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Research Paper

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 (10 nM) significantly ($P < 0.05$) 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 and function in sensory neurons and this is associated with enhanced neurite outgrowth and protection against indices of neuropathy.

1. Introduction

Peripheral 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

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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.

2. Materials and methods

2.1. 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 6 weeks 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.

2.2. 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.

2.3. 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 water at 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 h period.

2.4. 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 (2–5 μg 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), 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 Figs. 2, 4 and 7, and Supplemental Figs. 1 and 2 were obtained from one single blot with different exposure times.

2.5. 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 according to the manufacturer's instructions. Real-time quantitative RT-PCR was performed by using iQ™ SYBR® Green Supermix with an iQ5 cycler system (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′-ATCTGCAATCCA TCGAGGACC-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′.

2.6. Mitochondrial respiration in cultured neurons

To measure the basal level of mitochondrial oxygen consumption,