Impaired adenosine monophosphate-activated protein kinase signalling in dorsal root ganglia neurons is linked to mitochondrial dysfunction and peripheral neuropathy in diabetes

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Mitochondrial dysfunction occurs in sensory neurons and may contribute to distal axonopathy in animal models of diabetic neuropathy. The adenosine monophosphate-activated protein kinase and peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) signalling axis senses the metabolic demands of cells and regulates mitochondrial function. Studies in muscle, liver and cardiac tissues have shown that the activity of adenosine monophosphate-activated protein kinase and PGC-1α is decreased under hyperglycaemia. In this study, we tested the hypothesis that deficits in adenosine monophosphate-activated protein kinase/PGC-1α signalling in sensory neurons underlie impaired axonal plasticity, suboptimal mitochondrial function and development of neuropathy in rodent models of type 1 and type 2 diabetes. Phosphorylation and expression of adenosine monophosphate-activated protein kinase/PGC-1α signalling in sensory neurons underlie impaired axonal plasticity, suboptimal mitochondrial function and development of neuropathy in rodent models of type 1 and type 2 diabetes. Phosphorylation and expression of adenosine monophosphate-activated protein kinase/PGC-1α and mitochondrial respiratory chain complex proteins were down-regulated in dorsal root ganglia of both streptozotocin-diabetic rats and db/db mice. Adenoviral-mediated manipulation of endogenous adenosine monophosphate-activated protein kinase activity using mutant proteins modulated neurotrophin-directed neurite outgrowth in cultures of sensory neurons derived from adult rats. Addition of resveratrol to cultures of sensory neurons derived from rats after 3–5 months of streptozotocin-induced diabetes, significantly elevated adenosine monophosphate-activated protein kinase levels, enhanced neurite outgrowth and normalized mitochondrial inner membrane polarization in axons. The bioenergetics profile (maximal oxygen consumption rate, coupling efficiency, respiratory control ratio and spare respiratory capacity) was aberrant in cultured sensory neurons from streptozotocin-diabetic rats and was corrected by resveratrol treatment. Finally, resveratrol treatment for the last 2 months of a 5-month period of diabetes reversed thermal hypoalgesia and attenuated foot skin intraepidermal nerve fibre loss and reduced myelinated fibre mean axonal calibre in streptozotocin-diabetic rats. These data suggest that the development of distal axonopathy in diabetic neuropathy is linked to nutrient excess and mitochondrial dysfunction via defective signalling of the adenosine monophosphate-activated protein kinase/PGC-1α pathway.
Introduction

Structural changes in peripheral nerve of patients with diabetic neuropathy include microangiopathy, segmental demyelination and Wallerian degeneration that lead to loss of both myelinated and unmyelinated fibres (Yagihashi, 1996; Malik et al., 2005; Said, 2007). In recent years, the retraction of distal axons of sensory neurons located in the epidermis and characterized as intraepidermal nerve fibres has been developed as a minimally invasive means of assessing early stages of peripheral neuropathy in diabetes and other neurodegenerative diseases (Kennedy et al., 1996; Yagihashi, 1996; Malik et al., 2005; Quattrini et al., 2007; Ebenezer et al., 2011). Animal models of diabetic neuropathy reveal similar intraepidermal nerve fibre pathology and in common with humans, exhibit concurrent sensory disorders such as thermal hypoalgesia and tactile allodynia (Christianson et al., 2003, 2007; Mizisin et al., 2008; Jolivalt et al., 2008; Francis et al., 2009). While many mechanisms downstream of impaired insulin signalling and/or hyperglycaemia have been proposed as contributing to nerve dysfunction in diabetes (reviewed in Calcutt et al., 2009), how metabolic disorders lead to structural pathology remains unresolved.

Mitochondrial dysfunction has been proposed as a central mediator of development of many diabetic complications (Nishikawa et al., 2000; reviewed by Sivitz and Yorek, 2010) and there is accumulating evidence that neuronal mitochondria are impaired. For example, the inner membrane potential of mitochondria in sensory neuron cultures derived from streptozotocin-diabetic rats is depolarized (Srinivasan et al., 2000; Huang et al., 2003, 2005a, b; Akude et al., 2011) and lumbar dorsal root ganglia (DRG) from diabetic rats exhibit reduced respiratory chain activity that correlates with the downregulation of select proteins within the respiratory chain complexes (Chowdhury et al., 2010; Akude et al., 2011). Impaired mitochondrial function in the nerves of diabetic animals may therefore lead to the apparent paradox of cellular energy depletion despite elevated availability of glucose substrate.

Studies in muscle, liver and cardiac tissues have shown that mitochondrial performance in the face of fluctuating metabolic demands on cells is regulated by the adenosine monophosphate-activated protein kinase (AMPK), silent information regulator T1 (SIRT1) and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) signalling axis. Thus, under nutrient starvation, this pathway is activated to enhance ATP production (Puigserver et al., 1998; Feige and Auwerx, 2007) by direct binding of AMP to AMPK upon a rise in the cellular AMP/ATP ratio (Hardie, 2008). Mice expressing a dominant-negative form of AMPK did not increase mitochondrial biogenesis in response to energy deprivation in skeletal muscle (Zong et al., 2002). Once activated, AMPK switches on catabolic pathways to produce ATP while simultaneously shutting down energy-consuming anabolic processes. This is achieved, in part, via activation of PGC-1α whose activity is regulated both by its expression level and by post-translational modifications involving AMPK-directed phosphorylation and sirtuin-regulated deacetylation (Rodgers et al., 2008). The latter provides a positive feedback loop in which SIRT1 elevates AMPK activity through deacetylation of liver kinase B1 (LKB1), the upstream kinase that phosphorylates and activates AMPK (Lan et al., 2008), and AMPK activity enhances SIRT1 activity via elevation of NAD⁺ (Bao and Sack, 2010).

There is a strong overlap in the genes transcriptionally regulated by AMPK and those by PGC-1α, suggesting that PGC-1α may be an important mediator of AMPK-induced gene expression linked to mitochondrial respiratory chain function and glucose metabolism (Feige and Auwerx, 2007; Jager et al., 2007). PGC-1α is a potent coactivator of a plethora of transcription factors that influence whole-body energy expenditure, including myocyte enhancer factor-2, forkhead O-box (FOXO) transcription factors and nuclear receptors such as peroxisome proliferator-activated receptors and oestrogen-related receptors (Vega et al., 2000; Michael et al., 2001; Puigserver et al., 2003; Feige and Auwerx, 2007). PGC-1α consequently co-ordinately regulates gluconeogenesis, glycolysis, lipogenesis, peroxisomal and mitochondrial fatty acid oxidation, and mitochondrial respiratory efficiency. Over-expression of PGC-1α increases mitochondrial number and function (Lin et al., 2002). Conversely, deletion of PGC-1α results in abnormal glucose homeostasis and decreased mitochondrial function (Handschin et al., 2007).

High glucose concentration and/or nutrient excess leads to downregulation of AMPK activity in several cell types (da Silva Xavier et al., 2003; Mountjoy and Rutter, 2007; Eid et al., 2010). Decreased PGC-1α levels have been reported in skeletal muscle from insulin-resistant and type 2 diabetes mellitus patients (Mootha et al., 2003; Patti et al., 2003; Richardson et al., 2005). However, activity of the AMPK/SIRT1/PGC-1α signalling axis has not been widely studied in neurons. The naturally occurring polyphenol resveratrol offers an interesting tool for testing the consequences of manipulating this pathway in neurons as it augments AMPK phosphorylation and promotes axon outgrowth in neuronal cell cultures (Dasgupta and Milbrandt, 2007). Moreover, systemic treatment with resveratrol improved some indices of neuropathy in type 1 diabetic rats (Kumar et al., 2007; Sharma et al., 2009), but its mechanism of action and impact on neuronal structure remain unknown. Given the incomplete understanding of the effect of diabetes on the AMPK/PGC-1α pathway and mitochondrial function in the PNS, we have determined the impact of diabetes on this pathway in DRG sensory neurons, studied the mechanistic role of AMPK in neurite outgrowth, performed a detailed bioenergetics analysis of sensory neurons in streptozotocin-diabetic rodents and characterized the ability of resveratrol to protect from diabetic neuropathy.
Materials and methods

Induction, treatment and confirmation of diabetes

Male Sprague-Dawley rats (275–325 g) and Swiss Webster mice (25–30 g) were used as models of type 1 diabetes after delivery of a single intraperitoneal injection of 75–85 mg/kg or two injections of 90 mg/kg streptozotocin (Sigma), respectively. Db/db mice, a model of type 2 diabetes, became diabetic spontaneously at 4–6 weeks of age and remained diabetic for 30 weeks. Resveratrol (3,5,4′-trihydroxy-trans-stilbene; Sigma) was administered by oral gavage daily at 5 mg/kg to a subgroup of streptozotocin-diabetic rats after 9 weeks of diabetes and given for a further 9 weeks. Animals were euthanized and tissue collected after 4, 14, 18, 22 or 30 weeks of diabetes. Non-fasting blood glucose concentration was measured using the Alphatrak glucometer (Abbott). Animal procedures followed guidelines laid down by the University of Manitoba Animal Care Committee using the Canadian Council of Animal Care guidelines.

Adult rat dorsal root ganglia sensory neuron culture

Sensory neurons were isolated and dissociated from the DRG of adult male Sprague-Dawley rats as described (Huang et al., 2003; Zherebetskaya et al., 2009). Cells were plated onto poly-d-L-ornithine hydrobromide and laminin-coated 25 mm glass cover slips for microscopy studies (Electron Microscopy Sciences), NUNC plastic tissue culture multi-well dishes for immunoblot analysis or custom 24-well plastic dishes for assessing mitochondrial bioenergetics using a Seahorse Biosciences XF24 Analyser. Neurons were grown in defined Hams F-12 medium with N2 additives (no insulin), supplemented with a cocktail of sub-optimal doses of neurotrophic factors: 0.1 ng/ml nerve growth factor, 1.0 ng/ml glial cell line-derived neurotrophic factor, 0.1 ng/ml neurotrophin-3 and 0.1 nM insulin (all obtained from Sigma). In all studies neurons from control rats were cultured in the presence of 10 mM α-glucose and 10 nM insulin and neurons from diabetic rats were maintained in medium containing 25 mM α-glucose.

Viral transduction of AMP-activated protein kinase mutants in cultured sensory neurons

Adult sensory neurons from control or diabetic rats maintained in the presence of neurotrophic factors as described above were infected with adenovirus carrying dominant negative mutants of AMPK α1- or α2-subunits (DN1 or DN2) or constitutively active AMPK (ad-AMPK-CA), respectively. The ad-AMPK-CA and dominant-negative adenoviral constructs were delivered at 20 pfu/cell and the control adenoviral construct was delivered at 10 pfu/cell. Cultures were allowed to attach/grow for 1 day, incubated with adenovirus for 3 h and the media was changed. Neurite outgrowth was determined 48 h after infection. The constructs were kind gifts from Dr Jason Dyck, University of Alberta, Canada (Jacobs et al., 2007).

Quantification of neurite outgrowth

For assessing neurite outgrowth, the cultures were either imaged live for green fluorescent protein (GFP) fluorescence (for adenovirus infected cells) or fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 15 min at room temperature then permeabilized with 0.3% Triton X-100 in phosphate-buffered saline for 5 min. Fixed cells were then incubated in blocking buffer (Roche) diluted with foetal bovine serum and 1.0 mM phosphate-buffered saline (1:1:3) for 1 h and rinsed three times with phosphate-buffered saline. Primary antibody used was against neuron-specific β-tubulin isotype III (1:1000; from Sigma Aldrich). Plates were incubated at 4°C overnight. The following day, the cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody (Jackson Immunoresearch Laboratories) for 1 h at room temperature. Slides were mounted and imaged using a Carl Zeiss axioskope-2 upright fluorescence microscope equipped with an AxioCam camera. Images were captured with Axiovision software. For live cell fluorescence imaging for GFP a Carl Zeiss LSM510 confocal microscope was used and bright field images collected simultaneously. For both approaches, fluorescent images were captured and the mean pixel area as a measure of axon outgrowth determined using ImageJ software (adjusted for the cell body signal). All values for total neurite outgrowth were adjusted for neuronal number. In this culture system the level of total neurite outgrowth has been previously validated to be directly related to an arborizing form of axonal plasticity and homologous with in vivo collateral sprouting (Smith and Skene, 1997).

Western blotting for AMP-activated protein kinase and PGC-1α

Lumbar DRG from mice or rats were rinsed in ice-cold solution containing sucrase-Tris–EDTA buffer (250 mM sucrose, 10 mM Tris–HCl, 1 mM EDTA pH 7.4), then homogenized with a polytron homogenizer (IKA PowerGen 125, Fisher Scientific Limited) using 4 × 7.5 s grinding pulses at 30 s intervals. Cultured sensory neurons were harvested after 2 h of treatment and then homogenized in ice-cold stabilization buffer containing: 0.1 M PIPES, 5 mM MgCl₂, 5 mM EGTA, 0.5% Triton X-100, 20% glycerol, 10 mM NaF, 1 mM PMSE and protease inhibitor cocktail (Fernyhough et al., 1999). The protein content was measured according to the method of Bradford (1976). DRG homogenate or lysate from DRG cell culture (7.5–10 μg) were resolved on a 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis gel [6% for phosphorylated acetyl coenzyme A (acytely CoA) carboxylase] and electroblotted onto nitrocellulose membrane. Blots were then blocked in 5% non-fat milk containing 0.05% Tween-20, rinsed in phosphate-buffered saline (pH 7.4) and incubated with antibodies to the following proteins: phosphorylated AMPK (on Thr 172, P-AMPK; 1:500, Santa Cruz Biotechnology Inc.; Cell Signalling Technology), total AMPK (T-AMPK; 1:500, Cell Signalling Technology), PGC-1α (1:500, Santa Cruz Biotechnology), phosphorylated acetyl CoA carboxylase (P-ACC; 1:2000, Abcam), cytochrome c oxidase subunit IV (COX IV; 1:1000, Mitosciences), NADH dehydrogenase (ubiquinone) iron-sulphur protein 3 (NDUFS3; 1:1000, Mitosciences) and ATP synthase β-subunit (1:2000, Mitosciences). Total extracellular regulated protein kinase (T-ERK; 1:2000, Santa Cruz Biotechnology) was probed as a loading control (previous studies have shown that the expression of this protein does not change in DRG from diabetic rats). The blots were rinsed, incubated in Western blotting Luminol Reagent (Santa Cruz Biotechnology) or ECL Advance (GE Healthcare) and imaged using a Bio-Rad Fluor-S image analyser (Bio-Rad).
Determination of enzymatic activities of mitochondrial complexes and citrate synthase

Measurements of enzymatic activities from DRG homogenates were performed using a temperature controlled Ultrospec 2100 UV–visible spectrophotometer equipped with Biochrom Swift II software (Biopharmacia Biotech). Complex I activity was measured as rotenone-sensitive NADH:cytochrome c reductase activity. DRG homogenates were subjected to three freeze-thaw cycles to disrupt mitochondrial membranes and permit access of substrates. Freshly prepared assay buffer (50 mM K-phosphate pH 7.4, 1 mM KCN, 100 µM NADH) and 20 µg protein of DRG were added to the cuvette and preincubated for 3 min at 25°C. After the addition of 100 µM oxidized cytochrome c, the reaction was followed for 2 min at 550 nm. The reduction of oxidized cytochrome c was assayed by an increase in absorbance. The reaction was followed for two more minutes after the addition of 25 µM rotenone to allow calculation of the rotenone-sensitive Complex I activity (Powers et al., 2007). Complex IV activity was measured at 25°C by monitoring the absorbance decrease of reduced cytochrome c at 550 nm. The reaction was started by addition of 40 µM reduced cytochrome c into 50 mM phosphate buffer containing 10 µg of DRG protein solubilized with 0.02% lauryl maltoside (Chowdhury et al., 2007). The activity of the Krebs cycle enzyme, citrate synthase, was determined at 25°C in medium containing 150 mM Tris–HCl (pH 8.2), 0.02% lauryl maltoside, 0.1 mM dithionitrobenzoic acid and 20 µg DRG protein. The reaction was initiated by the addition of 100 µM acetyl CoA and changes in absorbance at 412 nm were measured for 2 min. This value was subtracted from the rate obtained after addition of 0.05 mM oxaloacetic acid (Chowdhury et al., 2005).

Assessment of mitochondrial membrane potential in cultured neurons

Sensory neurons were loaded with 3.0 nM tetramethyl rhodamine methyl ester (TMRM; Molecular probes) for 1 h and the fluorescence signal in the axons detected with a Carl Zeiss LSM510 confocal microscope (100× 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 (Nicholls, 2006). Antimycin A and oligomycin (Sigma) were injected into the culture media to a final concentration of 10 µM and 1 µM, 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 (Zherebitskaya et al., 2009).

Measurement of mitochondrial respiration in cultured dorsal root ganglia from rat

An XF24 Analyser (Seahorse Biosciences) was used to measure bioenergetics function in neurons. The XF24 creates a transient 7 µl chamber in specialized 24-well microplates that allows for oxygen consumption rate to be monitored in real time (Hill et al., 2009). Culture medium was changed 1 h before the assay to unbuffered Dulbecco’s modified Eagle’s medium, (pH 7.4) supplemented with 1 mM pyruvate (Gibco), and 10 mM d-glucose. Neuron density in the range of 2500–5000 cells per well gave a linear oxygen consumption rate. Oligomycin (1 µM). FCCP; carbonyl cyanide4-((trifluoromethoxy)phenylhydrazone (range of 0.1–1.0 µM) and rotenone (1 µM) + antimycin A (1 µM) were injected sequentially through ports in the Seahorse Flux Pak cartridges. Each loop was started with mixing for 3 min, then delayed for 2 min and oxygen consumption rate measured for 3 min. This allowed determination of the basal level of oxygen consumption, the amount of oxygen consumption linked to ATP production, the level of non-ATP-linked oxygen consumption (proton leak), the maximal respiration capacity and the non-mitochondrial oxygen consumption (Hill et al., 2009; Brand and Nicholls, 2011). Oligomycin inhibits the ATP synthase leading to a build-up of the proton gradient that inhibits electron flux and reveals the state of coupling efficiency. Uncoupling of the respiratory chain by FCCP injection reveals the maximal capacity to reduce oxygen. Finally, rotenone + antimycin A were injected to inhibit the flux of electrons through complexes I and III, and thus no oxygen was further consumed at cytochrome c oxidase. The remaining oxygen consumption rate determined after this intervention is primarily non-mitochondrial. Following oxygen consumption rate measurement the cells were immediately fixed and stained for β-tubulin III as described above. The plates were then inserted into a Cellomics ArrayScan-VTI HCS Reader (Thermo Scientific) equipped with Cellomics ArrayScan-VTI software to determine total neuronal number in each well. Data are expressed as oxygen consumption rate in picomole per minute for 1000 cells.

Determination of thermal sensitivity, intraepidermal and subepidermal nerve profiles

Hind paw thermal response latencies were measured as previously described (Beiswenger et al., 2008). In short, rats and mice were placed in plexiglass cubes on top of the thermal testing device (UARDG). The heat source was placed directly 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 four times at 5 min intervals and the median value of trials 2–4 used to represent the response latency for that paw. At the end of the experiment, animals were sacrificed under anaesthesia and the plantar skin of the hind paw removed to 4% paraformaldehyde, fixed overnight at 4°C then transferred to 0.1 M phosphate buffer before preparation for quantification of nerve profiles as described elsewhere (Beiswenger et al., 2008). Briefly, samples were passed through an ethanol gradient, Histo-Clear, xylene and paraffin before cutting 6 µm sections and staining with an antibody to PGP 9.5 (1:1000, Chemicon International or Biogenesis Ltd), followed by incubation with a secondary biotinylated antibody and a peroxidase-conjugated avidin–biotin enzyme complex (Vector Laboratories). Specificity was confirmed by omitting the primary antibody on selected sections. Protein gene product 9.5 (PGP 9.5) immunoreactive profiles were viewed using a light microscope and quantified by an observer unaware of the treatment groups. The total number of PGP 9.5 immunoreactive nerve profiles throughout the epidermis (intraepidermal nerve fibre) and papillary dermis (subepidermal nerveplexi) were calculated relative to the length of tissue examined.

Determining axonal calibre of myelinated nerve fibres

Tibial nerves were immersion fixed in 2.5% glutaraldehyde for 12 h, transferred to 0.1 M sodium phosphate buffer, dehydrated through an alcohol gradient, osmicated and embedded in araldite resin. Thick
Deficient AMPK and degeneration in neuropathy

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(1 μm) sections were stained with p-phenylene diamine before capture of digital images using a BH-2 light microscope, a U-PMTC video camera (both Olympus) and an LX-450 system digitizer (Optronics Engineering). Axonal area and perimeter were measured using NIH Image1.55. Each nerve section was sampled in a serpentine pattern such that the entire nerve was analysed with no overlapping fields. Axons cut as tangential or paranodal profiles were excluded from analysis, which incorporated ~650 (range 432–922) axons per nerve.

Data analysis

Data were subjected to two-tailed Student's t-tests or one-way ANOVA with post hoc comparisons using Tukey's or Dunnett's post hoc tests, as appropriate and indicated. Regression analysis was used with a one-phase exponential decay parametric test with the Fisher parameter (GraphPad Prism 4, GraphPad Software).

Results

The type 1 diabetic rodents did not suffer weight loss during the study but showed reduced weight gain after 4–22 weeks compared with their age-matched controls (Table 1). Type 2 db/db mice (30 weeks) were significantly heavier than their heterozygous littermate controls. Persistence of diabetes in the animals was verified by elevated non-fasting blood glucose. Streptozotocin-diabetic mice (Table 1) and rats (Fig. 9) exhibited marked thermal hypoalgesia as a sign of diabetic sensory neuropathy whereas db/db mice showed only a mild loss of thermal sensation at study end (Table 1).

Diabetes downregulates AMPK/PGC-1α and mitochondrial respiratory chain complexes in diabetic rodent dorsal root ganglia

To evaluate the impact of diabetes on the AMPK/PGC-1α pathway and mitochondria in diabetic rodents, protein expression of AMPK (total and phosphorylated), PGC-1α, mitochondrial proteins and respiratory complex activities were determined at various time-points in streptozotocin-diabetic Sprague-Dawley rats and Swiss Webster mice. In DRG of streptozotocin-diabetic Swiss Webster mice, expression of phosphorylated AMPK, total AMPK, PGC-1α and selective mitochondrial proteins (NDUFS3, COX IV, ATP-β) were unchanged at 4 weeks (data not shown). Following 8–14 weeks of diabetes, phosphorylated AMPK, PGC-1α (Fig. 1A and B) and NDUFS3, COX IV (Fig. 1C and D) were significantly reduced (data shown at 14 weeks) and these deficits were maintained for the length of the study (22 weeks: data not shown). The levels of phosphorylated acetyl CoA carboxylase, an endogenous substrate of AMPK, were also significantly suppressed in diabetic samples (Fig. 1A and B). In lumbar DRG of 5- to 6-month diabetic animals, similar reductions in phosphorylated AMPK and PGC-1α expression were observed in db/db mice and streptozotocin-diabetic rats (Fig. 2B and C). Enzymatic activities of mitochondrial complexes (Complex I and IV) and the Krebs cycle enzyme, citrate synthase, were determined in DRG of age-matched control and 4- and 14-week streptozotocin-diabetic mice (Table 2). The activity of mitochondrial complex I was assessed as rotenone-sensitive NADH-cytochrome c reductase (NCCR) when cytochrome c is the acceptor of electrons. Enzymatic activities of rotenone-sensitive NCCR (Complex I) and cytochrome c oxidase (Complex IV) as well as the Krebs cycle enzyme, citrate synthase, were unchanged at 4 weeks in streptozotocin-diabetic mice compared with controls, but significantly decreased by 14 weeks (Table 2). The relatively slow progression of these changes in gene expression and mitochondrial activity suggest that hyperglycaemia alone may not be driving phenotypic change within the DRG.

Manipulation of AMP-activated protein kinase alters levels of neurite outgrowth in sensory neurons

To assess the function of AMPK in neurite outgrowth, adult DRG neurons were maintained in culture and infected for 2 days with GFP-expressing adenoviral constructs carrying dominant-negative mutants of AMPKα1 or α2 (termed DN1 or DN2), constitutively active AMPK (ad-AMPK-CA) or GFP alone (Fig. 3A and B). Infection efficiency with all constructs was 50–70%. Cells expressing either of the two dominant-negative mutants had lower levels of neurotrophin-directed neurite outgrowth, as assessed by GFP fluorescence (Fig. 3A). Conversely, neurons derived from 4- to 5-month streptozotocin-diabetic rats transduced with ad-AMPK-CA exhibited significantly enhanced neurite outgrowth (Fig. 3B). It should be noted that under these culture conditions, with a background of low dose neurotrophic factors, that addition of 10 nM insulin to control cultures or raising glucose to 25 mM in diabetic cultures had no significant effect on neurite outgrowth in the presence or absence of resveratrol at 24-h in vitro (data not shown).

Resveratrol enhances neurite outgrowth in cultured diabetic neurons

Neurons isolated from the DRG of age-matched control or 4- to 5-month streptozotocin-diabetic rats were cultured under defined conditions and treated with a range of resveratrol concentrations for 2 h (Fig. 3C and D) or 24 h (Fig. 3E). Resveratrol had little effect on control neurons but significantly increased phosphorylation and expression of AMPK and increased neurite outgrowth in neurons from diabetic rats. To prove resveratrol was signalling via AMPK to enhance neurite outgrowth cultured DRG neurons from diabetic rats treated with resveratrol were infected with the dominant-negative construct, DN1-AMPK, or exposed to the specific inhibitor of AMPK, compound C. Both modes of inhibition effectively blocked resveratrol-induced neurite outgrowth (Fig. 4).

Mitochondrial inner membrane is depolarized in axons of diabetic neurons and normalized by resveratrol

Neurons isolated from the DRG of age-matched control or 4- to 5-month streptozotocin-diabetic rats were cultured ± 1 μM
resveratrol for 24 h to analyse mitochondrial inner membrane potential in axons using the dye TMRM. TMRM was used at a sub-quench concentration where a decrease in fluorescence signal intensity indicates reduced mitochondrial inner membrane potential (Nicholls, 2006). 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 at Complex III leading to mitochondrial depolarization whereas oligomycin inhibits the ATP synthase 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 neurons from diabetic rats (Fig. 5A and B). 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. Resveratrol significantly prevented mitochondrial depolarization in the axons of neurons from diabetic rats (Fig. 5).

Cellular bioenergetics profile is abnormal in cultured neurons from diabetic rats and corrected by resveratrol

To assess the cellular bioenergetics profile in sensory neurons derived from DRG of age-matched control and 4- to 5-month streptozotocin-diabetic rats, the oxygen consumption rate was measured in DRG neurons cultured for 24 h using the Seahorse Biosciences XF24 analyser. Neurons were treated sequentially with oligomycin followed by uncoupler FCCP to remove the pH gradient and enable maximal rates of electron transport to occur and then treated with a combination of rotenone and antimycin A to block respiratory electron flux at complexes I and III. Oxygen consumption rate induced by the uncoupler FCCP (0.3–1.0 μM) in cultured neurons from diabetic rats (Fig. 6B) as well as per cent related to basal respiration (Fig. 6C) was significantly decreased compared with neurons from age-matched control rats (Fig. 6A and C) indicating impairment of maximal electron transport activity in the diabetic state. Respiratory control ratio (Fig. 6E) and spare respiratory capacity (Fig. 6F) were significantly depressed in cultured neurons from diabetic versus control rats but coupling efficiency was slightly higher in neurons from diabetic rats (Fig. 6D). Pie charts are presented summarizing the different processes contributing to oxygen consumption in the cultures. The comparison of charts between DRG neurons from control (Fig. 6G) and diabetic rats (Fig. 6H) indicate that the altered bioenergetics parameter of note was the elevated non-mitochondrial oxygen consumption in diabetic neurons (21 versus 6%). Neurons from DRG of diabetic rats that were cultured for 24 h with resveratrol (1 μM) treatment showed significantly improved absolute oxygen consumption rate, oxygen consumption rate related to basal respiration, respiratory control ratio and spare respiratory capacity (Fig. 7). Supplementary Fig. 1 shows that under the culture conditions with a background of low dose neurotrophic factors, addition of 10 nM insulin to control cultures or raising glucose to 25 mM in control or diabetic cultures had no significant effect on mitochondrial bioenergetics at 24 h in vitro.

Resveratrol treatment corrects abnormal AMP-activated protein kinase pathway signalling, reverses thermal hypoalgesia and prevents intraepidermal nerve fibre loss and reduced axonal calibre in streptozotocin-induced diabetic rats

Streptozotocin-diabetic rats treated with resveratrol between Weeks 9 and 18 of diabetes exhibited the same lower body...
weight and higher plasma glucose levels as seen in untreated diabetic animals (Table 1). Phosphorylation/expression of AMPK/PGC-1α, mitochondrial complexes (NCCR, COX) and the Krebs cycle enzyme, citrate synthase, were significantly decreased in DRG of streptozotocin-diabetic rats and prevented by treating diabetic rats with resveratrol (Fig. 8). Streptozotocin-diabetic rats developed significant (P < 0.05) thermal hypoalgesia after 8 weeks of diabetes and resveratrol treatment significantly (P < 0.01) reversed the thermal hypoalgesia by Weeks 15 and 17 of diabetes (Fig. 9A). Hind paw plantar skin from diabetic rats showed a significant (P < 0.01) decrease in both intraepidermal nerve fibre and subepidermal nerve plexi profiles compared with skin from control

**Figure 1** Expression of phosphorylated AMPK, PGC-1α and mitochondrial respiratory chain proteins is downregulated in DRG of 14-week streptozotocin-diabetic mice. Quantitative immunoblot analysis revealed reduced expression of phosphorylated AMPK (P-AMPK), PGC-1α, phosphorylated acetyl CoA carboxylase and mitochondrial proteins, NDUFS3 and COX IV in 14-week streptozotocin-diabetic (Db) Swiss Webster mice versus age-matched control (Ctrl). Total AMPK (T-AMPK), ATP synthase subunit β and T-ERK remain unchanged. Representative blots are shown in A and C. (B) Quantification of phosphorylated AMPK, total AMPK, PGC-1α expression in db/db mice. (C) Quantification of the same proteins from DRG obtained from streptozotocin (STZ)-induced diabetic rats. All data have been normalized to T-ERK levels. Values are mean ± SEM of n = 4–6. *P < 0.05 versus control by Student’s t-test.

**Figure 2** Expression of phosphorylated AMPK and PGC-1α is reduced in DRG of db/db mice and streptozotocin-diabetic rat. (A) Representative immunoblots of DRG from db/db mice and (B) quantification of phosphorylated AMPK, total (T)-AMPK and PGC-1α expression in db/db mice. (C) Quantification of the same proteins from DRG obtained from streptozotocin (STZ)-induced diabetic rats. All data have been normalized to T-ERK levels. Values are mean ± SEM of n = 4–6. *P < 0.05 versus control by Student’s t-test.
AMPK regulates neurite outgrowth in sensory neurons derived from DRG of normal rats and resveratrol enhances phosphorylation/expression of AMPK and elevates neurite outgrowth in neurons derived from DRG from streptozotocin-diabetic rats.

(A) Manipulation of AMPK and neurite outgrowth with dominant-negative AMPK in normal neurons. Data are significantly different from control (GFP) by non-parametric Mann–Whitney U-test (*\(P < 0.01\)).

(B) Expression of constitutively active ad-AMPK-CA in DRG neurons derived from control or diabetic rats and cultured for 24 h with either 10 mM D-glucose with 10 nM insulin (control) or 25 mM D-glucose with zero insulin (diabetic). Values are mean ± SEM; \(n = 3\). *\(P < 0.05\) versus GFP control (Student’s t-test; and confirmed using a non-parametric test).

(C) Expression of phosphorylated AMPK, (D) total AMPK and (E) total neurite outgrowth of DRG neurons from age-matched control or streptozotocin-diabetic rats cultured with resveratrol (0–3 \(\mu M\)) for 2 h (C and D) or 24 h (E). Neurons were cultured in defined Hams F12 medium supplemented with N2 additives and a suboptimal dose range cocktail of neurotrophins. Values are mean ± SEM of \(n = 3\). *\(P < 0.05\) versus control (one-way ANOVA with Dunnett’s post hoc test; and confirmed using a non-parametric test).

Table 2 Enzymatic activities of mitochondrial respiratory chain complexes and citrate synthase activity in DRG of streptozotocin-diabetic mice

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>4 weeks Control</th>
<th>4 weeks Diabetic</th>
<th>14 weeks Control</th>
<th>14 weeks Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
<td>5–6</td>
<td>6–8</td>
<td>6–8</td>
</tr>
<tr>
<td>NADH-cytochrome c reductase</td>
<td>59.55 ± 5.26</td>
<td>61.37 ± 11.34</td>
<td>48.84 ± 4.31</td>
<td>34.58 ± 4.11*</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>509.70 ± 54.51</td>
<td>479.72 ± 51.36</td>
<td>466.98 ± 24.54</td>
<td>367.72 ± 31.78*</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>198.25 ± 9.24</td>
<td>189.23 ± 12.87</td>
<td>152.97 ± 5.24</td>
<td>129.86 ± 4.96*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.

*\(P < 0.05\) versus control (Student’s t-test).

Enzymatic activities of Complex I assessed as rotenone-sensitive portion of NCCR. All activities are expressed as nanomole per minute per milligram protein.
rats (Fig. 9B and C). Resveratrol treatment significantly increased intraepidermal nerve fibre profiles in diabetic rats ($P < 0.01$ versus untreated diabetic rats); although values remained significantly ($P < 0.01$) lower than controls. A similar pattern was seen for subepidermal nerve plexi in resveratrol-treated rats, except that subepidermal nerve plexi values were not significantly different from either controls or untreated diabetic rats. The mean axonal diameter of myelinated axons in the tibial nerve of streptozotocin-diabetic rats was significantly ($P < 0.05$) reduced compared with controls and this was prevented by resveratrol (control, $4.62 \pm 0.05 \mu m$; diabetic, $4.40 \pm 0.05 \mu m$; diabetic + resveratrol, $4.58 \pm 0.08 \mu m$; means ± SEM, $n = 9–10$; $P < 0.05$ by Dunnett’s for control versus diabetic).

**Discussion**

The findings in this study indicate a link between impaired AMPK/PGC-1α pathway signalling and mitochondrial respiratory chain dysfunction in DRG sensory neurons from streptozotocin-diabetic...
neurite outgrowth and this process was enhanced by resveratrol in cultured neurons derived from diabetic rats. Finally, resveratrol treatment reversed the clinical and pathological features of diabetic neuropathy, namely thermal hypalgesia, intraepidermal nerve fibre loss and reduced myelinated fibre axonal calibre, in streptozotocin-diabetic rats.

Adenoviral-mediated delivery of mutant AMPK proteins to adult sensory neurons in vitro revealed that AMPK was a key positive modulator of neurite outgrowth, a finding that is consistent with the suggestion that axon-producing neurons have a high demand for ATP through the need to drive growth cone motility (Bernstein and Bamburg, 2003). When using resveratrol to induce AMPK, it was notable that normal adult neurons were unresponsive with regard to AMPK expression or neurite outgrowth. In contrast, neurons derived from diabetic rats were responsive to resveratrol, which significantly increased expression and phosphorylation of AMPK and elevated neurite outgrowth. Previous studies in Neuro-2a cells and embryonic sensory neurons have shown that resveratrol raised AMPK phosphorylation, enhanced markers of mitochondrial biogenesis and promoted neurite outgrowth via an LKB1-dependent but SIRT1-independent mechanism (Dasgupta and Milbrandt, 2007). Adult sensory neurons do not express detectable levels of SIRT1 (S. K. Roy Chowdhury et al., unpublished data) but do express LKB1 and mitochondrial SIRT3, which can also be activated by resveratrol (Dasgupta and Milbrandt, 2007; Kim et al., 2011). It appears that AMPK modulation of neurite outgrowth is context specific and the reduced expression/activation of AMPK in neurons from diabetic rats at the time of plating makes these cells particularly responsive to the AMPK stimulatory effects of resveratrol.

Studies in muscle, liver and cardiac tissues have revealed that expression of AMPK and PGC-1α are decreased under diabetic conditions, presumably as a result of nutrient excess (Puigserver et al., 1998; Feige and Auwerx, 2007). In the present study, we extended these observations to sensory neurons of the DRG, which showed decreased expression of phosphorylated AMPK, PGC-1α and mitochondrial proteins such as NDUF53 and COX IV. The activities of mitochondrial respiratory chain enzymes and citrate synthase were also decreased after 14 weeks of diabetes in mice, as we have recently reported using stable isotope labelling by amino acids in cell culture (SILAC)-based proteomic analysis (Akude et al., 2011). In peripheral nerve, intracellular glucose concentration can accumulate to toxic levels under diabetic conditions since glucose uptake is insulin-independent and controlled by glucose transporter-driven equilibration of external glucose with intracellular glucose (Simpson et al., 2008; Tomlinson and Gardiner, 2008). Hyperglycaemia represents nutrient excess and drives a lower AMP/ATP ratio that triggers reduced phosphorylation and deactivation of AMPK (Feige and Auwerx, 2007; Hardie, 2008). Our present data support the proposal that hyperglycaemia-mediated deactivation of AMPK is instigating the ‘Crabtree effect’ (Ilsen, 1961) through modulation of PGC-1α and the downstream epigenetic control of mitochondrial biogenesis, since ATP resources are sufficient to allow deactivation of oxidative phosphorylation and reliance on glycolytic anaerobic metabolism. There is physiological evidence to support this hypothesis, as studies in diabetic rats and humans have demonstrated that rodents. Phosphorylation/expression of the AMPK/PGC-1α axis and the neuronal mitochondrial bioenergetics profile were impaired in DRG of streptozotocin-diabetic rats and this was prevented by resveratrol treatment. In addition, this study demonstrates that activity of the AMPK/PGC-1α pathway modulates
Peripheral nerve exhibits resistance to ischaemic conduction block (Seneviratne and Peiris, 1968, 1969) that has been attributed to an inherent increased reliance on anaerobic metabolism (Low et al., 1985). However, studies in lens (Obrosova et al., 1997), retina (Ola et al., 2006) and cardiac tissue (Trueblood and Ramasamy, 1998) of animal models of type 1 diabetes show that aspects of glycolytic pathway function are depressed. Excess intracellular glucose in peripheral nerve is also metabolized by aldose reductase, causing increased flux through the polyol pathway (Oates, 2008; Ido et al., 2010). Within the DRG there is polyol build-up in diabetes (Llewelyn et al., 1991) but no evidence of aldose reductase expression in neurons (Jiang et al., 2006). However, satellite cells express this enzyme (Jiang et al., 2006) and there is constitutive expression of aldehyde reductase in neurons (E. Zherebitskaya et al., unpublished data). Furthermore, cultured neurons derived from diabetic rats and treated with 25 mM glucose exhibit reduced reactive oxygen species production when exposed to the inhibitor of sorbitol dehydrogenase, SDI-158, confirming the presence of components of the polyol pathway (Akude et al., 2011). Therefore, enhanced sorbitol oxidation within the DRG could lead to a diminished NAD⁺/NADH ratio, as measured in nerve (Obrosova et al., 1999; Stevens et al., 2000), lowering available NAD⁺ levels resulting in reduced SIRT1 or SIRT3 activity (Guarente, 2006; Schwer et al., 2009). In addition, SIRT3 expression is downregulated in DRG in diabetes (S. K. Roy Chowdhury et al., unpublished observation), further depressing the SIRT signalling pathway. Consequently, lowered AMPK phosphorylation may occur in tandem with suboptimal SIRT3 activity resulting in diminished phosphorylation/deacetylation of PGC-1α and its impaired expression and transcriptional activity (Rodgers et al., 2008; Kim et al., 2011). Our results consistently reveal lowered PGC-1α expression in DRG in diabetes and we propose this will lead to suboptimal transcription of a range of genes including NRF-1, NRF-2α and Tfam, which are key modulators of mitochondrial respiratory chain protein expression and biogenesis (Feige and Auwerx, 2007; Jager et al., 2007; Rodgers et al., 2008).

Our recent findings that diabetes induces mitochondrial abnormalities and dysfunction including reduced respiration rates, respiratory enzymatic activities and mitochondrial proteome expression in sensory neurons (Chowdhury et al., 2010; Akude...
et al., 2011) are consistent with reports of similar disorders in hearts of streptozotocin-diabetic rats and in skeletal muscle of patients with type 2 diabetes (Kelley et al., 2002; Lashin et al., 2006; Mogensen et al., 2007; Yang et al., 2009). The current study extends these findings to reveal that diabetes impairs key elements of cellular bioenergetics. Seahorse Biosciences XF24 analysis permitted measurement of cell respiratory control, recording rate of ATP production, proton leak, coupling efficiency, maximum respiratory rate, respiratory control ratio and spare respiratory capacity (Brand and Nicholls, 2011). In sensory neurons maintained in vitro, carefully titrated uncoupled rates of mitochondrial electron flux reported the maximum activity of respiratory chain function and substrate oxidation. Measurements of oxygen consumption rate in the presence of uncoupler revealed that the maximal electron transport capacity was significantly depressed in sensory neurons from streptozotocin-diabetic rats. Reduced spare respiratory capacity, especially in neurons that have variable ATP demands, limits their ability to meet energetic needs and renders the cells more susceptible to secondary stressors (Hill et al., 2009; Brand and Nicholls, 2011). Diabetes also blunted the maximal oxygen consumption rate and significantly lowered spare respiratory capacity suggesting that the cells were energetically stressed and that mitochondrial workload was increased. Measurements of mitochondrial inner membrane potential also provided additional information about the functional status of active mitochondria in axons. Assessment of polarization status of the inner mitochondrial membrane in the presence of antimycin A + oligomycin in axons of sensory neurons revealed that the rate of mitochondrial depolarization was more rapid in axons of normal neurons compared with diabetic neurons, and this loss of polarization was prevented by resveratrol. These data complement the XF 24 analyser results and reveal that defects in mitochondrial function detected by our

Figure 8  AMPK/PGC-1α pathway and mitochondrial activity is improved by resveratrol treatment in DRG of streptozotocin-diabetic rats. (A) Typical blots and (B) charts in which phosphorylated AMPK, total AMPK and PGC-1α expression have been presented relative to T-ERK level. Values are group mean ± SEM, n = 6–7. (C) Enzymatic activities of Complex I assessed as rotenone-sensitive portion of NADH-cytochrome c reductase (NCCR, n = 8–9), cytochrome c oxidase (COX, n = 10–11) and citrate synthase (CS, n = 10–11).

*P < 0.05 versus Ctrl and Db + RESV, **P < 0.05 versus Db and Db + RESV (one-way ANOVA with Tukey’s post hoc test).

Ctrl = control; Db = diabetic; Db + RESV = diabetic treated with resveratrol.
bioenergetics analysis were not due to a general loss of mitochondrial number or mass in the cultured cells.

Downregulation of AMPK/PGC-1α in DRG cells during hyperglycaemia is a maladaptive process that has the potential to contribute to axonal structural pathology, such as retraction of distal nerve endings. The peripheral terminals of sensory neurons located in the epidermis constitutively express proteins normally associated with neuronal growth and plasticity such as GAP-43 (Beiswenger et al., 2008; Cheng et al., 2010) and the continuous plasticity required of axon terminals within an environment undergoing continuous turnover requires substantial amounts of ATP (Bernstein and Bamburg, 2003). Any defect in mitochondrial function is therefore likely to have a profound influence on axons, with the most active terminal regions being particularly vulnerable. Indeed, loss of function of mitochondrial proteins, such as bcl-w or mitofusin-2, results in a length-dependent sensory neuropathy that closely resembles the neurodegeneration observed in diabetes (Balogh et al., 2007; Misko et al., 2010; Courchesne et al., 2011).

Axonal plasticity that maintains sensory innervation of the epidermis therefore requires a sufficient supply of actively respiring mitochondria. In highly polarized cells such as neurons, this may create a unique metabolic problem in specific cellular compartments. The hyperglycaemia-induced restriction of the AMPK/PGC-1α axis in neurons during leads to suboptimal functioning of mitochondria with diminished reserve capacity that has the potential to trigger retraction of distal axon terminals.

Our studies suggest that enhancing the AMPK/PGC-1α axis in neurons should protect from indices of distal degenerative neuropathy and this was demonstrated for structural disorders of both myelinated and unmyelinated fibres. Although we cannot yet discount other reported properties of resveratrol, such as its anti-oxidant capacity (Kumar et al., 2007), previous work by ourselves and others has shown that impaired mitochondrial function in sensory neurons and Schwann cells is not linked to elevated

Figure 9  Thermal hypoalgesia and loss of foot skin nerves in streptozotocin-diabetic rats were corrected by resveratrol. (A) Paw thermal withdrawal latency of age-matched control and streptozotocin-diabetic rats. Data are group mean ± SEM (n = 8). *P < 0.05 compared with diabetic; **P < 0.05 versus other groups. PGP 9.5 immunoreactive intraepidermal nerve fibre (IENF) (B) and subepidermal nerve plexi (SNP) (C) profiles in hind paw plantar skin. Data are group mean ± SEM (n = 10–11). *P < 0.01 versus control and **P < 0.01 versus diabetic by ANOVA with Dunnett’s post hoc test. (D) Representative images of PGP 9.5-positive staining of intraepidermal nerve fibre (red arrows) and SNP (yellow arrows) in the plantar skin of the hind paw of a control and a diabetic rat. Scale bar = 40 μm.
reactive oxygen species generation (Chowdhury et al., 2010; Zhang et al., 2010; Akude et al., 2011). The bioenergetics profile of neurons from streptozotocin-diabetic rats in the present study did indicate a higher rate of non-mitochondrial oxygen consumption and previous work has shown signs of high glucose-induced oxidative stress in axons of diabetic neurons (Zherebitskaya et al., 2009). This could be indicative of high rates of oxygen consuming processes within the mitochondria and/or cytoplasm of diabetic axons that contribute to enhanced oxidative stress, such as augmented activity of NAD(P)H oxidase (Block et al., 2009; Eid et al., 2010). Nevertheless, our present findings in diabetic rats are consistent with impairment of the AMPK/PGC-1α axis as being a central component of the pathogenic cascade linking hyperglycaemia with structural indices of diabetic neuropathy. Our results highlight the utility of resveratrol as a tool to manipulate the AMPK pathway and identify novel drug targets in animal models of diabetes. The value of resveratrol itself as an oral therapy for diabetic neuropathy in humans remains to be determined.

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Supplementary material

Supplementary material is available at Brain online.

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