr>
<td>Valine, leucine and isoleucine degradation</td>
<td>1.00 x 10^{-3}</td>
<td>3.6</td>
<td>111</td>
</tr>
<tr>
<td>Fatty acid metabolism</td>
<td>1.25 x 10^{-3}</td>
<td>2.6</td>
<td>192</td>
</tr>
<tr>
<td>Phenylalanine metabolism</td>
<td>1.25 x 10^{-3}</td>
<td>2.8</td>
<td>109</td>
</tr>
<tr>
<td>Ketone body biology</td>
<td>2.51 x 10^{-3}</td>
<td>10.5</td>
<td>19</td>
</tr>
<tr>
<td>Fatty acid elongation</td>
<td>5.01 x 10^{-3}</td>
<td>4.4</td>
<td>45</td>
</tr>
<tr>
<td>Induction of apoptosis by HIV1</td>
<td>6.31 x 10^{-3}</td>
<td>4.6</td>
<td>65</td>
</tr>
</tbody>
</table>

Significance provides the confidence of the association as identified by the p value of the Fisher exact test. The ratio provides the percent of proteins associated with the pathway that underwent a significant change. The total genes column refers to all known genes to be linked to the pathway (not necessarily identified by the proteomic screen).
Table 3. Effect of diabetes and insulin therapy on representative proteins annotated to oxidative phosphorylation and mitochondrial dysfunction. Values shown are the mean and the asterisk (*) indicates proteins that showed a significant change in expression. Percent change represents effect of insulin treatment on the protein expression ratio measured from diabetic rats. Please see Supplemental Table 1 for more complete information. ¶ confirming (8; 16); § confirming (29).

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Protein Description</th>
<th>Diabetic</th>
<th>Diabetic + Insulin</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP5C1</td>
<td>ATP synthase, F1 complex, gamma</td>
<td>0.90</td>
<td>1.29*</td>
<td>143</td>
</tr>
<tr>
<td>ATP5D</td>
<td>ATP synthase, F1 complex, delta subunit</td>
<td>0.69*</td>
<td>0.92</td>
<td>133</td>
</tr>
<tr>
<td>ATP5F1</td>
<td>ATP synthase, F0 complex, subunit B1</td>
<td>0.92</td>
<td>1.31*</td>
<td>142</td>
</tr>
<tr>
<td>ATP5I</td>
<td>ATP synthase, F0 complex, subunit E</td>
<td>0.75*</td>
<td>0.81</td>
<td>108</td>
</tr>
<tr>
<td>COX2</td>
<td>cytochrome c oxidase subunit 2</td>
<td>0.82</td>
<td>1.06</td>
<td>129</td>
</tr>
<tr>
<td>COX4I1</td>
<td>cytochrome c oxidase subunit IV isoform 1 ¶</td>
<td>0.71*</td>
<td>0.89</td>
<td>125</td>
</tr>
<tr>
<td>COX5A</td>
<td>cytochrome c oxidase subunit Va</td>
<td>0.83</td>
<td>1.28*</td>
<td>154</td>
</tr>
<tr>
<td>CPT1A</td>
<td>carnitine palmitoyltransferase 1A (liver)</td>
<td>0.51*</td>
<td>0.77</td>
<td>172</td>
</tr>
<tr>
<td>CYCS</td>
<td>cytochrome c, somatic</td>
<td>0.56*</td>
<td>0.9</td>
<td>160</td>
</tr>
<tr>
<td>HSD17B10</td>
<td>hydroxysteroid (17-beta) dehydrogenase 10</td>
<td>0.71*</td>
<td>1.14</td>
<td>160</td>
</tr>
<tr>
<td>ND4</td>
<td>NADH dehydrogenase, subunit 4 (complex I)</td>
<td>0.43*</td>
<td>0.56*</td>
<td>130</td>
</tr>
<tr>
<td>NDUFA10</td>
<td>NADH dehydrogenase 1 α subcomplex 10 §</td>
<td>0.73*</td>
<td>0.79</td>
<td>108</td>
</tr>
<tr>
<td>NDUFA13</td>
<td>NADH dehydrogenase 1 alpha subcomplex, 13</td>
<td>0.35*</td>
<td>0.59*</td>
<td>168</td>
</tr>
<tr>
<td>NDUFB10</td>
<td>NADH dehydrogenase 1 beta subcomplex, 10</td>
<td>0.94</td>
<td>1.4*</td>
<td>148</td>
</tr>
<tr>
<td>NDUFS1</td>
<td>NADH dehydrogenase Fe-S protein 1</td>
<td>0.78</td>
<td>0.91</td>
<td>116</td>
</tr>
<tr>
<td>NDUFS2</td>
<td>NADH dehydrogenase Fe-S protein 2</td>
<td>0.48*</td>
<td>0.54</td>
<td>113</td>
</tr>
<tr>
<td>NDUFS3</td>
<td>NADH dehydrogenase Fe-S protein 3 ¶</td>
<td>0.64*</td>
<td>0.84</td>
<td>131</td>
</tr>
<tr>
<td>NDUFS8</td>
<td>NADH dehydrogenase Fe-S protein 8</td>
<td>0.91</td>
<td>1.34*</td>
<td>147</td>
</tr>
<tr>
<td>PRDX3</td>
<td>peroxiredoxin 3</td>
<td>0.87</td>
<td>1.26*</td>
<td>145</td>
</tr>
<tr>
<td>PRDX5</td>
<td>peroxiredoxin 5</td>
<td>0.74*</td>
<td>0.86</td>
<td>116</td>
</tr>
<tr>
<td>SOD2</td>
<td>superoxide dismutase 2, mitochondrial ¶</td>
<td>0.73*</td>
<td>1.08</td>
<td>148</td>
</tr>
<tr>
<td>UQCRAC1</td>
<td>ubiquinol-cytochrome c reductase 1</td>
<td>0.87</td>
<td>1.26*</td>
<td>145</td>
</tr>
</tbody>
</table>
Diabetes alters the DRG mitochondrial proteome and function.

Figure 1
Diabetes alters the DRG mitochondrial proteome and function.

Figure 2

A

B

C

Diabetic ratio vs Diabetic + Insulin ratio
Diabetes alters the DRG mitochondrial proteome and function.

**Figure 3**

**A**
Oxygen consumption of DRG

**B**
TMRM fluorescence in axons

**C**
Mitochondrial depolarization

**D**
TMRM fluorescence (AUC)
Diabetes alters the DRG mitochondrial proteome and function.

Figure 4

A  Oligomycin-induced hyperpolarization

B  control
C  control + 10min
D  diabetic
E  diabetic + 10min

F  Axonal Mitosox red signal

G  Mitosox red - AUC
Diabetes alters the DRG mitochondrial proteome and function.

Figure 5

(E) DCF fluorescence

(F) # of HNE +ve puncta per 100 μm of axon

(G) SDI-158 (10 μM)

(H) DCF fluorescence (AUC)