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Insulin Prevents Aberrant Mitochondrial Phenotype in Sensory Neurons of Type 1 Diabetic Rats

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Abstract

Diabetic neuropathy affects approximately 50% of diabetic patients. Down-regulation of mitochondrial gene expression and function has been reported in both human tissues and in dorsal root ganglia (DRG) from animal models of type 1 and type 2 diabetes. We hypothesized that loss of direct insulin signaling in diabetes contributes to loss of mitochondrial function in DRG neurons and to development of neuropathy. Sensory neurons obtained from age-matched adult control or streptozotocin (STZ)-induced type 1 diabetic rats were cultured with or without insulin before determining mitochondrial respiration and expression of mitochondrial respiratory chain and insulin signaling-linked proteins. For *in vivo* studies age-matched control rats and diabetic rats with or without trace insulin supplementation were maintained for 5 months before DRG were analyzed for respiratory chain gene expression and cytochrome *c* oxidase activity. Insulin (10nM) significantly ($P<0.5$) increased phosphorylation of Akt and P70S6K by 4-fold and neurite outgrowth by 2-fold in DRG cultures derived from adult control rats. Insulin also augmented the levels of selective mitochondrial respiratory chain proteins and mitochondrial bioenergetics parameters in DRG cultures from control and diabetic rats, with spare respiratory capacity increased by up to 3-fold ($P<0.05$). Insulin-treated diabetic animals exhibited improved thermal sensitivity in the hind paw and had increased dermal nerve density compared to untreated diabetic rats, despite no effect on blood glucose levels. In DRG of diabetic rats there was suppressed expression of mitochondrial respiratory chain proteins and cytochrome *c* oxidase activity that was corrected by insulin therapy. Insulin elevates mitochondrial respiratory chain protein expression

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Author contributions:

MRA performed the majority of work including primary neuron cell culture, Western blotting, real-Time PCR and immunocytochemistry. DRS and NAC maintained, treated, and performed behavioral, electrophysiological and biochemical assays on groups of rats. MRA, SKR and MGS performed Seahorse analysis of mitochondrial function and cell counting. MRA and SKR carried out the mitochondrial enzymatic activities. MRA, NAC and DRS analyzed data. DRS and MRA designed experiments. MRA contributed to write the first draft of paper. PF designed experiments, analyzed data and wrote and edited the paper.

and function in sensory neurons and this is associated with enhanced neurite outgrowth and protection against indices of neuropathy.

Keywords

axon regeneration; bioenergetics; diabetic neuropathy; dorsal root ganglia; mitochondrial function; neurotrophin

Introduction

Neuropathy, the most common complication of diabetes, is characterized by distal dying-back of nerve fibers combined with impaired axon regeneration (Vinik et al., 2016; Zochodne, 2016). Oxidative stress, defective insulin signaling, neurotrophic factor deficiency, dyslipidemia and aberrant neurovascular interactions have all been proposed as contributors to pathogenesis of diabetic neuropathy (Calcutt et al., 2008; Davidson et al., 2010; Vincent et al., 2009; Yagihashi, 2016; Zochodne, 2016). Other than an improvement in indices of diabetic neuropathy by tight glycemic control in persons with type 1 diabetes (Nathan et al., 1993), there are no promising therapies for diabetic or other peripheral neuropathies, many of which display some degree of mitochondrial dysfunction (Bennett et al., 2014; Cashman and Hoke, 2015).

The high energy consumption of neurons requires fine control of mitochondrial function (Chowdhury et al., 2013; Fernyhough, 2015) and the growth cone motility required to maintain fields of innervation consumes 50% of ATP supplies in neurons due to high rates of actin treadmilling (Bernstein and Bamburg, 2003). Unmyelinated axons are more energetically demanding than myelinated axons, consuming 2.5–10-fold more energy per action potential (Wang et al., 2008). There is mounting evidence that diabetes suppresses mitochondrial function in dorsal root ganglia (DRG) (Chowdhury et al., 2010; Freeman et al., 2016; Ma et al., 2014; Roy Chowdhury et al., 2012; Sas et al., 2016; Urban et al., 2012). We have previously proposed that hyperglycemia-induced down-regulation of the AMP-activated protein kinase (AMPK)/peroxisome proliferator-activated receptor γ co-activator 1-α (PGC-1α) signaling axis can result in axon degeneration and failure to regenerate (Calcutt et al., 2017; Chowdhury, et al., 2013; Fernyhough, 2015; Roy Chowdhury, et al., 2012). However, there is also a growing appreciation that hyperglycemia is not the sole initiating factor in the pathogenesis of diabetic neuropathy. A number of authors have presented data indicating that loss of direct insulin signaling contributes to diabetic neuropathy and retinopathy (Ishii, 1995; Reiter and Gardner, 2003; Zochodne, 2016). For example, providing systemic insulin at low levels or injecting insulin adjacent to the sciatic nerve can prevent deficits in sensory and motor nerve conduction velocity (NCV) in streptozotocin (STZ)-induced diabetic rats independent of correction of hyperglycemia (Brussee et al., 2004; Huang et al., 2003; Singhal et al., 1997). Local injection of insulin to the skin, or topical application to the cornea also enhances sensory nerve fiber density in diabetic rodents (Chen et al., 2013; Guo et al., 2011). In humans, the local application of insulin can enhance nerve recovery in carpal tunnel syndrome in patients with type 2 diabetes (Ozkul et al., 2001).

The potential for direct neurotrophic and neuroprotective actions of insulin is supported by reports that neurons express the appropriate proteins to facilitate responses to insulin exposure. Insulin receptors (IRs) and receptor substrate scaffolds (IRS1, IRS2) are expressed by sensory neurons and activate signal transduction pathways that modulate neurite outgrowth and axonal plasticity (Fernyhough et al., 1993; Grote et al., 2013; Huang et al., 2005; Singh et al., 2012). In neurons, the insulin receptor pathway activates several messengers that include the important survival kinase, phosphatidylinositide 3-kinase (PI3-K), that is directly associated with, and activated by, IRS-1 and induces Akt activation (Grote, et al., 2013; Huang, et al., 2005; Kim et al., 2011). P70S6K is a serine/threonine kinase that acts downstream of the PI-3K/Akt pathway to regulate survival and growth of neurons. Upon phosphorylation on the T389 site, P70S6K is activated and triggers protein synthesis via activation of S6 ribosomal protein (Chung et al., 1994). Neurons are therefore under direct regulatory control by insulin and impaired insulin signaling in diabetes provides a parallel pathogenic mechanism to hyperglycemia.

In the present study, we tested the hypothesis that exogenous insulin could correct mitochondrial dysfunction in adult rat sensory neurons under hyperglycemic conditions using both *in vitro* and *in vivo* models.

Materials and methods

Animals

Male Sprague-Dawley rats (275–325 g) were used as a model of type 1 diabetes after delivery of a single intraperitoneal injection of 90 mg/kg STZ (Sigma, St Louis, MO, USA). Insulin implants (Linplant, Linshin Canada Inc., Canada) were injected subcutaneously into the nape of the neck of a subgroup of STZ-induced diabetic rats after approximately 4 weeks of diabetes and at monthly intervals thereafter. Fasting blood glucose concentration was monitored weekly using the AlphaTRAK glucometer (Abbott) to ensure that insulin therapy did not alter hyperglycemia. At the end of 5 months, blood glucose, glycated haemoglobin (HbA1C) and body weight were recorded before tissue collection. Animal procedures were approved by the University of Manitoba Animal Care Committee.

Hind paw thermal sensitivity test in adult rats

Hind paw thermal response latencies were measured using a Hargreaves apparatus (UARD, La Jolla, CA, USA) as previously described (Jolivalt et al., 2016). Briefly, rats were placed in plexiglass cubicles on top of the thermal testing system. The heat source was placed below the middle of one of the hind paws and latencies of the paw withdrawal to the heat source were automatically measured. Response latency of each paw was measured three times at 5 min intervals.

Adult DRG sensory neuron culture

DRGs were isolated and dissociated using previously described methods (Calcutt, et al., 2017). Neuron-enriched cells were cultured in Hams F12 media supplemented with Bottenstein's N2 without insulin (0.1 mg/ml transferrin, 20 nM progesterone, 100 μM putrescine, 30 nM sodium selenite 0.1 mg/ml BSA; all additives were from Sigma, St Louis,

MO, USA; culture medium was from Caisson labs, USA). DRG neurons from control rats were cultured in the presence of 5 mM D-glucose and DRG neurons derived from STZ-induced diabetic rats with 25 mM D-glucose and zero insulin. Porcine insulin powder (Sigma, St Louis, MO, USA) dissolved in PH=2 and different doses (10 or 100 nM) were used as treatments. No neurotrophins were added to any DRG cultures. In this culture system there is approximately 5% cell loss over a 24 hr period.

Quantitative Western blotting for insulin signaling and mitochondrial proteins

Rat DRG neurons were harvested from culture or isolated intact from adult rats and then homogenized in ice-cold lysis buffer containing: 0.1 M Pipes, 5 mM $MgCl_2$, 5 mM EGTA, 0.5% Triton X-100, 20% glycerol, 10 mM NaF, 1 mM PMSF, and protease inhibitor cocktail. Proteins were assayed using DC protein assay (BioRad; Hercules, CA, USA). The samples (2–5μg total protein/lane) were resolved and separated via 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After separation, proteins were transferred to a nitrocellulose membrane (Bio-Rad, CA, USA) using Trans-Blot Turbo Transfer System (Bio-Rad, CA, USA) and immunoblotted with specific antibodies against pP70S6K T389 (1:1000, Cell Signaling Technology), pAkt S473 (1:1000, Santa Cruz, TX, USA), Total OXPHOS (1:2000, MitoSciences, Abcam, USA), Porin (1:1000, Abcam), β-actin (1:1000, Santa Cruz Biotechnology) and total-ERK (1:1000, Santa Cruz Biotechnology). Of note, total protein bands were captured by chemiluminescent imaging of the blot after gel activation (TGX Stain-Free™ FastCast Acrylamide Solutions, Bio-Rad, CA, USA) in addition to use of T-ERK and porin levels for target protein normalization. The secondary antibodies were HRP-conjugated goat anti-rabbit IgG (H+L) or donkey anti-mouse IgG (H+L) from Jackson ImmunoResearch Laboratories, PA, USA. The blots were incubated in ECL Advance (GE Healthcare) and imaged using a Bio-Rad ChemiDoc image analyzer (Bio-Rad). All raw data signals for each antibody were normalized to T-ERK (in vitro work) or total protein (in vivo work) from the same blot. Please note that electron transport chain protein bands in Figures 2, 4 and 7, and supplemental Figures 1 and 2 were obtained from one single blot with different exposure times.

Real-Time PCR of DRG samples

RNA was extracted from previously frozen tissue samples using TRIzol® Reagent (Invitrogen). RNA samples underwent DNase treatment and reverse transcription by using the iScript™ gDNA Clear cDNA Synthesis Kit and iQ cycler system (Bio-Rad, CA, USA) according to the manufacturer's instructions. Real-time quantitative RT-PCR was performed by using iQ™ SYBR® Green Supermix (Bio-Rad, CA, USA). The ΔΔCt method was used to quantify gene expression. The expression of porin gene was used for normalization. Primer sequences for gene expression analysis are listed as follows: *SDHB*-Forward: 5′-ATCTGCAATCCATCGAGGACC-3′, *SDHB*-Reverse: 5′-AGCGAT AAGCCTGCATGAGAA-3′, *MT-CO1*-Forward: 5′-TCCAGATGCTTACACCACATGA-3′, *MT-CO1*-Reverse: 5′-AGTTGAGGAGTAGGAAATTGAGAGT-3′, *VDAC1* (porin) -Forward: 5′-GCTTTTCGGCCAAAGTGAACA-3′, *VDAC1* (porin) -Reverse: 5′-CGCATTGACGTTCTTGCCAT-3′.

Mitochondrial respiration in cultured neurons

To measure the basal level of mitochondrial oxygen consumption, the ATP-linked oxygen consumption (proton leak), the maximal respiration, the spare respiratory capacity and the non-mitochondrial oxygen consumption, an XF24 Analyzer (Seahorse Biosciences, Billerica, MA, USA) was used (Brand and Nicholls, 2011). In short, DRG culture medium was changed 1hr before the assay to unbuffered DMEM (Dulbecco's modified Eagle's medium, pH 7.4) supplemented with 1mM pyruvate, and 5 mM D-glucose. For diabetic rat DRG cultures, 25 mM D-glucose was used. Four mitochondrial complex inhibitors including oligomycin (1 μM), FCCP (1 μM) and rotenone (1 μM) + antimycin A (1 μM) were injected sequentially through ports in the Seahorse Flux Pak cartridges. Oligomycin acts as an irreversible ATP synthase inhibitor, FCCP as an uncoupler, rotenone as Complex I inhibitor, and antimycin A as an inhibitor of Complex III of the mitochondrial electron transport system. The XF24 machine which creates a transient 7μl chamber in specialized 24-well microplates, allowed real time recording of live cell mitochondrial oxygen consumption rate (OCR). After OCR measurement, cells were fixed with 4% paraformaldehyde and stained for β-tubulin III which specifically labels sensory neurons. Plates were then placed into a Cellomics Arrayscan-VTI HCS Reader (Thermo Scientific, Pittsburgh, PA, USA) equipped with Cellomics Arrayscan-VTI software to facilitate a full neuronal count of each well. OCR measures from each well were normalized to neuronal count for that specific well. Data are presented as OCR per 5000 cells.

Immunocytochemistry for β-tubulin III for neurite outgrowth in DRG cultures

DRG neurons were removed from culture and fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min at room temperature then permeabilized with 0.3% Triton X-100 in PBS for 5 min. Cells were incubated with blocking buffer for 1 h and with neuron-specific -tubulin III primary antibody (1:1000; from Sigma Aldrich) overnight. Following three washes with PBS, cells were incubated with fluorescein isothiocynate-conjugated secondary antibody (1:1000, Jackson ImmunoResearch Laboratories) for 1 h at room temperature. Slides were mounted using mounting medium with DAPI and imaged by a Carl Zeiss Axioscope-2 upright fluorescence microscope equipped with AxioVision3 software. The fluorescent signal was collected as total pixel area for neurites and was measured by ImageJ software and normalized to number of cell bodies to calculate total neurite outgrowth per neuron.

Nerve density in footpads

The plantar dermis and epidermis of the hind paw were removed and placed in 4% paraformaldehyde. Tissue was processed to paraffin blocks, cut as 6μm sections, immunostained using an antibody to PGP 9.5 (1:1000, Biogenesis Ltd. Poole, UK) and the number of immunoreactive intra-epidermal fiber s(IENF) and sub-epidermal nerve profiles (SNP) per unit length quantified under blinded conditions by light microscopy (Jolivalt, et al., 2016).

Enzymatic activity of respiratory Complex IV in DRG

Enzymatic activity of cytochrome *c* oxidase (a subunit of Complex IV of the mitochondrial electron transport system) was measured by a temperature controlled Ultrospec 2100 UV–

visible spectrophotometer equipped with Biochrom Swift II software (Biopharmacia Biotech). Briefly, 0.02% lauryl maltoside was mixed with 10 μg DRG homogenates and incubated for 1 min before addition of 40 μM reduced cytochrome *c* and 50 mM KPi to the mixture. The resulting absorbance decrease of reduced cytochrome *c* at 550 nm was monitored for 2min (Roy Chowdhury, et al., 2012).

Statistical analysis

Data were analyzed using two-tailed Student's t-tests or one-way ANOVA followed by Tukey's or Dunnett's post hoc tests, as appropriate and indicated (GraphPad Prism 4, GraphPad Software). A P value < 0.05 was considered to be significant.

Results

Insulin augments pAkt (short-term), pP70S6K and neurite outgrowth (long-term) in cultured DRG neurons from normal adult rats

Sensory neurons derived from a normal adult rat were maintained *in vitro* and exposed to insulin at the physiologically relevant concentration of 10 nM. Akt was activated within 15 min of exposure to insulin while P70S6K exhibited enhanced phosphorylation on residue T389 within 2h (Figure 1A, B). To confirm that insulin was acting as a neurotrophic factor for adult sensory neurons, DRG-derived neurons were treated with insulin (1, 10 and 100 nM) for 24h. At doses of 10 nM and 100 nM, but not 1 nM, insulin significantly ($P<0.001$ and $P<0.0001$, respectively) enhanced total neurite outgrowth (Figure 1C, D).

Insulin up-regulates mitochondrial gene expression and function in cultured neurons derived from normal adult rats

Mitochondrial respiratory complex proteins, including the NDUFB8 subunit of Complex I and ATP5A subunit of Complex V, were significantly ($P<0.05$ and 0.01 respectively) up-regulated in cultures of DRG neurons derived from control rats 24h after treatment with insulin (Figure 2). Subunits of Complex II and IV were also raised 2-fold at 24h when compared to the untreated control group but this enhancement was not statistically significant. Insulin treatment elevated OCR (Figure 3A) and bioenergetic parameters such as basal respiration, maximal respiration and spare respiratory capacity were significantly ($P<0.05$) augmented 2h after insulin delivery (Figure 3B).

Insulin enhances mitochondrial gene expression and function in cultured neurons derived from a diabetic rat

To investigate if diabetes impedes insulin driven induction of mitochondrial gene expression and mitochondrial function, we analyzed expression of mitochondrial proteins in DRG neurons derived from STZ-induced diabetic rats cultured in the presence of 25mM D-glucose. Mitochondrial gene expression of subunits of Complexes II, IV and V was up-regulated within 24h of exposure to 10 nM insulin (Figure 4). This stimulatory effect of insulin was also observed in a complementary experiment where DRGs were derived from age-matched control and diabetic rats at the same time, cultured, and the diabetic culture was treated with/without insulin for 24h (Supplemental Figure 1). The same dose of insulin enhanced respiration in cultured sensory neurons derived from diabetic rats (Figure 5A) with

both basal and maximal respiration significantly (P<0.001 and 0.01 respectively) increased at 6h, but not 2h (Figure 5B), indicating a delayed response compared to cells from control rats (Figure 3B).

Insulin therapy prevents thermal hypoalgesia and partially corrects dermal nerve loss in diabetic rats

Type 1 diabetic rats showed significant hyperglycemia, elevated HbA1c and reduced weight gain after 5 months when compared with their age-matched controls (Table 1). Insulin treatment of diabetic rats significantly improved body weight (P<0.001 vs untreated diabetic) but did not alter terminal blood glucose or HbA1c levels (Table 1). STZ-diabetic rats exhibited marked thermal hypoalgesia that was prevented by insulin (Figure 6A). The footpads from untreated diabetic rats exhibited significant (P<0.01) loss of IENF and SNP profiles (Figure 6B, C). Insulin partially attenuated nerve loss, most notably in the SNP, where values did not differ significantly from age-matched control animals.

Decreased mitochondrial respiratory protein expression and activity in DRG of diabetic animals is corrected by insulin

DRG derived from age-matched control, diabetic, and insulin-implanted diabetic rats were homogenized and subjected to mRNA and protein expression analysis. Western blotting showed a significant decreased expression in subunits of mitochondrial Complexes II, IV and V proteins in DRG of diabetic rats compared with age matched controls (Figure 7A, B). In insulin-implanted animals, levels of Complex IV and V proteins in DRG were raised compared with untreated diabetic and were not significantly different from age-matched control - except for Complex II protein where the increase was less dramatic (Figure 7A, B). Similar findings were seen when the data was normalized to the mitochondrial protein, porin (Supplemental Figure 2). Insulin also significantly up-regulated mRNA for the MTCO1 subunit of Complex IV compared to both control (P<0.001) and diabetic (P<0.01) rats (Figure 8A, Supplemental Figure 3). Complex II-SDHB mRNA levels were not different between groups. Enzymatic activity of cytochrome *c* oxidase was significantly (P<0.001) reduced in the DRG of diabetic animals relative to controls and this decrease was partially prevented by insulin treatment (Figure 8B and Supplemental Figure 4).

Discussion

Insulin signaling was identified in sensory neurons of adult rat by detection of Akt and P70S6K activation, confirming earlier work (Grote, et al., 2013; Huang, et al., 2005). We used a physiologically relevant concentration (10 nM) of insulin in all cell culture experiments to minimize confounding effects deriving from potential cross-occupation of insulin-like growth factor receptors by excess insulin (Benyoucef et al., 2007; Kleinridders, 2016; Recio-Pinto and Ishii, 1988). Other reported consequences of insulin stimulation of sensory neurons include increased transcription and synthesis of cytoskeletal proteins, such as tubulin and neurofilament (Fernyhough et al., 1989; Wang et al., 1992) that are essential for axonal growth, regeneration and structural stability. Demonstration that exogenous insulin also dose-dependently enhanced neurite outgrowth of sensory neurons is consistent

with prior studies (Fernyhough, et al., 1993; Recio-Pinto et al., 1986) and highlights the direct neurotrophic properties of insulin in sensory neurons.

Studies in liver, muscle and cardiac cells have suggested that insulin can modulate mitochondrial phenotype. For example, *in vitro* studies on the liver of alloxan-induced diabetic rats and de-pancreatized cats revealed depressed mitochondrial oxygen consumption and ATP production that was restored by addition of insulin (Hall et al., 1960). More recently, a 20min exposure of skeletal muscle myotubes to 10 and 100 nM insulin increased respiratory control ratio and coupling efficiency of oxidative phosphorylation (Nisr and Affourtit, 2014). The effects of insulin are also apparent *in vivo*. Acute infusion of insulin in healthy humans resulted in higher ATP production, increased mitochondrial proteins including MT-CO1 and NADH dehydrogenase subunit IV at mRNA and protein levels, and elevated citrate synthase and COX enzymatic activities as measured in biopsies of skeletal muscle cells (Stump et al., 2003). Conversely, knockout of insulin receptor or PI3-K signaling in mouse myocardial tissue triggered maladaptive responses in mitochondria including reduced expression of genes and proteins related to oxidative phosphorylation (Boudina et al., 2009; O'Neill et al., 2007). We have now extended these studies to adult sensory neurons by demonstrating that a physiologic concentration of insulin raised the expression of mitochondrial electron transport chain proteins and enhanced respiration *in vitro*. Moreover, exogenous insulin had similar effects on sensory neurons derived from diabetic rats and maintained under hyperglycemic conditions, although the time course of OCR enhancement was delayed. This delay could perhaps reflect the inhibitory effect of hyperglycemia, as we have previously shown that diabetes, via hyperglycemia mediated nutrient excess, down-regulates expression of mitochondrial proteins and respiration (Chowdhury et al., 2011). Insulin appears to circumvent the effects of hyperglycemia without lowering glucose levels *per se*.

It is well established that insulin triggers transcription and translation within cells via both the PI3-K/Akt and MAPK/ERK pathways (Cheng et al., 2010). AMPK is a sensor of energy status that augments mitochondrial function and adaptability (Hardie et al., 2012). Insulin signaling via Akt down-regulates this activity in cardiac tissue (Gamble and Lopaschuk, 1997), in part, through phosphorylation on the ST loop of AMPKα at Ser 485/491 (in rats) (Hawley et al., 2014; Valentine et al., 2014). Insulin interaction with the AMPK pathway is multifaceted (Towler and Hardie, 2007) and adipocytes maintained under conditions of ongoing fatty acid metabolism, insulin can activate AMPK over a period of 40 min via elevation in the AMP/ATP ratio (Hebbachi and Saggerson, 2012; Liu et al., 2010). In hippocampal neurons, compounds that activate the insulin receptor also mobilize the AMPK-SIRT signaling axis with associated enhancement of mitochondrial function (Barhwal et al., 2015). Insulin can also boost intracellular Ca^{2+} ion concentration (Contreras-Ferrat et al., 2014) which could trigger activation of an upstream activator of AMPK, namely Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ) (Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005). Thus, insulin signaling and coordinated activation of AMPK could augment mitochondrial function in sensory neurons. Insulin signaling via the Akt/IKK/NF-κB (Mauro et al., 2011; Saleh et al., 2013; Salminen and Kaarniranta, 2010) and the PI3-K pathway (by-passing Akt involvement) (O'Neill, et al.,

2007) could also regulate mitochondrial function. The putative involvement of all these pathways requires further study.

We have previously reported that by 22 weeks of type 1 diabetes, rat sensory neurons showed impaired mitochondrial respiration, reduced mitochondrial Complexes I and IV enzymatic activities and reductions in expression of selective mitochondrial respiratory chain proteins compared with controls (Chowdhury, et al., 2010). Insulin therapy in a subgroup of STZ-diabetic rats for the final 4 weeks improved mitochondrial respiration but also that corrected hyperglycemia so that it was not feasible to determine whether the effect was due to insulin per se or secondary to glycemic control. Our present findings show that protein expression of subunits of mitochondrial Complexes II, IV and V and activity of Complex IV were recovered in insulin-implanted diabetic animals without correcting the hyperglycemic state. This supports actions of insulin that are not mediated by glycemic control.

Down-regulation of specific mitochondrial OXPHOS proteins and Complex activities in diabetic conditions has been demonstrated by other investigators. Skeletal muscle biopsies from type 2 diabetic patients showed a significant reduction in mRNA levels of multiple subunits of Complexes I, II, III, IV and V proteins (Patti et al., 2003). Protein levels of OXPHOS subunits (I, II, III, and IV) were significantly repressed in cardiac mitochondria of $Ins2^{+/-}$ Akita mice (C57BL/6) compared to control mice (Bugger et al., 2009). In kidney cortex in mouse models of type 1 and type 2 diabetes components of Complexes I–IV were suppressed as well as activity of Complex IV (Dugan et al., 2013; Sas, et al., 2016). Reduced expression of NADH dehydrogenase Fe-S protein 3 (Complex I-NDUFS3) and MT-CO1 (Complex IV) together with the enzymatic activities of NADH-cytochrome c reductase (Complex I) and cytochrome c oxidase (Complex IV) in STZ-diabetic rodent DRGs vs. control has been reported in our previous studies (Akude et al., 2011; Chowdhury, et al., 2010; Roy Chowdhury, et al., 2012). In the current study we had significant down-regulation of Complex IV subunits and activity. It can be inferred from all these studies that mitochondrial OXPHOS protein expression, especially within Complex IV and its activity, is depressed under diabetic conditions. Finally, to illustrate the complexity of interactions in diabetes in the nervous system proteomic studies of distal sciatic nerve of type 1 diabetic rats reveal elevations in specific components of the mitochondrial electron transport chain (Freeman, et al., 2016).

Studies with a variety of neurotrophic growth factors reveal that mitochondrial function is under the control of an array of signal transduction pathways. For example, in adult sensory neurons IL-1β, IL-17A and ciliary neurotrophic factor all enhance neurite outgrowth in hand with augmentation of mitochondrial function (Habash et al., 2015; Saleh et al., 2013; Saleh, et al., 2013). These growth factors utilize multiple signal transduction pathways, include NF-kB and JAK/STAT, to directly stimulate mitochondrial function. NT3 most likely signals through Akt to modulate mitochondrial polarization in sensory neurons from control or STZ-diabetic rats (Huang, et al., 2005). Insulin and IGF-1 share very similar transduction pathways, PI3K/Akt and MAPK, which could lead to protein synthesis and transcription initiation, respectively. IGF-1 possibly by activating these two main transduction pathways promotes growth and impedes mitochondrial aberration and cell death mediated by caspase

activation in SH-SY5Y cell cultures exposed to high glucose concentration (Leinninger et al., 2004). For the first time insulin can be added to this group of neurotrophic proteins. Insulin has previously been demonstrated to modulate mitochondrial membrane potential (Huang, et al., 2003; Huang, et al., 2005) and the current study reveals that insulin has the potential to control mitochondrial function via up-regulation of mitochondrial electron transport system protein expression and Complex activity. It is possible that insulin is optimizing responses to neurotrophic factors. For example, insulin was shown to up-regulate high affinity NGF binding in the human neuroblastoma SH-SY5Y neuronal cell line (Recio-Pinto et al., 1984). All experiments in the present were performed in the absence of exogenous neurotrophic factors (no exogenous growth factors were added to the culture) so this eventuality seems less likely. However, we cannot exclude the possibility that the cultured neurons may be secreting neurotrophins such as BDNF (Acheson et al., 1995; Tonra et al., 1998) whose function may be augmented by insulin via enhanced $p75^{NTR}$ and/or trkB interaction. Further studies will dissect the potential for direct vs indirect actions of insulin on mitochondrial function.

Loss of background levels of systemic insulin and insulin-like growth factors has long been suspected of contributing to the development of diabetic neuropathy (Ishii, 1995) and recent studies have reported that insulin can activate several signal transduction pathways to enhance nerve repair in diabetes (Grote, et al., 2013; Zochodne, 2016). The present study supports this concept and demonstrates that insulin modulates mitochondrial function and thus may contribute to nerve repair. As all in vitro studies were performed with 10 nM insulin, we are confident that signal transduction via the insulin receptor accounts for the positive effects on mitochondrial function. Interpretation of the in vivo data is not so straightforward. Given that insulin can cross-occupy IGF type 1 receptors at concentrations above 30 nM (Recio-Pinto and Ishii, 1988) it cannot yet be discounted that some of the in vivo efficacy of insulin is a consequence of stimulation of IGF receptor signaling. Ongoing studies are addressing this issue (manuscript in preparation). Future work will attempt to identify the signal transduction pathway activated by IGF-1 to modulate mitochondrial function, for example Akt vs AMPK. It will be interesting to see if this pathway is shared with NT-3, cytokines or IGF-1 and a major goal will be to characterize key components of the mitochondrial respiratory chain, e.g. proteins such as MTCO1 within Complex IV, and sites of regulation that may be commonly modulated by these pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Insulin promoted mitochondrial gene expression/function in DRG neuron cultures
- Insulin raised spare respiratory capacity in mitochondria of cultured neurons
- In vitro expression of proteins of the respiratory chain were up-regulated by insulin
- Insulin implants partially or completely prevented indices of neuropathy in type 1 diabetic rats
- In diabetic rats mitochondrial protein mRNA/protein in DRG were normalized by insulin
- Insulin up-regulated respiratory chain activity in DRG of diabetic rats

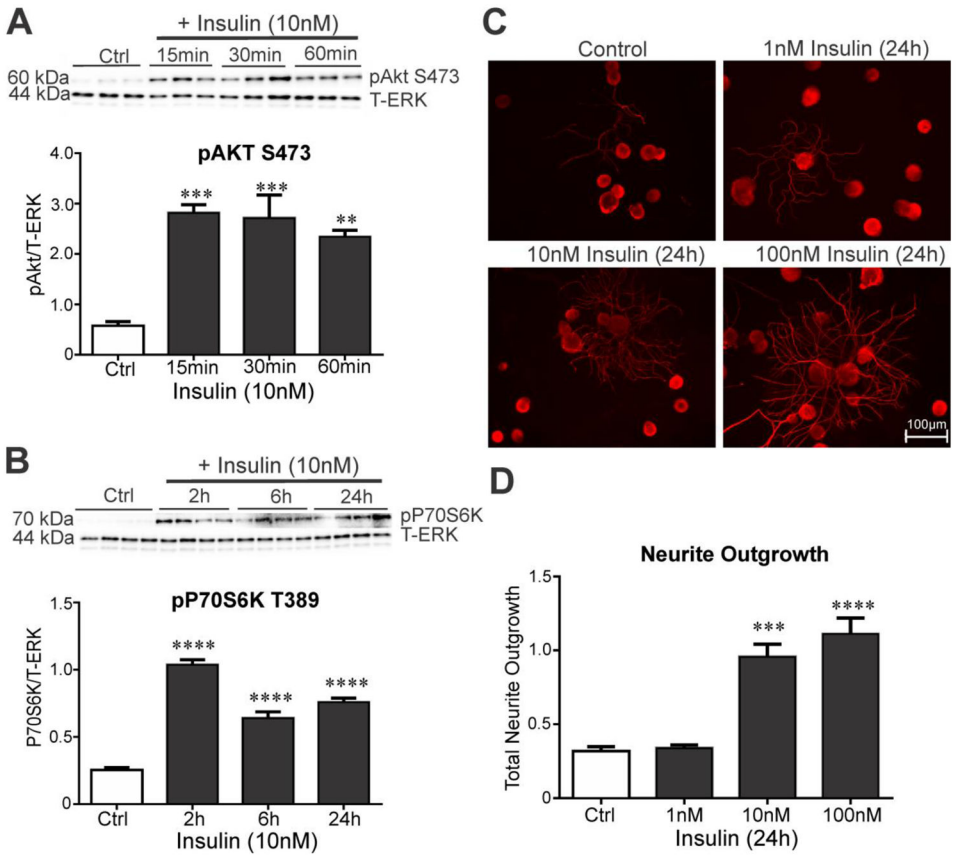

Figure 1. Insulin treatment increases the expression of pAkt, pP70S6K and elevates neurite outgrowth in DRG neurons

DRG neurons derived from adult control rats were grown in the absence of neurotrophic factors and under defined conditions then treated with/without 10 nM insulin for (A) 15–60min or (B) 2–24h. Lysates were collected and subjected to Western blotting. In (C,D) DRG neuron cultures from adult control rats were treated with/without 1 nM, 10 nM and 100 nM insulin for 24h then fixed and immunostained for β-tubulin III. In (A,B) Western blot data were normalized to T-ERK. In (D) total neurite outgrowth data is presented relative to neuron number. Data are mean ± SEM of N=3–4 replicates; **= $p<0.01$ or ***= $p<0.001$ or ****= $p<0.0001$ vs ctrl by one-way ANOVA with Dunnett's *post-hoc* test.

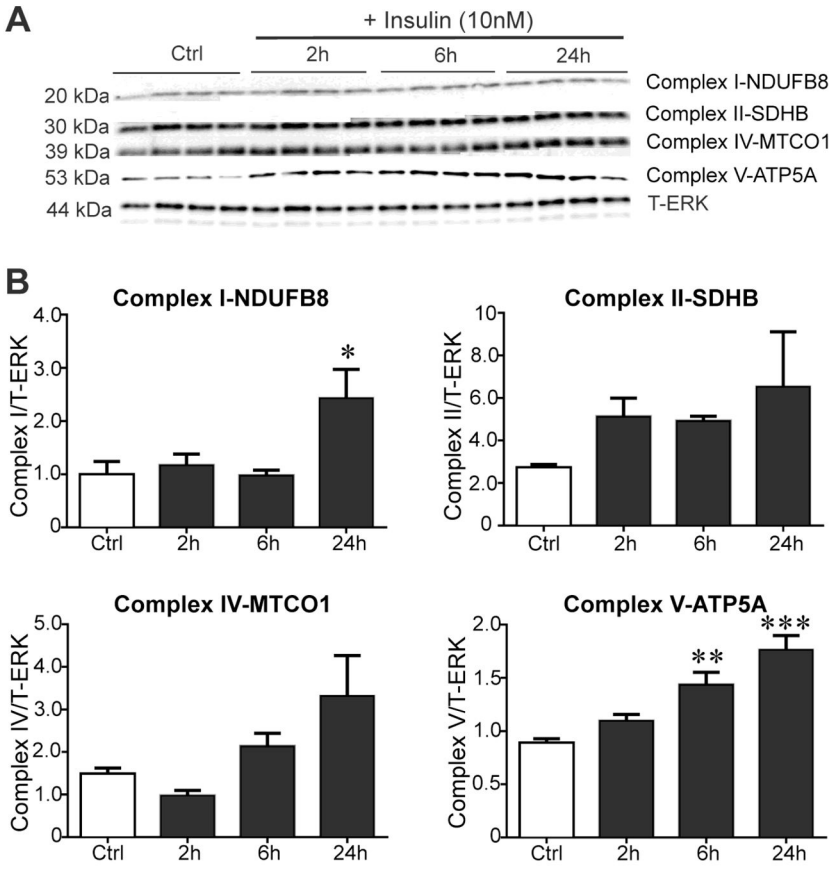

Figure 2. Insulin treatment increases the expression of electron transport chain proteins

DRG neurons derived from adult control rats were treated with/without 10 nM insulin for 2–24 h and lysates subjected to Western blotting. Specific proteins from each respiratory Complex were quantified and expressed relative to T-ERK. Complex III-UQCRC2 subunit protein was not detectable. Data are mean ± SEM of N=4 replicates; *= $p<0.05$ or **= $p<0.01$ or ***= $p<0.001$ or ****= $p<0.0001$ vs ctrl by one-way ANOVA with Dunnett's *post-hoc* test.

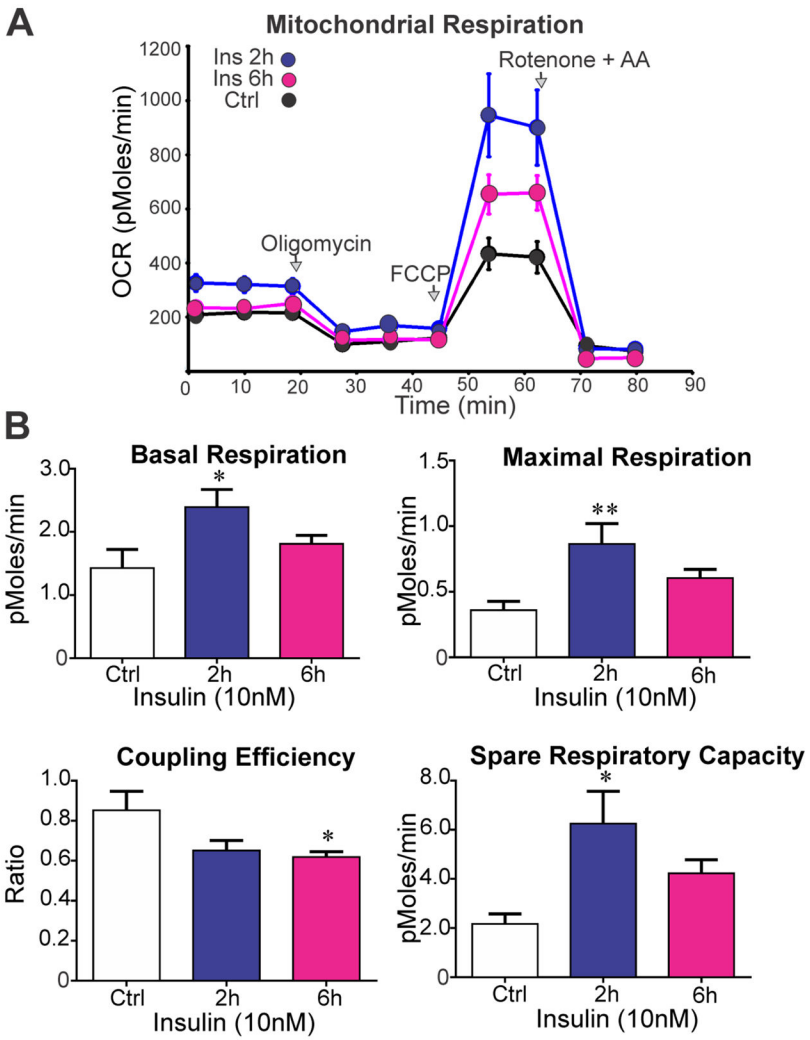

Figure 3. Insulin enhances mitochondrial respiration in cultured adult sensory neurons

DRG neurons derived from adult control rats were maintained overnight and then were treated with/without 10 nM insulin for 2 and 6 h. The culture plate was then inserted into the Seahorse XF24 Analyzer and oligomycin, FCCP and rotenone+AA (antimycin A) added sequentially. Data were normalized to cell number per well derived from immunocytochemical counting using the Seahorse software prior to statistical analysis. Data are presented as OCR in pmoles/min per 5000 cells. Data are mean ± SEM of N=5 replicates; *= $p<0.05$ or **= $p<0.01$ vs ctrl by one-way ANOVA with Dunnett's *post-hoc* test.

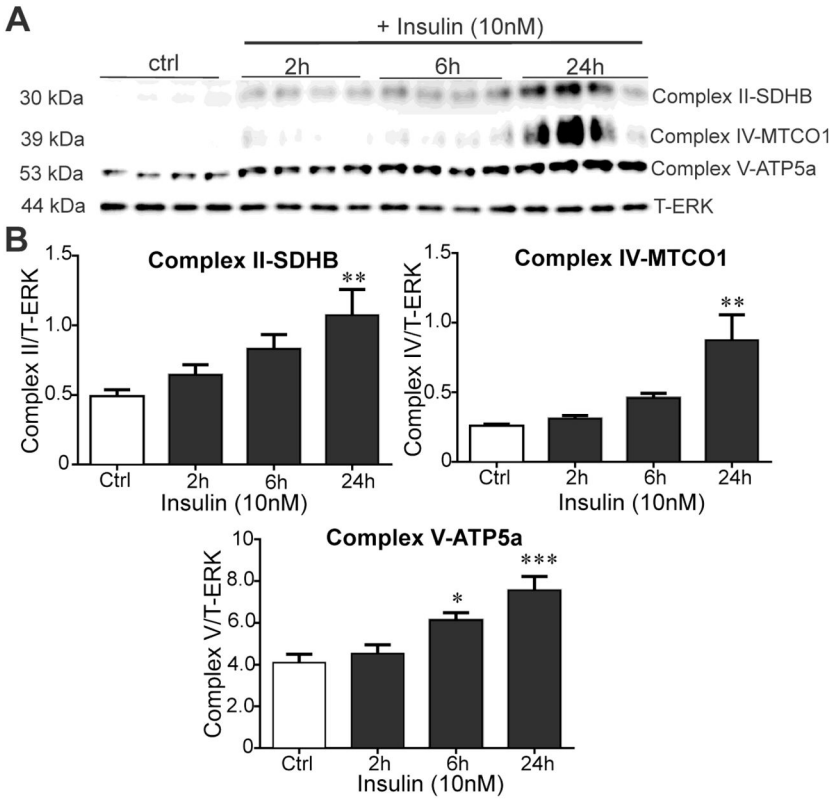

Figure 4. Insulin treatment increases the expression of electron transport chain proteins in DRG cultures from a diabetic rat

DRG neurons derived from adult STZ-diabetic rats were cultured in the presence of 25mM glucose, and treated with/without 10 nM insulin for 2–24h. Complex I and III subunit proteins were not detectable. Protein band intensity was normalized to T-ERK. Data are mean ± SEM of N=4 replicates; *= $p<0.05$ or **= $p<0.01$ or ***= $p<0.001$ vs ctrl by one-way ANOVA with Dunnett's *post-hoc* test.

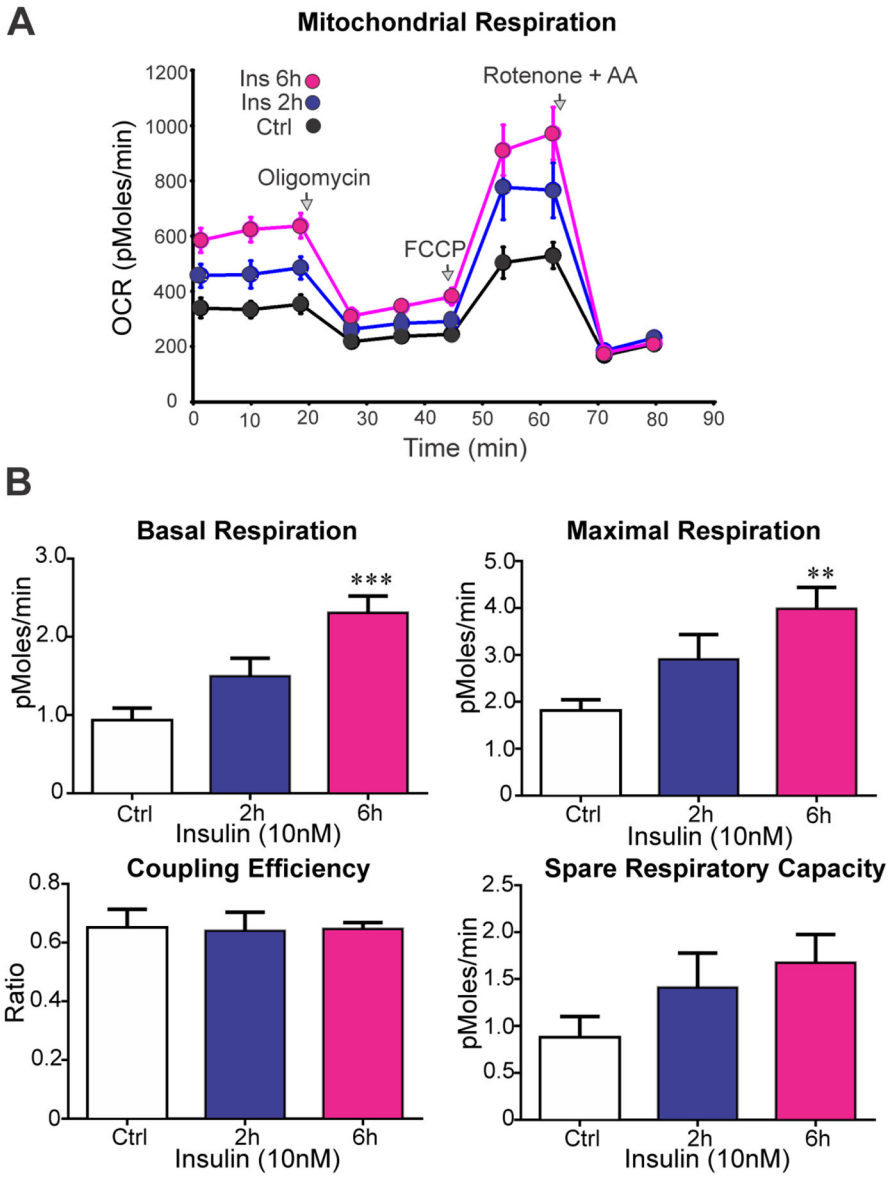

Figure 5. Insulin enhances mitochondrial respiration in cultured DRG neurons from a diabetic rat

DRG neurons derived from adult STZ-diabetic rats were cultured in the presence of 25mM glucose, and treated with/without 10 nM insulin for 2 and 6 h. Data were normalized to cell number using Seahorse software prior to statistical analysis. Data are presented as OCR in pmole/min per 5000 cells. Data are mean ± SEM of N=5 replicates; **= $p<0.01$ or ***= $p<0.001$ vs ctrl by one-way ANOVA with Dunnett's *post-hoc* test.

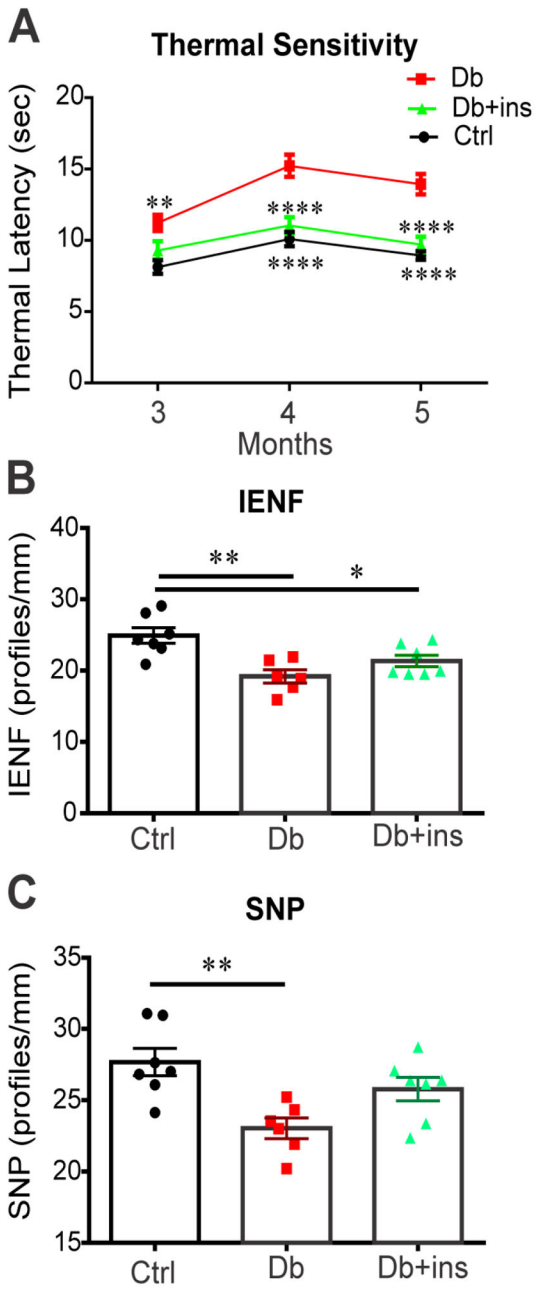

Figure 6. Insulin implants prevented thermal hypoalgesia and sub-epidermal neural plexus (SNP) loss in diabetic rats

Three groups of animals: control (Ctrl), diabetic (Db) and insulin-implanted diabetic (Db +ins) rats were tested for their thermal response latency (A). **= $p<0.01$ vs. control and ****= $p<0.0001$ vs. diabetic; analyzed by two-way ANOVA with Tukey's *post-hoc* test. IENF (B) and SNP (C) profiles in plantar hind paw skin (from the three groups of animals which were maintained for 5 months) were also measured; *= $p<0.05$ or **= $p<0.01$ or ****= $p<0.0001$; analyzed by one-way ANOVA with Tukey's *post-hoc* test. Data are mean ± SEM of N=6–7 animals.

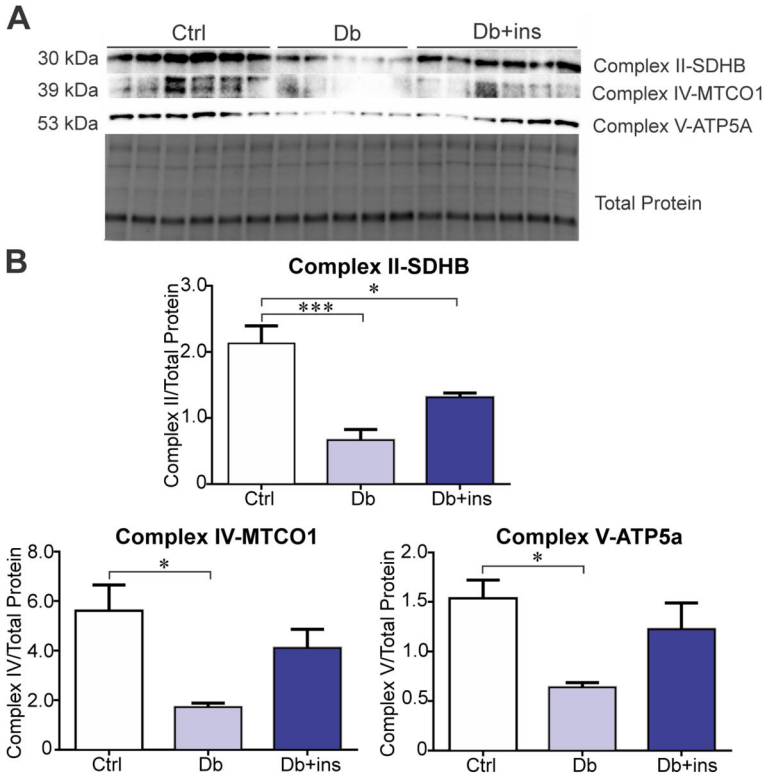

Figure 7. Insulin corrects deficits in mitochondrial protein expression in DRG of diabetic animals

DRG tissues from control (Ctrl), diabetic (Db) and insulin-implanted diabetic (Db+ins) rats were isolated and subjected to Western blotting. Complex I and III subunit proteins were not detectable. Western blot band intensity of these proteins was normalized to total protein bands of the same blot. Data are mean ± SEM of N=5–6 animals; *= $p<0.05$ or ***= $p<0.001$ analyzed by one-way ANOVA with Tukey's *post-hoc* test.

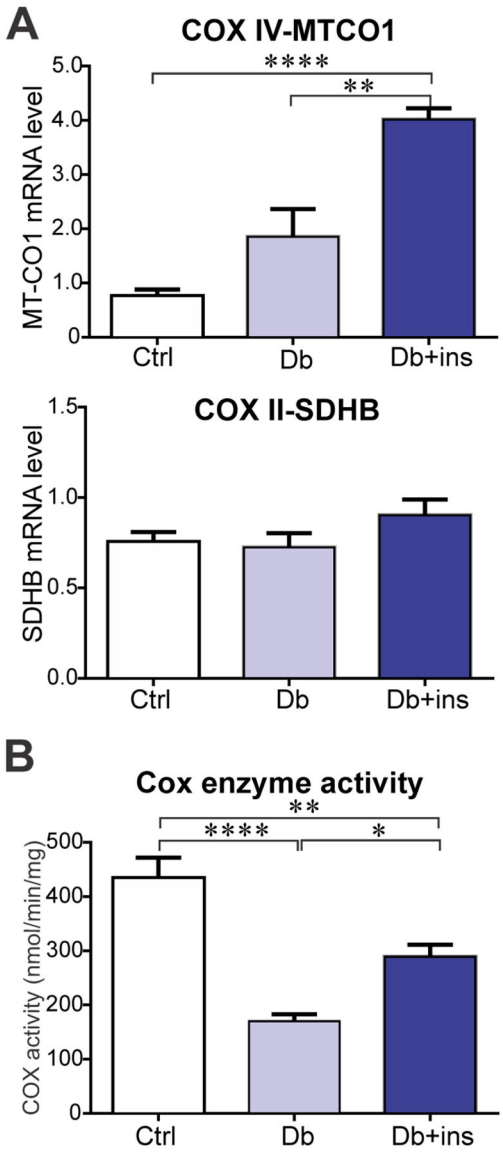

Figure 8. Increased MT-CO1 mRNA expression and restored cytochrome c oxidase (Cox) activity in insulin-implanted diabetic rats

A) DRG tissues from control (Ctrl), diabetic (Db) and insulin-implanted diabetic (Db+ins) rats were used for Real-Time PCR assay. Complex II-SDHB and Complex IV-MT-CO1 mRNA levels were calculated relative to porin mRNA levels using delta dela Ct method. Data are mean ± SEM of N=5–6 animals (duplicate test tubes). B)Tissues were used for cytochrome *c* oxidase enzymatic activity assay. Data are in nmol/mim/mg tissue and are mean ± SEM of N=5 animals. *= $p<0.05$, **= $p<0.01$ and ****= $p<0.0001$; analyzed by one-way ANOVA with Tukey's *post-hoc* test.

Table 1

Insulin-treated diabetic rats exhibited no change in their blood glucose and HbA1c compared to untreated diabetic rats.

Group	N	Body weight (g)	Blood glucose (mmol/L)	HbA1c (%)
Control	**6**	725.6 ± 21.0*	8.1 ± 0.6	5.1 ± 0.1
Diabetic	**6**	395.2 ± 22.0	38.9 ± 2.1*	11.3 ± 0.7*
Insulin-implanted diabetic	**6**	589.6 ± 14.3*¶	35.5 ± 1.7*	12.7 ± 0.3*

Animals were maintained for 5 months. Blood glucose, HbA1C and body weight were recorded at tissue collection. Data are mean ± SEM of N=6; ¶= $p<0.001$ vs. control and *= $p<0.0001$ vs. other groups by one-way ANOVA with Tukey's *post-hoc* test.