Development of Selective Axonopathy in Adult Sensory Neurons Isolated from Diabetic Rats: Role of Glucose-induced Oxidative Stress

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ABSTRACT

Objective. Reactive oxygen species (ROS) are pro-oxidant factors in distal neurodegeneration in diabetes. We tested the hypothesis that sensory neurons exposed to type 1 diabetes would exhibit enhanced ROS and oxidative stress and determined if this stress was associated with abnormal axon outgrowth.

Research Design and Methods. Lumbar dorsal root ganglia (DRG) sensory neurons from normal or 3-5 month streptozotocin (STZ)-diabetic rats were cultured with 10mmol/l or 25-50mmol/l glucose. Cell survival and axon outgrowth were assessed. ROS were analyzed using confocal microscopy. Immunofluorescent staining detected expression of manganese superoxide dismutase (MnSOD), adducts of 4-hydroxy-2-nonenal (4-HNE) and MitoFluor green dye detected mitochondria.

Results. DRG neurons from normal rats exposed to 25-50mmol/l glucose did not exhibit oxidative stress or cell death. Cultures from diabetic rats exhibited a 2-fold (P<0.001) elevation of ROS in axons after 24 hrs in 25mmol/l glucose compared with 10mmol/l glucose or mannitol. Perikarya exhibited no change in ROS levels. Axonal outgrowth was reduced by approximately 2-fold (P<0.001) in diabetic cultures compared with control, as was expression of MnSOD. The antioxidant 1mmol/l N-acetyl-cysteine lowered axonal ROS levels, normalized aberrant axonal structure and prevented deficits in axonal outgrowth in diabetic neurons (P<0.05).

Conclusions. DRG neurons with a history of diabetes expressed low MnSOD and high ROS in axons. Oxidative stress was initiated by high [glucose] in neurons with a STZ-induced diabetic phenotype. Induction of ROS was associated with impaired axonal outgrowth and aberrant dystrophic structures that may precede or predispose the axon to degeneration and dissolution in human diabetic neuropathy.
Diabetic sensory polyneuropathy in humans and animal models is associated with a spectrum of structural changes in peripheral nerve that includes microangiopathy, axonal degeneration, segmental demyelination, and ultimately loss of both myelinated and unmyelinated fibers (1; 2). High glucose concentrations induce toxicity and cell death in sensory neurons and this triggers diabetic neuropathy through loss of nerve fibers (3). Cultured embryonic dorsal root ganglion (DRG) sensory neurons were exposed to high, non-physiological, concentrations of glucose that induced oxidative stress by increasing production of reactive oxygen species (ROS) and this was associated with mitochondrial dysfunction which resulted in programmed cell death (4-6).

Morphological studies have provided a variety of results in relation to sensory neuron survival in animal models of diabetes. Long term studies of 9 months in streptozotocin (STZ)-diabetic mice revealed a significant loss of sensory neurons (7). In STZ-diabetic rats of up to 12 month duration no significant loss of adult lumbar DRG neurons was observed (8; 9). Additionally, in 4 month diabetic BB rats there was no DRG sensory neuron cell death (10), however, by 10 months there was progressive neuronal loss, but prominent only in the small DRG neuron population and not involving apoptosis (11). At the same time there was a significant decrease in numbers of myelinated and unmyelinated fibers, but no evidence of structural changes in mitochondria in DRG sensory neurons (11). In STZ-diabetic mice where loss of small neurons was also occurring there was no sign of activation of pro-apoptotic markers p38, caspase-3, and phosphorylated c-jun (12).

Sural nerve from humans with diabetic neuropathy assessed using quantitative morphometry have significant endoneurial microangiopathy, early structural abnormalities in Schwann cells in myelinated fibers and degeneration and loss of unmyelinated and myelinated fibers, however, in intact axons mitochondria appeared structurally normal (1; 13). In addition, studies performed on post-mortem samples from type 2 diabetic patients have shown the occurrence of dystrophic changes in axon terminals and within the DRG and autonomic ganglia but no evidence for significant neuronal cell loss (14; 15).

These results show that in vivo, in animals and humans, the impact of diabetes on sensory neuron survival are discordant with the in vitro studies demonstrating toxic effects of high glucose concentration leading to apoptosis. We hypothesized that the underlying reason for this discrepancy was the use of embryonic sensory neurons for in vitro glucose toxicity studies (3). Cultured embryonic sensory neurons have phenotypic differences with adult sensory neurons and are dependent on neurotrophic factor-derived support for survival (16). Therefore, the aim of this study was to compare responses of adult DRG sensory neurons from age matched control and 3-5 month STZ-diabetic rats exposed to high glucose concentration. To this end, the effect of high glucose concentration on oxidative stress and neuronal survival and axonal morphology was assessed.

**RESEARCH DESIGN AND METHODS**

**DRG sensory neuron cultures.** DRG sensory neurons from adult Sprague-Dawley male rats were isolated and dissociated using a described method (17-19). Rats were age matched control or 3-5 month STZ-diabetic that included cohorts receiving insulin implants (LinShin Canada, Inc., North Scarberough, ON, Canada; 2 Linplant implants placed subcutaneously). Rats were made diabetic with a single i.p. injection of 75
mg/kg STZ (Sigma, St Louis, MO). Endpoints for body weight, plasma glucose and HbA1c are presented in Table 1 (supplementary section). Cells were plated onto poly-d-L-ornithine and laminin-coated 12-well plates (Nunclon Surface, Ottawa, Ontario) for the neuronal survival study, 25 mm glass cover slips (Electron Microscopy Sciences, Hatfield, PA, German glass #1) for neurite outgrowth and for immunocytochemistry and 35 mm glass bottom dishes for ROS measurement (Bioscience Tools, San Diego, CA, Cat# W20). Neurons were cultured in Ham’s F12 medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS, HyClone, Logan, UT) for long term cultures (>5 days). For short term cultures neurons were grown in defined medium with modified Bottenstein and Sato’s N2 medium containing: 0.1 mg/ml transferrin, 20 nmol/l progesterone, 100 µmol/l putrescine, 30 nmol/l sodium selenite, 1 mg/ml BSA and 0.01 mmol/l cytosine arabinoside (the modified N2 formulation used did not include insulin; all obtained from Sigma). Neuronal survival was quantified by established methods (20; 21). Viable neurons were counted before experimental treatment and at time points following treatment. Neurons that died in the intervals between examination points were absent, and the viability of the remaining neurons was assessed by morphological criteria. Neurons with membranes and soma with a smooth round appearance were viable, whereas neurons with fragmented or distended membranes and vacuolated soma were nonviable. Four images were collected from each well and from the same place using marked grids on each well bottom at 1, 4, 7, 21 and 28 days using a light microscope (Nikon Diaphot, phase contrast). Survival was confirmed by trypan blue exclusion at the last time point. For neurite outgrowth measurements images of cultures were collected from 10 random fields from each well. The longest axon and total number of neurons and number of intersects of their neurites with a vertical grid were counted using a morphometric approach and using SigmaScan Microsoft software. The total number of intersects per one neuron was taken as the parameter of total axon outgrowth as described (22).

**Reactive oxygen species (ROS) measurement.** ROS levels were detected using real time fluorescence microscopy on a Carl Zeiss LSM510 inverted confocal microscope using two dye-based approaches: (i) dihydrorhodamine 123 (DHR 123) and (ii) 5-(and-6)-chloromethyl-2’7’-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA). Neurons were loaded with 5 µmol/l of DHR123 (Sigma, in 100% EtOH) or 1.2 µmol/l CM-H2DCFDA (Molecular Probes, in 100% anhydrous DMSO) for 30 or 15 min respectively at 37°C, then washed 3x with F-12 and visualized using the argon laser (at 2 % power). For DHR123 excitation (max) was 505 nm and emission (max) was 534 nm, and for CM-H2DCFDA excitation (max) was 488 nm and emission collected above 510 nm. Images were collected and analyzed as pixel intensity at the level of the axon or cell body. For axonal signals all axons in each field were assessed and using the Carl Zeiss software package a line was placed along the length of the axon and pixel intensity collected per µm of axon. Control experiments were performed to confirm that the DHR123 and CM-H2DCFDA signals were sensitive to antioxidant (N-acetyl-cysteine) or pro-oxidant (H2O2) treatment (not shown).

**Immunocytochemistry for activated caspase 3, 4-hydroxy-2-nonenal (4-HNE), manganese superoxide dismutase (MnSOD) and phosphorylated neurofilament H (NFH).** DRG neurons were fixed with 4% paraformaldehyde in phosphate buffer at pH 7.4 for 15 min followed by permeabilizing with 0.3 % Triton X-100 in
PBS. Nonspecific binding was blocked by incubation with blocking reagent combined with FBS and 1.0 mol/l PBS in proportion 3:1:1 (Roche, Indianapolis, IN, Cat# 1 096 176) for 1 hr at room temperature and washed with PBS three times. Cells were then incubated with antibodies to β-tubulin isotype III (Sigma, Oakville, ON, 1:1000), caspase 3 (Chemicon, Temecula, CA, 1:100), (E)-4-hydroxy-2-nonenal adducts (anti-4-HNE adducts PAb) (Alexis Biochemicals, San Diego, CA, 1:500), MnSOD (StressGen, Ann Arbor, MI; 1:300) and phosphorylated neurofilament H (NFH) (SMI-31; Covance, Berkeley, CA, 1:500). For localization of mitochondria live cultures of DRG neurons were treated for 15 min with 200 nmol/l MitoFluor Green dye (Molecular Probes, Eugene, OR) before being fixed for immunostaining for anti-NFH. Primary antibodies were incubated with slides overnight in a humidified chamber followed by FITC- and CY3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, 1:250) for 3 hrs at room temperature. Fluorescence signals were examined and quantified using a Carl Zeiss Axioscop-2 mot microscope with AxioVision 3 software and equipped with FITC, CY3, DAPI filters, and AxioCam camera.

**Data analysis.** Because of the technically difficult nature of the cultures and the image acquisition not all images were collected in a blinded fashion although a random approach was taken for image capture from each well. Where appropriate, data were subjected to one-way ANOVA with posthoc comparison using Tukey’s test (GraphPad Prism 4, GraphPad Software Inc., San Diego, CA). In all other cases standard two-tailed unpaired t-Test with Welch’s correction was performed, which does not assume equal variances with significance levels of p=0.05 using GraphPad Prism 4.

**RESULTS**

Initial experiments were designed to test the influence of high concentration of glucose (50 mmol/l) on survival of sensory neurons in culture, the induction of apoptosis and markers of oxidative stress. Sensory neurons from adult control rats were grown up to 4 wks in F12 medium with 10% FBS. Healthy neurons were phase bright and by 1wk cultures exhibited high numbers of non neuronal cells including Schwann cells, satellite cells and fibroblasts (Fig.1A and B). High glucose concentration had no effect on survival of neurons at any time and did not affect axon outgrowth at 1 day (Fig.1C and insert). Surviving DRG neurons did decline over time and had diminished by 50% by the 28th day; however, there was no effect of 50 mmol/l glucose on this process. Oxidative stress and/or induction of apoptosis was assessed by staining for adducts of 4-HNE and caspase 3 activation. There was no effect of 50 mM glucose on caspase 3 or 4-HNE adduct expression in neuronal perikarya at 2 or 4 weeks (Fig.1D and E). However, there was a small (10-20%), but statistically significant, increase of activation of caspase 3 and 4-HNE adduct expression in 4 week versus 2 week group of DRG neurons; this effect was not perturbed by 50 mmol/l glucose.

A second set of experiments tested if high glucose concentration induced oxidative stress in neuronal perikarya or axons in cultures of adult sensory neurons under defined conditions in F12 + modified N2 medium. To assess oxidative stress a real time video microscopy approach was taken using the fluorescent dye CM-H_2DCFDA. This dye is believed to give a read out of levels of cellular ROS, including peroxynitrite, hydrogen peroxide and hydroxyl radicals. Neurons were grown 24 hours under control (10 mmol/l glucose) or high glucose (25 mmol/l). Fig. 1 (supplementary section) shows no effect of 25
mM glucose on CM-H$_2$DCFDA intensity in neuronal perikarya (Fig. 1A supp.) or axons (Fig. 1B supp.). High [glucose] did not elevate 4-HNE adducts in cell body (Fig. 1C supp.) or axon (Fig. 1D supp.).

We next determined if cultured neurons from STZ-diabetic rats were also refractory to high [glucose]. Neurons from age matched control rats were grown in defined F12 + modified N2 medium under low (10 mmol/l) glucose with 10 nmol/l insulin, whereas neurons from 3-4 month STZ-diabetic rats were grown in F12 + modified N2 medium with 25 mmol/l glucose with no insulin support (to attempt to mimic diabetes in vivo). Cell survival of neurons from normal and diabetic rats over a 4 day period and under defined conditions were identical. Fig. 2 shows the phase contrast images of sensory neurons from control (Fig. 2A; upper and lower panels) and STZ-diabetic rats (Fig. 2B) after 4 days in culture. Diabetic DRG neurons exhibited abnormal morphological changes in neurite outgrowth, which were characterized by less axonal outgrowth (Fig. 2C) and appearance of phase dark axonal swellings and beading along neurites (see inset in Fig. 2B lower panel; white arrows). Separate cultures from control or STZ-diabetic rats were grown for 1 day and assessed for level of ROS in cell bodies and axons using CM-H$_2$DCFDA (Fig. 3A, B and E) or DHR123 (Fig. 3C, D and F) fluorescence imaging. ROS levels in cell bodies did not differ between control and diabetic neurons (not shown). Axonal ROS levels in neurons from STZ-diabetic rats showed at least a 2-fold elevation compared to low [glucose] (Fig. 5E). High [mannitol] revealed an intermediate level of ROS production in axons of sensory neurons from STZ-diabetic rats was measured after 3 days of culture (Fig. 5C and D). High [glucose] induced a significant increase in 4-HNE adduct accumulation in axons compared with group with 10 mmol/l glucose and mannitol (Fig. 5F). Neurons isolated from STZ-diabetic rats treated for final month with insulin that significantly lowered hyperglycemia (see Table 1 – supplementary data) did not respond to high glucose with elevated ROS or 4-HNE accumulation in axons (Fig. 5E and F). Studies were also performed where STZ-diabetic neurons were treated with 10 nM insulin in presence of low or high [glucose], under such conditions there was no impact on ROS or 4-HNE levels of the insulin treatment (data not shown). In addition, an alternative control was used for
mannitol, 15 mmol/l L-glucose, and results were the same as seen with mannitol.

Experiments now determined if oxidative stress was causally linked to aberrant axonal structure in neurons derived from STZ-diabetic rats. The antioxidant, N-acetyl cysteine (NAC), was tested for its ability to lower ROS. Cultures from control rats were assessed for ROS using DHR123 imaging and treated acutely with 1 mmol/l NAC (Fig. 6). Fig. 6A-D show that NAC rapidly neutralized ROS levels. In a separate experiment STZ-diabetic cultures were plated for 1 day in the presence or absence of 1 mmol/l NAC and effect on axonal outgrowth determined; NAC was clearly able to enhance levels of total axonal outgrowth (Fig. 6E). Cultures of sensory neurons from control or STZ-diabetic rats were maintained for 3 days (at this time point diabetic neurons exhibit 4-HNE staining – see Fig. 5C and D). During the last 24 hours of culture control and STZ-diabetic neurons were treated with 1 mmol/l NAC for a further 24 hours. After 4 days all groups of cultures were treated with MitoFluor Green dye to specifically stain mitochondria and then fixed and immunostained for phosphorylated NFH. Fig. 7A and B show control neurons with normal axonal structure and uniform axonal localization of mitochondria. Cultures from STZ-diabetic rats demonstrated abnormal accumulations of mitochondria in axons that colocalized with phosphorylated NFH staining (Fig. 7C and D; white arrows). Treatment with 1 mmol/l NAC significantly lowered the number of axonal swellings in normal and STZ-diabetic cultures (Fig. 7E-G).

Increased ROS under high glucose concentration in diabetic neurons may reflect an impaired capacity to scavenge free radicals. Therefore, the effect of diabetes and high glucose on expression of MnSOD was assessed in isolated mitochondrial preparations from DRG and in cultured sensory neurons. In Fig. 9 is presented Western blot data demonstrating that MnSOD expression was reduced by 32% (P<0.005) in mitochondria isolated from DRG of STZ-diabetic rats and this was prevented by insulin therapy. Fig. 8A and B shows axonal staining of MnSOD of sensory neurons from age matched control rats cultured for 1 day under 10 mmol/l glucose. Results were similar under 25 mmol/l glucose (images not shown). Neurons from STZ-diabetic rats cultured under 10 mmol/l glucose for 1 day exhibited diminished immunostaining for MnSOD (Fig. 8C and D), however, if cultured in the presence of 25 mmol/l glucose then no alteration in MnSOD expression was observed (Fig. 8E and F). When cultures were maintained for 3 days in vitro the ability of 25 mmol/l glucose to maintain or raise MnSOD expression was significantly impaired (Fig. 8G and H).

**DISCUSSION**

This work demonstrates for the first time that high glucose concentration can induce oxidative stress and axonal degeneration in adult sensory neurons, but only if these neurons exhibit a STZ-induced diabetic phenotype. Neurons from normal rats did not exhibit oxidative stress or cell death under high glucose concentrations. High glucose concentration in neurons from diabetic rats triggered elevated ROS levels and adducts of 4-HNE specifically in axons, with the perikarya being unaffected, and this distally directed process of oxidative stress resulted in axonal swellings and axon degeneration. This neurodegenerative process may have been triggered or exacerbated by impaired antioxidant defenses, as evidenced by the diminished expression of MnSOD in axons. However, these neurons did not undergo any process of cell death. The appearance and content of the axonal swellings closely resembled dystrophic axons observed in human disease.
Diabetic neuropathy in cultured DRG neurons

Previous studies on cultures of embryonic sensory neurons have shown that high glucose concentration triggers oxidative stress, activation of apoptosis and death of neurons (4-6). *In vitro* studies by Russell et al and Feldman et al used glucose concentrations of 45-50 mmol/l and higher and showed using an array of markers of oxidative stress and apoptosis that these indices were enhanced in cultured embryonic neurons (3-6). Instead we found that survival of adult sensory neurons from lumbar DRGs of normal or diabetic rats did not change under high glucose concentration. This supports previous in vitro studies on sensory neuron cultures from adult rat and mouse (dissociated and explant) where exposure to a hyperglycemic environment failed to induce neuronal death over 1 to 8 days (23; 24). In addition, embryonic sensory neurons given time to mature in vitro for several weeks also become resistant to the apoptosis-inducing effects of high [glucose] (25). Differences in culture conditions between embryonic and adult preparations may play a role here, and in particular the presence of non-neurons in the mature and adult cultures may be protective to hyperglycemia.

However, this glucose-insensitivity of adult rat sensory neurons with respect to cell survival is in good agreement with *in vivo* studies on experimentally diabetic rodents (8-10; 12; 26). In the STZ and BB rat models of type 1 diabetes there is no loss of DRG neurons until 10-12 months of disease and by this time there is already extensive distal axon loss (9-11; 27). There are clearly a range of effects of STZ-induced diabetes on neuronal survival and fiber loss in rodent models, depending upon the rodent and/or strain. Clearly in rat models, either *in vitro* or *in vivo*, neuronal cell loss is not a feature of the pathogenesis that underlies distal axon loss. It should be noted that sensory neurons under diabetic conditions can express markers of apoptosis, e.g. activated caspase-3, but these indices are dissociated from cell death (8). These varied effects of the diabetic state on neuronal survival in the animal models must be judged with caution given the findings that neuronal cell loss in the human disease is not a major feature of the pathology. Studies on post-mortem lumbar DRG and sympathetic ganglia derived from type 2 diabetic patients show ultrastructure of the neuronal perikarya to be abnormal with signs of shrinkage and axonal dystrophy, however, no evidence of any cell loss (14; 15). This lack of clear neuronal cell loss occurs while there is overt distal axonal loss in the epidermis (28-30) and sural nerve (1; 2; 31) of type 1 and 2 patients.

The key finding of the current work is that high [glucose] induced oxidative stress in axons of sensory neurons isolated from STZ-diabetic rats. This effect was not due to osmotic stress and the presence or absence of insulin in the culture was irrelevant to the development of neurodegeneration. The failure of glucose to enhance such processes in normal neurons clearly reveals the importance of the ‘STZ-induced diabetic phenotype’ of neurons which we propose provides susceptibility of these neurons to glucose-induced metabolic stress. Whether such neurons have a metabolic memory related to previous high [glucose]-related stress is impossible to ascertain at this juncture. A more profitable line of reasoning relates to growth factor starvation and alterations in the phenotype of the neuron. During 3-4 months of STZ-diabetes the loss of neurotrophic support from insulin, glial cell line-derived neurotrophic factor, nerve growth factor and neurotrophin-3 has been well described and it is known that these growth factors combine to modulate sensory neuron gene expression (32-35). Of particular interest is the ability of insulin and neurotrophin-3 to correct abnormalities in mitochondrial function and calcium homeostasis (17; 18; 36). Therefore, in the cultures from diabetic rats studied herein it is
feasible that the ability to respond to glucose-induced oxidative stress was impaired as a consequence of sub-optimal mitochondrial function and dyshomeostasis of Ca^{2+} (37). We believe abnormalities in Ca^{2+} homeostasis leading to raised intracellular Ca^{2+} concentration in neurons in diabetes is important because this key molecular marker of neurodegeneration appears more rapidly in the sensory neurons with the longest axons (36). We know of no other molecular marker of disease that is specifically associated with lumbar DRG sensory neurons with the longest axons. This abnormal Ca^{2+} homeostasis could be due to global gene expression changes including affects on ER resident proteins (e.g. SERCA2) (38), in addition diabetes may impair expression of proteins associated with antioxidant defenses (e.g. MnSOD and thioredoxin-interacting protein) (39) and growth factor receptors (e.g. the p75NTR receptor) (40). Furthermore, sensitivity of sensory neuron axon outgrowth to extracellular matrix proteins is known to alter under stress, e.g. neurons become more sensitive to fibronectin at the expense of laminin following axonal injury (41). These changes in cellular phenotype could combine to sensitize the cell to high [glucose] and/or changes in growth factor support.

The axonal swellings observed in diabetic neurons were comprised of accumulation of phosphorylated NFH and mitochondria – as seen in dystrophic axonal swellings in autonomic ganglia and nerve, lumbar DRG and epidermal nerve fibers of skin in humans with diabetic neuropathy (14; 15; 42). We hypothesize that axonal swellings are sites of mitochondrial and structural protein (e.g. NFH) accumulation due to blockade of their axonal transport and the outcome is a dearth of functioning mitochondria and structural proteins at distal axonal sites. The absence of structural proteins and ATP generating organelles in the distal axon, where high [ATP] is required for optimal axonal plasticity in the form of axon sprouting and regeneration (43), will trigger pathological conditions that lead to an absence of efficient axonal plasticity. This process may precede or predispose the axon to degeneration and dissolution and distal axonal degeneration will follow. A key aspect is that the neurodegenerative process within the epidermis caused by the ‘STZ-induced diabetic state’ will not be repaired through normal regenerative processes since these repair functions will also be targeted. The diabetic state as envisaged would include toxic effects of high [glucose] in addition to abnormalities in neurotrophic support.

We believe these degenerative processes proceed more vigorously in the axon than the perikaryal region of sensory neurons and this explains the distal dying back nature of diabetic sensory polyneuropathy. The axonal environment during axon sprouting and regeneration would have a very high ATP demand, in fact, 50% of all ATP synthesized is required for growth cone motility during axon sprouting and regeneration (43). Also, ROS scavenging systems, primarily MnSOD may not be as efficient in the axon due to reduced expression. Fig.8 shows that sensory neurons with a STZ-diabetic phenotype can mount a MnSOD-based antioxidant defense in axons in response to high glucose, however, by 3 days in vitro the up-regulation of MnSOD expression shows signs of failing and may contribute to enhanced oxidative stress and associated aberrant axon structure (Fig.8H). These events may combine to place an additional load on mitochondrial bioenergetics not seen in the perikarya and sensitize the physiology of the organelle to elevations in Ca^{2+} and high [glucose].

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FIG. 1. High glucose concentration does not impair adult sensory neuron survival or induce oxidative stress. In (A) and (B) are shown phase contrast images of 2wk cultures of adult DRG sensory neurons grown in defined F12 + 10 % FBS medium with and without 50 mmol/l D-glucose. Note the phase bright neuronal perikarya and phase dark non-neuronal cells (mostly fibroblasts and Schwann cells). In (C) is shown levels of survival of DRG neurons for control (10 mmol/l D-glucose, open box) or 50 mmol/l D-glucose (closed box) over a 4 week period. Cell numbers were assessed by morphology under a phase contrast microscope. Values are means ± SEM, n=4 replicate cultures. The insert graph shows no difference in total axonal outgrowth at 24 hours. Values are means ± SEM, n=3 replicate cultures. In (D) and (E) are presented levels of 4-HNE adduct expression or caspase 3 activation in neuronal perikarya in arbitrary units of fluorescence intensity at 2 and 4 weeks of culture. Values are means ± SEM, n = 85–108 neurons; * p<0.05.
FIG. 2. Cultured sensory neurons from STZ-diabetic rats exhibit abnormal morphology and reduced levels of axon outgrowth. In (A) upper and lower panels are shown phase contrast images of neurons derived from normal rats at 4 days in culture in defined F12 + modified N2 medium with 10 nmol/l insulin. In (B) upper and lower panels are shown images of cultures derived from 3-4 month STZ-diabetic rats and grown in defined media with 25 mmol/l glucose and no insulin. White arrows show areas of abnormal axonal structure highlighted by swelling and beading. The insert shows a X3 image exhibiting the phase dark axonal swellings. White arrowheads are rare swellings in normal neurons. Black arrowheads are non-neurons. In (C) is quantification of axonal outgrowth, longest axon, after 1 and 4 days of cultures from control or STZ-diabetic rats. Values are means ± SEM, n = 58–97 axons; * p<0.001.
FIG. 3. Axons of sensory neurons from STZ-diabetic rats exhibit elevated level of ROS. In (A) and (C) are images of ROS levels in axons at 24 hours of adult DRG neuron culture from control rats. Cultures were stained for ROS using CM-H$_2$DCFDA (A) or DHR 123 (C) dye and cells were grown in defined F12 + modified N2 medium with 10 nmol/l insulin. In (B) and (D) are presented axonal ROS level at 24 hours of adult DRG neuron cultures from 3-4 month STZ-diabetic rats. Cells were grown in defined F12+N2 medium with 25 mmol/l glucose and without insulin. Solid arrows show axons that were quantified for ROS level. Open arrow indicates a non-neuronal source of ROS. Quantification of ROS accumulation in axons of DRG neurons from control and STZ-diabetic rats is shown in bar graph derived from CM-H$_2$DCFDA (E) or DHR 123 (F) staining. Values are means ± SEM, n = 55-64 axons; * p<0.001.
FIG. 4. Axons of neurons of STZ-diabetic rats exhibit swellings that includes adducts of 4-HNE. (A), (C) and (E) represent merged immunofluorescent images stained for 4-HNE (green) and neuron-specific β-tubulin III (orange) at 4 days of sensory neuron culture from control rats (blue nuclei stained with DAPI). Cells were grown in F12 + modified N2 defined medium with 10 mmol/l D-glucose and with 10 nmol/l insulin. (B), (D) and (F) show merged immunofluorescent images of cultures from STZ-diabetic rats grown in F12 + modified N2 defined medium with 25 mmol/l D-glucose and without insulin. White arrows show 4-HNE adduct accumulation in axon swellings in STZ-diabetic neurons. Red arrows show β-tubulin III accumulation in rare swellings without 4-HNE. Yellow arrows indicate non-neurons. Asterisks indicates perikarya strongly labeled for 4-HNE.
FIG. 5. High glucose concentration elevates ROS and adducts of 4-HNE in axons of sensory neurons from STZ-diabetic rats. (A) and (B) display images of sensory neuron cultures, which were assessed for ROS in axons at 1 day using CM-H$_2$DCFDA at (A) 10 mmol/l D-glucose or (B) 25 mmol/l D-glucose or 15 mmol/l D-mannitol (with 10 mmol/l glucose; image not shown) in defined F12 + modified N2 medium. (E) Quantification of ROS accumulation in axons of DRG cultures from control, diabetic or diabetic treated with insulin animals using CM-H$_2$DCFDA emission. Values are means ± SEM, n = 44-57 axons. * p<0.05 vs 25 mmol/l glucose. (C) and (D) show immunofluorescent images of accumulation of adducts 4-HNE in axons in sensory neuron cultures after 3 days in defined F12+N2 medium with (C) 10 mmol/l D-glucose or (D) 25 mmol/l D-glucose (individual puncta are indicated by white arrows; 15 mmol/l mannitol; image not shown). (F) displays levels of accumulation of puncta of adducts of 4-HNE in axons. Values are means ± SEM, n = 3-6 replicate cultures; ** p<0.05 vs other groups (one-way ANOVA with Tukeys posthoc comparison).
FIG. 6. Treatment with antioxidant N-acetyl cysteine lowers ROS levels in axons and elevates axon outgrowth. ROS levels, measured using DHR123, in axons (A) before and (B) after 10 min of treatment with 1 mmol/l NAC (asterisk indicates perikarya of neuron). (C) and (D) show real time imaging data of ROS levels in axons (C) before and (D) after 1 mmol/l NAC treatment. Values are means ± SEM, n = 4-6 axons. (E) presents total axon outgrowth for STZ-diabetic neurons cultured for 24 hours with/without 1 mmol/l NAC. Values are mean ± SEM, n=3 replicate cultures; * p<0.05.
FIG. 7. Axonal swellings in neurons from STZ-diabetic rats exposed to high glucose represent accumulations of mitochondria and phosphorylated NFH which are eliminated by antioxidant treatment. In left column of immunofluorescent images is Mitofluor green staining; the right column exhibits phosphorylated NFH staining (orange). Double staining is yellow. DRG sensory neuron cultures were derived from age matched control or 3-4 month STZ-diabetic rats and maintained in defined media for 4 days. STZ-diabetic rat cultures were grown in 25 mmol/l glucose without insulin. During the final 24 hr of culture time selected control or diabetic cultures were treated with 1 mmol/l NAC. (A) and (B) control, (C) and (D) STZ-diabetic and (E) and (F) STZ-diabetic culture treated for 24 hr with 1 mmol/l NAC. White arrows indicate areas of co-localization of mitochondria and phosphorylated NFH. (G) Quantification of number of axonal swellings in each treatment group. Values are mean ± SEM, n=3 replicate cultures; * p<0.05.
FIG. 8. Effect of STZ-diabetes on expression of MnSOD in axons of cultured sensory neurons. Lumbar DRG sensory neurons were isolated from age matched normal or 5 month STZ-diabetic rats and cultured with 5 mmol/l (control) or 25 mmol/l glucose for 1 or 3 days. Cells were fixed and immunofluorescently co-stained for β-tubulin III (red; neuron-specific) (A, C and E show double stain) and MnSOD (green; B, D and F show single stain). Charts in G (1 day) and H (3 days) show MnSOD expression in axons assessed by fluorescence intensity at X100 on confocal microscope. Values are means ± SEM (n = 65-94 axons). *P 0.05 vs other groups and **P <0.05.
FIG. 9. MnSOD expression is reduced in mitochondria of DRG from STZ-diabetic rats. DRG from 5 month control, STZ-diabetic or insulin treated diabetic rats were homogenized in mitochondrial isolation buffer containing: 10 mmol/l HEPES (pH 7.4), 200 mmol/l mannitol, 70 mmol/l sucrose, and 1 mmol/l EGTA. The DRG homogenate was centrifuged at 800 x g for 10 min at 4°C followed by centrifugation of the supernatant at 8000 x g for 15 min and mitochondrial pellet collected. Mitochondrial protein preparations (5 µg/lane) were resolved on a 12% SDS-PAGE gel and electroblotted (30V, 16 hr) onto nitrocellulose membrane. Blots were then blocked in 5 % nonfat milk containing 0.05 % Tween overnight at 4°C, rinsed in phosphate buffered saline (pH 7.4) and then incubated with rabbit anti-MnSOD polyclonal antibody (1:2000 dilution) overnight at 4°C. Extracellular signal-regulated kinase (ERK, Covance, Berkeley, CA; 1:2000) was probed as a loading control (previously shown not to change in diabetes in DRG). The blots were rinsed, incubated in Super Signal West Pico (Pierce Biotechnology, Rockford, IL) and imaged using the BioRad Fluor-S image analyzer. (A) shows the blots and (B) shows the chart where MnSOD signal has been presented relative to total ERK levels. Values are means + SEM (n=6). *P<0.005 vs control; **P=0.056 vs diabetic + insulin (one-way ANOVA with Tukeys).