

# Diabetes regulates mitochondrial biogenesis and fission in mouse neurons

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Received: 4 August 2009 / Accepted: 2 September 2009 / Published online: 22 October 2009  
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## Abstract

**Aims/hypothesis** Normal mitochondrial activity is a critical component of neuronal metabolism and function. Disruption of mitochondrial activity by altered mitochondrial fission and fusion is the root cause of both neurodegenerative disorders and Charcot–Marie–Tooth type 2A inherited neuropathy. This study addressed the role of mitochondrial fission in the pathogenesis of diabetic neuropathy.

**Methods** Mitochondrial biogenesis and fission were assayed in both in vivo and in vitro models of diabetic neuropathy. Gene, protein, mitochondrial DNA and ultrastructural analyses were used to assess mitochondrial biogenesis and fission. **Results** There was greater mitochondrial biogenesis in dorsal root ganglion neurons from diabetic compared with non-diabetic mice. An essential step in mitochondrial biogenesis is mitochondrial fission, regulated by the mitochondrial fission protein dynamin-related protein 1 (DRP1). Evaluation of diabetic neurons in vivo indicated

small, fragmented mitochondria, suggesting increased fission. In vitro studies revealed that short-term hyperglycaemic exposure increased levels of DRP1 protein. The influence of hyperglycaemia-mediated mitochondrial fission on cell viability was evaluated by knockdown of *Drp1* (also known as *Dnm1l*). Knockdown of *Drp1* resulted in decreased susceptibility to hyperglycaemic damage.

**Conclusions/interpretation** We propose that: (1) mitochondria undergo biogenesis in response to hyperglycaemia, but the increased biogenesis is insufficient to accommodate the metabolic load; (2) hyperglycaemia causes an excess of mitochondrial fission, creating small, damaged mitochondria; and (3) reduction of aberrant mitochondrial fission increases neuronal survival and indicates an important role for the fission–fusion equilibrium in the pathogenesis of diabetic neuropathy.

**Keywords** Diabetic neuropathy · Mitochondrial biogenesis · Mitochondrial fission

**Electronic supplementary material** The online version of this article (doi:10.1007/s00125-009-1553-y) contains supplementary material, which is available to authorised users.

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## Abbreviations

DRG	Dorsal root ganglion
DRP1	Dynamin-related protein 1
COX-IV	OxPhos complex IV subunit I
FIS1	Fission 1 protein
miRNA	Micro RNA
mtDNA	Mitochondrial DNA
PGC-1 $\alpha$	Peroxisome proliferator activated receptor-coactivator $\alpha$
ROS	Reactive oxygen species

## Introduction

Mitochondrial damage is central to the pathophysiology of both inherited and acquired neuropathies [1, 2]. In diabetic

neuropathy, physiological abnormalities of sensory neurons are directly initiated by hyperglycaemia [1, 3], though subsequent mechanisms leading to neuronal dysfunction are not yet fully characterised. Our laboratory has demonstrated the critical role of mitochondria in the progression of hyperglycaemia-mediated neuronal damage [4]. In vitro models of diabetic neuropathy reveal that hyperglycaemia overwhelms normal mitochondrial function in dorsal root ganglion (DRG) neurons, resulting in the production of reactive oxygen species (ROS). ROS in turn damage the mitochondrial electron transport apparatus and cellular proteins, lipids and DNA. Mitochondrial membrane depolarisation and the loss of ATP production, in particular, precede neuronal dysfunction [5, 6]. In parallel, DRG neurons from in vivo rodent models of diabetic neuropathy contain vacuolated mitochondria with disrupted cristae [7] with biochemical evidence of ROS [7].

Mitochondrial networks are dynamic, undergoing both mitochondrial biogenesis and mitochondrial fission [8]. The distinction between mitochondrial biogenesis and fission is based on whether or not there is replication of mitochondrial DNA (mtDNA) and an increase in mitochondrial mass. By definition, mitochondrial biogenesis involves complete mtDNA replication. In contrast, fission occurs in the absence of mtDNA replication, and the existing copies of mtDNA are simply divided between the new fissioned mitochondria [8, 9]. Thus, mitochondrial networks are dynamic and respond to metabolic signals by increasing the actual mass of the network (biogenesis) as well as dispersing the existing mitochondria into a larger network (fission).

Increased mitochondrial biogenesis is part of the cellular response to oxidative stress [10]. Mitochondrial biogenesis attenuates oxidative stress by increasing mitochondrial capacity to metabolise reducing equivalents. Cells undergoing mitochondrial biogenesis consume less oxygen, maintain mitochondrial membrane potential, and produce fewer ROS, which together decrease cellular damage and increase survival [11, 12]. Neurons transfected with the mitochondrial biogenesis-promoting protein peroxisome proliferator activated receptor-coactivator  $\alpha$  (PGC-1 $\alpha$ ) are protected against paraquat- or H<sub>2</sub>O<sub>2</sub>-induced oxidative stress [13]. Despite the neuroprotective effects of mitochondrial biogenesis, its role in diabetic neuropathy is not well characterised. Current theory suggests that increased mitochondrial biogenesis allows cells to accommodate increased energy loads, thereby promoting cellular viability [14].

While mitochondrial biogenesis increases mitochondrial mass, mitochondrial fission increases the actual number of mitochondria. Dynamin-related protein 1 (DRP1) is a GTPase that translocates from the cytosol to the outer mitochondrial membrane to mediate mitochondrial fission at sites known as mitochondrial scission sites [15, 16].

Fission 1 protein (FIS1) is anchored to the outer mitochondrial membrane and promotes fission in concert with DRP1 [17–19].

Previous work in our laboratory has implicated DRP1 in hyperglycaemia-mediated mitochondrial damage in sensory neurons [1]. We contend that mitochondria initially attempt to disperse the metabolic load of prolonged hyperglycaemia by maintaining a dynamic balance between mitochondrial biogenesis and fission. We hypothesise that mitochondrial networks are disrupted as ROS accumulate with loss of ATP generation and excessive mitochondrial fission, producing unstable mitochondrial networks and neural injury. As a first step in testing this hypothesis, the present study examined the role of mitochondrial biogenesis and fission in hyperglycaemia-induced neuronal damage using in vivo and in vitro models of diabetic neuropathy. We detected increased levels of mtDNA and biogenesis-related proteins in DRG neurons from a genetic murine model of type 2 diabetes with diabetic neuropathy; however, these mitochondria were significantly smaller and more electron-dense. The presence of small, fragmented mitochondria and increased levels of DRP1 suggested increased mitochondrial fission and was investigated further in vitro. Because DRP1 is essential for both mitochondrial fission and cell viability, we hypothesised that attenuation of DRP1 levels would decrease mitochondrial fission and improve short-term neuronal survival. Efficient knockdown of *Drp1* (also known as *Dnm1l*) in DRG neurons decreased glucose-mediated neuronal injury. Our data suggest that an imbalance in mitochondrial neural networks favouring mitochondrial fission in DRG neurons plays a role in the pathogenesis of diabetic neuropathy and that therapies that maintain mitochondrial neural networks could be beneficial in the treatment of diabetic neuropathy.

## Methods

**Diabetic mice** Diabetic (BKS.Cg-*m*<sup>+/+</sup>*Lepr*<sup>db</sup>/J, BKS-*db/db*) and control (BKS-*db*/+) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were housed in a pathogen-free environment with continuous access to food (Purina 5053 chow) and water on a 12 h light–12 h dark schedule, and cared for following the University of Michigan Committee on the Care and Use of Animals guidelines (approval no. 07675) [20].

At the end of the experimental period, glycated haemoglobin (HbA<sub>1c</sub>) was measured using a Glyco-Tek Affinity Column Kit (Helena Laboratories, Beaumont, TX, USA). Analyses and procedures were performed in compliance with established Animal Models of Diabetic Complications Consortium (AMDCC) protocols ([www.amdcc.org](http://www.amdcc.org), accessed 25 March 2003). At the 5-week time

point ( $n=9$  for each group), the BKS-*db/+* and BKS-*db/db* mice had an average weight of 18.5 and 23.5 g respectively and HbA<sub>1c</sub> was 4.3% and 5.3%; at 24 weeks weights were 26.5 and 52.2 g respectively and HbA<sub>1c</sub> was 7.6% and 13.0%.

DRGs were harvested at 5 and 24 weeks of age (1 and 20 weeks of diabetes). The mice were killed by sodium pentobarbital overdose. A blood sample (50  $\mu$ l) was collected for measurement of HbA<sub>1c</sub> (see above). DRGs were harvested by microdissection, immediately immersed in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$ . Protein was extracted using T-PER tissue protein extraction reagent (Pierce, Rockford, IL, USA).

**Embryonic DRG culture** Dissociated DRG neurons were isolated from embryonic day 15 Sprague–Dawley rat embryos and plated in NB+ medium supplemented with B27 (without antioxidants) and 1.4 mmol/l L-glutamine. NB+ medium was composed of Neurobasal medium (Invitrogen, Grand Island, NY, USA) + 30 nmol/l selenium, 10 nmol/l hydrocortisone, 10  $\mu$ g/ml transferrin, 10 nmol/l  $\beta$ -oestradiol, 10 ng/ml 2.5 S nerve growth factor, 7  $\mu$ mol/l aphidicolin and penicillin/streptomycin/neomycin (5,000 U/5 mg/10 mg/ml, respectively). After 24 h, cultures were re-fed with fresh NB+ without B-27 or L-glutamine. Cultures were used for experiments after 3 days, at which time more than 95% of the cells were neurons.

NB+ medium contained 25 mmol/l glucose (control medium), which is optimal for DRG neuron survival and neurite outgrowth [7]. High-glucose treatment medium consisted of control medium plus 20 mmol/l additional glucose to give a final concentration of 45 mmol/l glucose. This parallels the 1.4-fold increase in blood glucose concentration found in human patients with diabetes [21].

**In vitro incorporation of BrdU into mtDNA and BrdU visualisation** Cultures were prepared as above and plated on glass coverslips coated with 25  $\mu$ g/ml laminin. Two days after plating, neurons were incubated with 15  $\mu$ mol/l BrdU (Sigma-Aldrich, St Louis, MO, USA) for 6 h. Cells were fixed with 2% paraformaldehyde and permeabilised with 0.1% Triton X-100 for 10 min at room temperature, and DNA was denatured with 2 N HCl for 30 min at  $37^{\circ}\text{C}$ . Immunocytochemistry was performed by incubating with anti-BrdU (Vector Laboratories, Burlingame, CA, USA) at 1:50 overnight at  $4^{\circ}\text{C}$ . The BrdU signal was amplified using a horseradish peroxidase–goat anti-mouse antibody and Alexa Fluor 488 tyramide signal amplification kit (Invitrogen, Eugene, OR, USA) following the manufacturer's protocol. Coverslips were mounted with Prolong Gold containing DAPI (Invitrogen).

An Olympus FluoView500 confocal microscope was used to image fluorescent signals with a 60 $\times$  oil objective

and images were magnified 2 $\times$  with FluoView software (v5.0). Fluorescent signals were sequentially scanned at z-intervals of 0.225  $\mu$ m. Maximum projections of the three-dimensional data were used to show BrdU incorporation in mtDNA of representative neurons. BrdU-labelled mtDNA was analysed with Velocity software (v4.2; Improvion, Waltham, MA, USA). Z-series were deconvolved with fast restoration and cropped to isolate individual neurons. BrdU-labelled mtDNA was classified as follows: fill holes in objects; separate touching objects by 0.1 nm; and exclude objects by size (0.008–0.5 nm). The number of sites where BrdU was incorporated into mtDNA was counted per DRG soma.

**Transmission electron microscopy** Semi-thin and ultra-thin morphological analyses were performed as described [22]. Age-matched BKS-*db/db* and *db/+* mice were killed as described above and perfused with 4% paraformaldehyde in phosphate-buffered saline (0.1 mol/l, pH 7.2). DRGs were removed and postfixed in 0.12 mol/l phosphate buffer containing 2% glutaraldehyde followed by osmium tetroxide and embedded in Epon (Fluka, Buchs, Switzerland). Ultra-thin sections (100–120 nm thick) were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy. All ultrastructural analyses were performed in a blinded and non-biased manner from photomicrographs (magnification  $\times 8,000$ : *db/db* 41, *db/+* 42 photographs) using a Zeiss Leo 912 transmission electron microscope (Omega, Brattleboro, VT, USA). An average of 20 neurons per mouse were analysed. Each image represents a 7.5  $\mu\text{m}^2$  region of the cytoplasm. Cross-sections were randomly selected for analysis from BKS-*db/db* ( $n=2$ ) and *db/+* ( $n=2$ ) mice. Mitochondrial density (number of mitochondria/area) and diameter were assessed.

**Micro RNA knockdown of Drp1** Lentivirus containing micro RNA (miRNA) for human and mouse *Drp1* in the pGIPz vector (Open Biosystems, Huntsville, AL, USA) were produced through the University of Michigan Vector Core ([www.med.umich.edu/vcore/](http://www.med.umich.edu/vcore/)). DRG neurons were infected with a 1 $\times$  stock of virus on the day of plating, and the medium was changed from NB+ supplemented with B-27 and L-glutamine to NB+ without B-27, L-glutamine, and virus 1 day after plating/infection [1]. All viral infections were performed in accordance with the University of Michigan Institutional Biosafety Committee guidelines.

**Immunoblotting** Protein levels were determined using western immunoblotting on whole-cell lysates from DRG both in vivo (described above) and in vitro. DRG neurons were cultured as described above in 60 mm Biocoat collagen-coated plates (BD Biosciences, San Jose, CA,

USA). Neurons were exposed to control (25 mmol/l) or high glucose (45 mmol/l) media for 0 or 6 h, and prepared for western blotting according to our published protocol [23]. Equivalent amounts of protein were subjected to 12.5% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Pall, East Hills, NY, USA). The PVDF membranes were probed using antibodies against OxPhos complex IV subunit I (COX-IV, Invitrogen), PGC1- $\alpha$ ; (Cayman Chemical, Ann Arbor, MI, USA), DRP1 (Abnova, Walnut, CA, USA), cleaved caspase-3 (Cell Signaling, Danvers, MA, USA) and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) used at 1:1,000 dilution. Blots were developed using enhanced chemiluminescence and densitometry was performed with data normalised to actin [24].

**Mitochondrial DNA quantification** DNA was extracted using the Genelute Mammalian Genomic DNA Kit (Sigma) according to the manufacturer's instructions. Real-time PCR amplification and SYBR Green fluorescence detection were performed using the iCycler iQ Real-time Detection system (Bio-Rad, Hercules, CA, USA). The fluorescence threshold ( $C_t$ ) value was calculated by the iCycler iQ system software. Levels of mtDNA were measured by normalising the mitochondrial gene (cytochrome *b*) to the nuclear gene (glyceraldehyde-3-phosphate dehydrogenase, *Gapdh*). The  $2^{-\Delta\Delta C_t}$  method was used. A total of 10 ng genomic DNA was used for mtDNA and nuclear DNA markers, in a 25  $\mu$ l reaction containing  $1\times$  SYBR Green iCycler iQ mixture and 0.2  $\mu$ mol/l each of forward and reverse gene-specific primers. Primer sequences are listed in the Electronic supplementary material (ESM) Table 1.

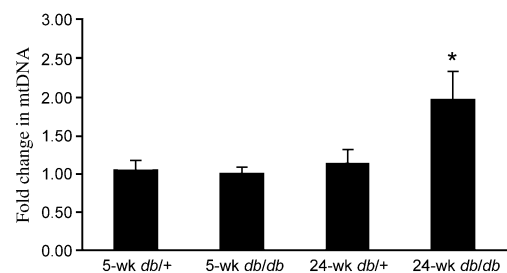
**RNA isolation and real-time PCR** Total RNA was extracted using an RNeasy Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed using the iScript cDNA Synthesis kit (Bio-Rad). Real-time PCR reactions were carried out in 96-well 0.2 ml PCR plates sealed with iCycler Optical Sealing tapes (Bio-Rad). Sequence information on all the primers is listed in ESM Table 2. The PCR amplification profile was as follows: 95°C for 5 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 1 min, extension at 72°C for 30 s and a final phase of 72°C for 5 min. The fluorescence threshold  $C_T$  value was calculated by the iCycler iQ system software and the levels were first normalised to the endogenous reference *Gapdh* ( $\Delta C_t$ ), then relative to the control group ( $\Delta\Delta C_t$ ) and expressed as  $2^{-\Delta\Delta C_t}$ . PCR product levels were expressed as mean  $\pm$  SEM and a two-sample equal variance *t* test was performed ( $n=4$  mice per condition).

## Results

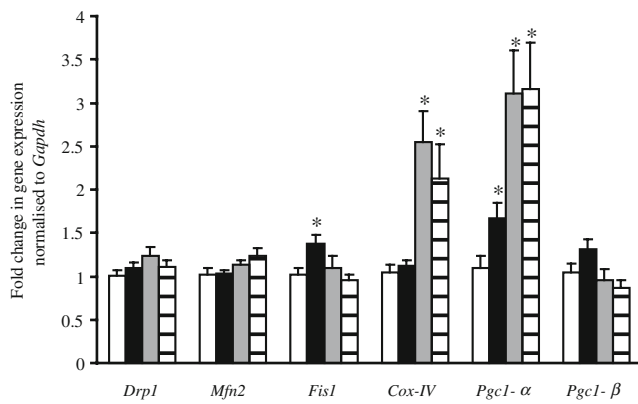
*Mitochondrial biogenesis is altered by hyperglycaemia in vivo* Diabetes results in significant disruption of mitochondrial structure and function [4, 7, 25] and regulates multiple genes related to mitochondrial metabolism and biogenesis [26] in DRG neurons. These observations were extended by examining mitochondrial biogenesis in mice with diabetic neuropathy. To avoid the potential confounding affects of streptozotocin [27], we examined a genetic model of type 2 diabetes that exhibits profound diabetic neuropathy [20, 28].

Replication of mtDNA is a necessary component of mitochondrial biogenesis and a commonly used marker for mitochondrial biogenesis [29]. mtDNA was measured in DRGs harvested from BKS-*db/db* and *db/+* mice at 5 and 24 weeks of age (1 and 20 weeks of diabetes). In Fig. 1, mtDNA levels in DRGs harvested at 5 weeks revealed no significant differences between BKS-*db/db* and *db/+* mice. In contrast, 24-week-old BKS-*db/db* DRGs contained twice the relative amount of mtDNA as age-matched *db/+* DRG.

The expression of six genes related to mitochondrial biogenesis was examined at the levels of transcription and translation in DRG harvested from BKS-*db/db* and *db/+* mice. These genes include those encoding the mitochondrial fission proteins DRP1 and FIS1, which form complexes on the outer mitochondrial membrane; the mitochondrial fusion protein mitofusin 2 (MFN2); the mtDNA-encoded protein COX-IV, a marker of mitochondrial mass; the mitochondrial biogenesis protein PGC1- $\alpha$  (involved in glucose metabolism); peroxisome proliferator-activated receptor gamma, coactivator 1 beta (PGC1- $\beta$ ), involved in lipid metabolism [30]; and an internal control (*Gapdh*). At 5 weeks, *Fis1* and *Pgc1- $\alpha$*  (also known as *Ppargc1a*) were significantly elevated ( $p<0.05$ ) in DRGs harvested from BKS-*db/db* neurons compared with those from *db/+* neurons (Fig. 2). At 24 weeks, *Cox-IV* (also known as *Cox1* or *Mt-co1*) and *Pgc1- $\alpha$*  in DRGs were elevated compared with 5-week-old mice, but were not affected by diabetes (Fig. 2). These data suggest that



**Fig. 1** Levels of mtDNA were increased in diabetic mice. mtDNA levels were determined by measuring cytochrome *c* DNA levels. Genomic DNA isolated from control (*db/+*) and diabetic (*db/db*) DRG neurons at 5 and 24 weeks (wk) was subjected to real-time PCR using the primers listed in ESM Table 1 ( $n=4$ )



**Fig. 2** Transcription of genes involved in mitochondrial biogenesis was significantly increased in the acute response to hyperglycaemia. RNA was extracted from DRGs of control (*db/+*) and diabetic (*db/db*) mice at 5 and 24 weeks of age. RT-PCR was used to analyse mRNA levels of genes involved in mitochondrial biogenesis (*Pgc1-α*, *Pgc1-β* [also known as *Ppargc1b*]), mitochondrial fission (*Drp1* and *Fis1*), mitochondrial fusion (*Mfn2*) and mitochondrial mass (*Cox-IV*). White columns, *db/+* mice aged 5 weeks; black, *db/db* mice aged 5 weeks; grey columns, *db/+* mice aged 24 weeks; hatched columns, *db/db* mice aged 24 weeks. \* $p < 0.05$

transcription of *Fis1* and *Pgc1-α* is altered as an acute response to hyperglycaemia, but not to prolonged hyperglycaemia.

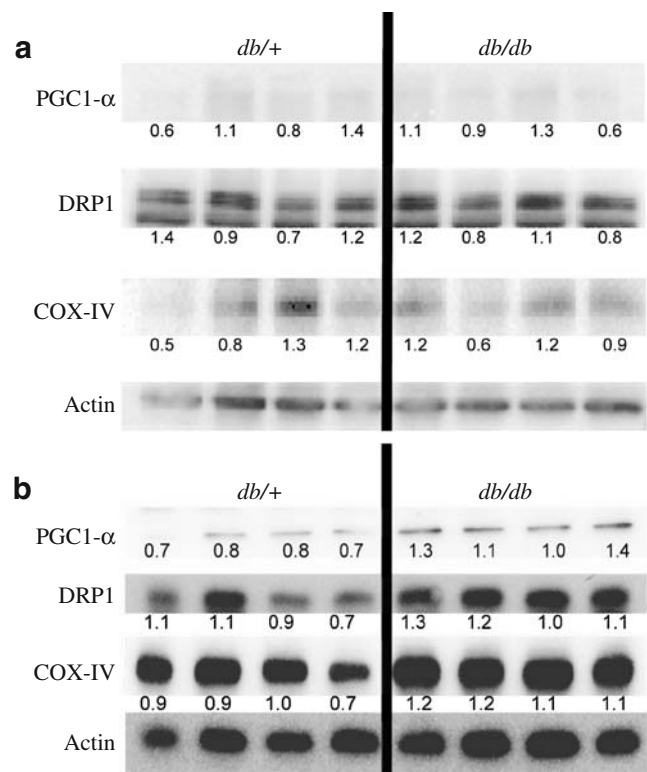
Interestingly, analysis of protein levels indicated that at 5 weeks, BKS-*db/db* and *db/+* DRGs exhibited no significant differences in PGC1- $\alpha$ , COX-IV and DRP1 (Fig. 3a). In contrast, DRGs from 24 week-old BKS-*db/db* DRGs demonstrated a 55% increase in PGC1- $\alpha$ , a 34% increase in COX-IV and a 21% increase in DRP1 compared with *db/+* DRGs (Fig. 3b). An increase in protein levels suggests the presence of mitochondrial biogenesis in diabetic (*db/db*) mice. To ensure that mitochondrial biogenesis was not ubiquitous throughout the BKS-*db/db* animals, skeletal muscle from 24-week-old BKS-*db/db* and *db/+* mice was analysed; no changes in DRP1 or COX-IV were found (data not shown).

**Prolonged hyperglycaemia leads to an increase in mitochondrial fission** Mitochondria localised within DRG cell bodies from BKS-*db/db* mice differed morphologically from *db/+* mitochondria. Mitochondria from BKS-*db/+* mice displayed normal, round morphology with discrete cristae and a contiguous matrix (Fig. 4a). In contrast, mitochondria of BKS-*db/db* mice appeared smaller, fragmented and asymmetrical (Fig. 4b–d). Mitochondrial membrane density and cristal structure were also altered. Mitochondria from BKS-*db/db* neurons showed signs of the inner membrane enclosing itself to form individual compartments. Based on the classification system of Sun *et al.* [31], mitochondria from BKS-*db/db* mice are characterised as normal-vesicular and vesicular. Further structural analy-

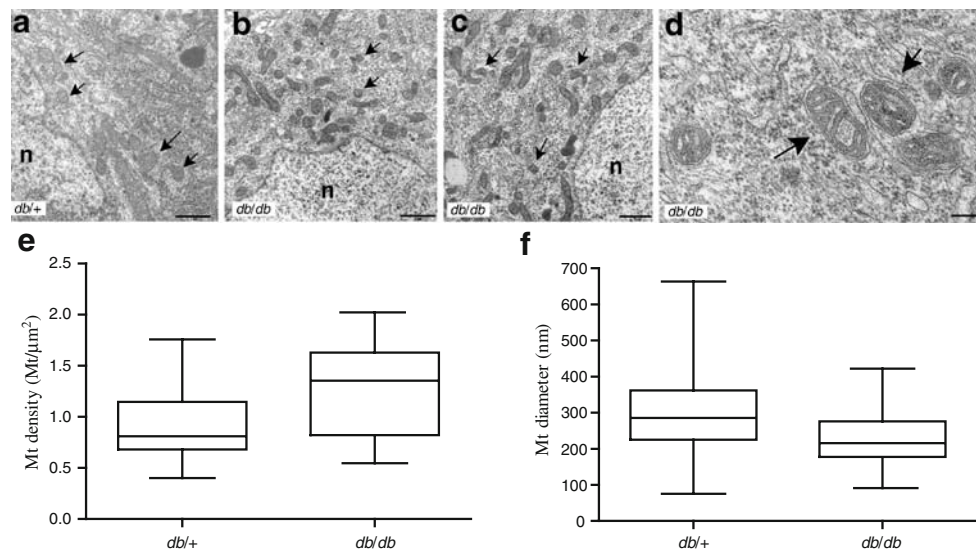
sis of the diabetic mitochondria indicated an energised state (activity in electron transport chain complexes) with dilated intercrystal spaces and increased matrix density (Fig. 4d).

A quantitative evaluation of mitochondria in randomly selected DRG neurons from 24-week-old BKS-*db/db* and *db/+* mice (Fig. 4) detected an increase in mitochondrial density ( $1.283 \pm 0.06$  mitochondria/ $\mu\text{m}^2$  in BKS-*db/db* vs  $0.9138 \pm 0.05$  mitochondria/ $\mu\text{m}^2$  in *db/+*;  $p < 0.0001$ ) (Fig. 4e). Mitochondria from BKS-*db/db* mice exhibited reduced diameter compared with *db/+* ( $219.6 \pm 3.3$  nm vs  $292.4 \pm 5.8$  nm; number of mitochondria=404–281;  $p < 0.0001$ ) (Fig. 4e). These results indicate that prolonged hyperglycaemia alters mitochondrial dynamics as well as morphology in vivo.

**Hyperglycaemia increases mitochondrial biogenesis and fission in DRG neurons in vitro** To further investigate hyperglycaemia-induced mitochondrial biogenesis, we examined DRG neurons under hyperglycaemic in vitro conditions and assessed mtDNA replication and protein expression. Dissociated DRG neurons were incubated in control (25 mmol/l) and high-glucose (45 mmol/l) medium



**Fig. 3** Prolonged hyperglycaemia significantly increased protein levels of PGC1- $\alpha$ , COX-IV and DRP1. **a**, **b** Immunoblots of mitochondrial proteins from DRG neurons of control (*db/+*) and diabetic (*db/db*) mice at age 5 weeks (**a**) and 24 weeks (**b**). Blots were analysed using antibodies against PGC1- $\alpha$ , DRP1, COX-IV and actin. Each lane represents DRG lysates from different animals. Numbers under the bands indicate density relative to actin



**Fig. 4** Hyperglycaemia leads to altered mitochondrial dynamics and morphology in DRG neurons in vivo. **a–d** Transmission electron micrographs of mitochondria from control (**a**) and diabetic (**b–d**) neurons. Arrows indicate mitochondria. **d** Mitochondria from DRG neuron from diabetic (*db/db*) mice show a slightly dilated intracristal space but the matrix is still dense. n, nucleus. Scale bars: **a–c**, 1  $\mu\text{m}$ ; **d**,

180 nm. **e** Quantitative analysis of mitochondria (Mt) in DRG neurons from 24-week-old mice shows an increase in the number of mitochondria in diabetic (*db/db*) compared with control (*db/+*) mice;  $p < 0.0001$ . **f** Mt from diabetic (*db/db*) mice showed reduced diameter compared with control (*db/+*);  $p < 0.0001$ ,  $n = 2$

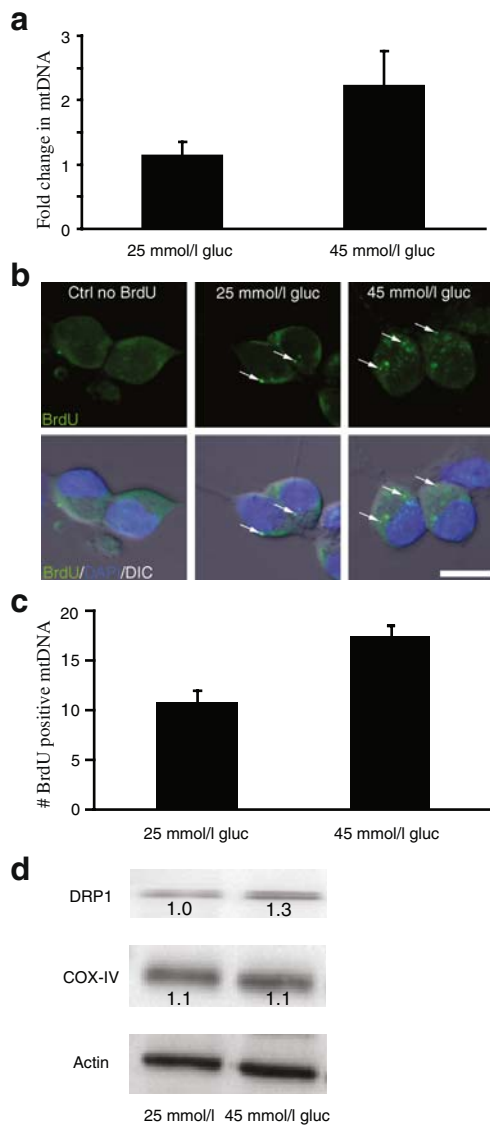
for 6 h and mtDNA was quantified (Fig. 5). A twofold increase in cytochrome *b* DNA was detected in DNA isolated from DRG neurons grown in high-glucose medium compared with control medium (Fig. 5a). Replication of mtDNA was also measured in individual DRG neurons by BrdU incorporation. DRG neurons exposed to high glucose for 6 h showed an increase in the number of sites where mtDNA was labelled with BrdU (Fig. 5b). The increase in BrdU-positive mtDNA in individual DRG neurons treated with high glucose was comparable to the increase measured by isolated DNA (Fig. 5c). Mitochondrial COX-IV protein levels were not changed following exposure to high glucose; however, there was an increase in DRP1 levels (Fig. 5d), in agreement with our previous studies [1]. Increased abundance of DRP1 suggests increased mitochondrial fission, while stable COX-IV abundance suggests no increase in mitochondrial mass. Together, these findings suggest that incomplete mitochondrial biogenesis or over-activity of mitochondrial fission occurs during short-term hyperglycaemia.

Mitochondrial fission may either promote or diminish the viability of stressed cells [32, 33]. To investigate the role of mitochondrial fission in cell viability under hyperglycaemic conditions, *Drp1* expression was decreased using lentivirus-administered miRNA (Fig. 6). Infection of DRG neurons with mouse anti-*Drp1* miRNA constructs produced knockdown of the *Drp1* expression compared with that in uninfected neurons cultured in low-glucose medium. Scrambled (non-specific) miRNA had no significant effect on *Drp1* expression. Cleaved caspase-3 was

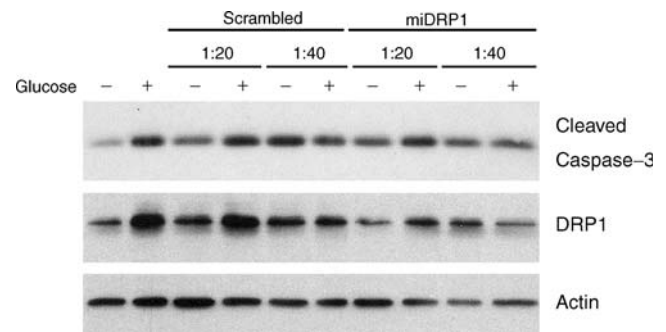
used to evaluate cell viability. Neuronal levels of cleaved caspase-3 were elevated in response to 6 h of exposure to the high-glucose condition compared with the normal glucose condition. Treatment of DRGs with scrambled miRNA increased cleaved caspase-3 activation under both normal and high-glucose conditions, indicating some damage occurred from the lentiviral infection. Neurons treated with *Drp1* miRNA showed a decrease in cleaved caspase-3 compared with lentivirus control vector infection. These data were corroborated using 20  $\mu\text{mol/l}$  of the specific DRP1 chemical inhibitor mdiv-1 [34], demonstrating that chemical inhibition of DRP1 reduces levels of cleaved caspase-3 in response to exposure to high glucose in vitro (data not shown).

## Discussion

Previous work in our laboratory documented the role of oxidative stress and mitochondrial dysregulation in type 1 and 2 rodent models and in cell culture models of diabetic neuropathy [4, 6, 20, 28]. The present study extended these investigations by examining the impact of hyperglycaemia on mitochondrial networks in vitro and in vivo and the balance between mitochondrial biogenesis and mitochondrial fission. Here we demonstrate (1) increased mitochondrial biogenesis in vivo and in vitro, i.e. an increase in measures of mtDNA replication; (2) an increase in mitochondrial fission with continued hyperglycaemia, as



**Fig. 5** Hyperglycaemia increases mitochondrial biogenesis and fission in DRG neurons in vitro. **a** Dissected and dissociated rat embryonic day 15 DRGs were incubated in the presence of control glucose (gluc) (25 mmol/l) or high-glucose medium (45 mmol/l) for 6 h. Quantification of cytochrome *b* DNA was used as a marker for mtDNA.  $n=3$ . **b** In vitro analysis of BrdU incorporation into mtDNA of DRG neurons under hyperglycaemia. DRG neurons were cultured in the presence (or absence; control [Ctrl], no BrdU) of BrdU and normal (25 mmol/l) or high glucose (45 mmol/l) for 6 h. BrdU incorporation into mtDNA (arrows) was visualised by immunocytochemistry with tyramide-amplified Alexa Fluor 488 green signal (top panels) and merged with nuclear staining (DAPI, blue) and differential interference contrast (DIC) images (lower panels). Cells were visualised using a laser scanning confocal microscope. Bar, 10  $\mu$ m. **c** mtDNA was measured by identifying the number of sites where BrdU-positive mtDNA were present in each DRG soma. In vitro cultures were incubated in control or high-glucose medium for 6 h.  $n=10$  per group. **d** Western blot analysis of in vitro cultures of DRG after exposure to control or treatment medium. Protein lysates were subjected to SDS-PAGE and analysed with antibodies against COX-IV, DRP1 and actin (internal control)



**Fig. 6** DRP1 promotes hyperglycaemia-induced cell death in sensory neurons. Cultured embryonic day 15 rat DRG neurons were lentivirally infected (1:20 or 1:40 dilution) with non-specific miRNA (scrambled) or *Drp1*-specific miRNA (miDRP1), or were not infected (control). Neurons were treated with high-glucose (+) or control (-) medium. Cell extracts were subjected to SDS-PAGE and analysed by western blotting with antibodies for cleaved caspase-3, DRP1 and actin (internal control)

assessed by an increase in the number of mitochondria in neurons from mice with diabetic neuropathy—these mitochondria are small and fragmented with a pattern of cristae that indicates dysfunction; and (3) that blocking mitochondrial fission in a short-term model of hyperglycaemia-mediated injury promotes neuronal survival. Our results suggest that neurons initially respond to hyperglycaemia by increasing both mitochondrial biogenesis and mitochondrial fission but, over time and with the accumulation of ROS, the balance favours mitochondrial fission, producing small aberrant mitochondria with reduced respiratory capacity.

Mitochondrial networks are dynamic and respond to external signals by increasing the actual mass of the network (biogenesis) as well as dispersing the existing mitochondria into a larger network (fission). This was explored by examining both mitochondrial biogenesis and fission in the sciatic nerve of diabetic (BKS-*db/db*) and non-diabetic (*db/+*) mice. Whole-nerve microarray studies [26] of mtDNA indicate that mitochondrial biogenesis occurs in mice with diabetic neuropathy and previous studies link mitochondrial biogenesis with increased metabolic load on mitochondria [35, 36]. We detected an increase in mtDNA indicative of mitochondrial biogenesis after 20 weeks of diabetes in the BKS-*db/db* mice. An increase in mitochondrial mass is also supported by the increased expression of mitochondrial-specific *Cox-IV* mRNA and protein. Upregulation of mitochondrial biogenesis as a means of accommodating the elevated glucose load is consistent with our hypothesis that neurons under hyperglycaemic conditions are subjected to metabolism-induced oxidative stress [37, 38]. In NT2 cells, a model system for central nervous system neurons, increased mitochondrial biogenesis is correlated with a reduction in mitochondrial oxidative stress [38]. Similarly, promotion of mitochondrial biogenesis in endothelial cells prevents

hyperglycaemia-induced oxidative stress [39]. These studies demonstrate a connection between mitochondrial biogenesis and the capacity to handle metabolic load. Our investigations demonstrate that, in peripheral sensory neurons, mitochondrial biogenesis is activated in response to diabetes. We propose that mitochondrial biogenesis is acting as a defence mechanism in response to hyperglycaemia-induced stress in diabetic DRG neurons.

Mitochondrial fission is a coordinate and/or alternative response to an increase in metabolic load. This strategy is frequently used by neurons under increased physiological stress. Increased mitochondrial fission is essential in hippocampal neurons, which require active mitochondrial fission for the formation, function and maintenance of synapses, processes that place a high metabolic demand on neurons [8, 40]. In DRG neurons, mitochondrial biogenesis probably acts as a protective mechanism against glucotoxicity; however, over time, the cell exhausts this capacity, resulting in mitochondrial fission without replication of mtDNA. In the DRG of the BKS-*db/db* mice, 20 weeks of diabetes induced an increase in the number of mitochondria but a marked decrease in mitochondrial size, indicating an increase in the rate of mitochondrial fission. These data suggest that the mitochondrial biogenesis occurring in diabetic neurons is insufficient to produce healthy mitochondria, that the balance is towards mitochondrial fission and that continued fission results in aberrant mitochondrial morphology. Damaged mitochondria demonstrate altered morphology, breakdown of cristae and loss of function [4, 25, 41–43]. Changes in mitochondrial morphology are classified into five categories, progressing from normal to vesicular to swollen, the swollen state coinciding with the beginning stages of cell death [31]. Our data document increased numbers of mitochondria with dysfunctional morphology corresponding to the intermediate stages of cell damage, normal-vesicular and vesicular. The increased density (darkened appearance) of the mitochondrial matrix indicates that the mitochondria have decreased electron transport complex activity. Taken together, our findings show that, in vivo, diabetic neurons undergo (1) mitochondrial biogenesis, albeit insufficient; (2) morphological changes of mitochondria, and (3) biochemical dysfunction of mitochondria.

To further explore the effects of hyperglycaemia on mitochondrial biogenesis and fission in neurons, DRG neurons were exposed to high-glucose medium in vitro. DRG neurons exhibited a significant increase in mtDNA; however, no change in mitochondria-specific proteins, such as *Cox-IV*, was detected. The increase in mtDNA without a simultaneous increase in mitochondrial protein is probably due to a combination of oxidative stress and temporal effects. Low levels of oxidative stress increase mtDNA copy number, whereas excess oxidative stress damages and

inhibits mitochondrial biogenesis [44]. The lack of a detectable increase in mitochondria-encoded proteins may be due to insufficient time for mitochondrial protein translation following DNA replication, or to damage of the mitochondrial translation machinery by hyperglycaemia-induced oxidative stress. Our previous studies of hyperglycaemia-induced oxidative stress reveal a biphasic response with early (up to 6 h) and late (after 12 h) changes in gene and protein expression [4]. Further studies comparing the timing of the antioxidant response and mitochondrial biogenesis and fission are under way to clarify this issue.

The increased number of mitochondria and their diminished size indicate that mitochondrial fission, a component of mitochondrial biogenesis, may be overactive in diabetic neurons. Regulation of the fission protein DRP1 in vivo and in vitro supports this idea. We hypothesised that depletion of DRP1 in embryonic DRG neurons would lead to increased viability. Depletion of DRP1 by miRNA had a rescuing effect on short-term neuronal glucotoxicity, as indicated by diminished cleaved caspase-3. Thus, in vitro, we found that inhibition of DRP1 diminished hyperglycaemia-mediated cellular dysfunction. Further confirmation of this idea comes from recent reports of altered DRP1 levels in cortical neurons from patients with Alzheimer's disease that correspond to changes in neuronal mitochondrial number and distribution [45]. Previous work has also shown that dysfunction in the fission–fusion machinery leads to inherited neuropathies [46, 47] and that inhibition of DRP1 renders cells resistant to dysfunction [32]. Collectively, these studies demonstrate the importance of the fission–fusion machinery in neuronal dysfunction and implicate imbalances in the fission–fusion machinery in the pathogenesis of diabetic neuropathy. Furthermore, DRP1 is in part regulated by post-translational modifications which facilitate the cellular distribution and/or stability of DRP1, which is important for the promotion of fission [48–50]

In summary, our findings suggest an increase in mitochondrial biogenesis and fission in response to hyperglycaemia in both in vivo and in vitro models of diabetic neuropathy. Although mitochondrial numbers increase in neurons from diabetic mice, these mitochondria are small and have a dysfunctional morphology, suggesting excessive upregulation of mitochondrial fission. Knockdown of *Drp1*, a key regulator of mitochondrial fission, rescues mitochondria from short-term glucotoxicity in vitro. Despite the short-term beneficial effects of *Drp1* knockdown, its role in the long-term development of diabetic neuropathy remains to be defined. Further studies are currently under way to dissect the role of mitochondrial biogenesis and fission events in glucotoxicity and to determine the efficacy of site-directed in vivo knockdown of *Drp1* in supporting neuronal survival in diabetic conditions.



**Acknowledgements** We would like to thank J Boldt for her expert secretarial assistance, K Sullivan for careful reading of the text and KM Haines for assistance with BrdU signal analysis. This work was supported by National Institute of Health T32 D07245, the Juvenile Diabetes Research Foundation Center for the Study of Complications in Diabetes, the Program for Neurology Research and Discovery (PNRD) and the A. Alfred Taubman Medical Research Institute. This work utilised the Morphology and Image Analysis Core of the Michigan Diabetes Research and Training Center funded by NIH5P60 DK20572 and UO1 DK076160 from the National Institute of Diabetes & Digestive & Kidney Diseases.

**Duality of interest** The authors declare that there is no duality of interest associated with this manuscript.

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