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Mitochondria in DRG neurons undergo hyperglycemic mediated injury through Bim, Bax and the fission protein Drp1

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Dorsal root ganglia (DRG) neurons degenerate in diabetic neuropathy (DN) and exhibit mitochondrial damage. We studied mitochondria of cultured DRG neurons exposed to high glucose as an in vitro model of DN. High glucose sequentially increases the expression, activation and localization of the pro-apoptotic proteins Bim and Bax and the mitochondrial fission protein dynamin-regulated protein 1 (Drp1). High glucose causes association of Drp1/Bax, similar to other apoptotic stimuli. Collectively, these events promote mitochondrial fragmentation and reduce mitochondrial number, suggestive of apoptotic mitochondrial fission. Drp1 is also upregulated in DRG from experimentally diabetic rats, suggesting a role for mitochondrial fission in DN. Insulin-like growth factor-I (IGF-I) protects high glucose-treated DRG neurons by preventing mitochondrial accumulation of Bim and Bax but does not modulate Drp1 expression or localization. We propose that mitochondria are compromised by convergence of Bim/Bax proteins with Drp1, which contributes to high glucose-induced injury in DRG neurons.

Keywords: Dorsal root ganglion; Glucose; Bim; Bax; Drp1; IGF-I; Neuron; Apoptosis; Mitochondria; Fission

Introduction

Mitochondrial dysfunction and oxidative stress are common themes among neurodegenerative diseases (Kang and Hamasaki, 2005; Zeevalk et al., 2005; Beal, 2005; Xu et al., 2004; Le et al., 2005). Degeneration of dorsal root ganglia (DRG) neurons in hyperglycemic conditions underlies diabetic neuropathy (DN), but the pathogenetic mechanism(s) involved are poorly understood (The Diabetes Control and Complications Trial Research Group, 1993; Kalichman et al., 1998). Our laboratory has postulated that high glucose levels promote mitochondrial dysfunction, oxidative stress and ultimately neuronal death (Russell et al., 2002; Vincent and Feldman, 2004; Vincent et al., 2004b, 2005). We have shown that high glucose induces mitochondrial membrane depolarization, cytochrome *c* release, caspase-9 and -3 activation and apoptosis (Leinninger et al., 2004a; Russell et al., 2002). Furthermore, insulin-like growth factor-I (IGF-I), which prevents oxidative stress and apoptosis signaling in DRG neurons (Leinninger and Feldman, 2005; Leinninger et al., 2004a,b), promotes regeneration and restores nerve function (Leinninger et al., 2004c). Yet, it remains unclear how high glucose initiates, and IGF-I interrupts, mitochondrial apoptotic events.

Mitochondrial regulation of apoptosis may occur through the action of Bcl proteins. Apoptotic stimuli induce pro-apoptotic Bcl proteins (e.g., Bax or BH3-only proteins) and promote Bax conformational re-arrangement that activates and targets it to mitochondria (Schinzel et al., 2004). Mitochondrial Bax forms membrane channels, leading to cytochrome *c* release, activation of caspase-9 and apoptosis (Kuwana et al., 2002). BH3-only proteins including Bim are "sensors" of apoptotic stress located upstream of Bax (Puthalakath and Strasser, 2002). Bim binds to Bax, inducing its activation, mitochondrial insertion and channel formation (Kuwana et al., 2005). IGF-I could inhibit glucose-induced damage in DRG neurons through Bim and Bax protein expression [as it does in the central nervous system (Chrysis et al., 2001; Linseman et al., 2002)] or by inhibiting mitochondrial localization (Ness et al., 2004; Shibata et al., 2002).

High glucose may also interrupt the balance of mitochondrial fission and fusion, which control mitochondrial morphology and number (Rube and van der Bliek, 2004). The GTPases Mitofusin 1, Mitofusin 2 and Opa1 control fusion, while dynamin-regulated protein 1 (Drp1) and Fis1 mediate fission (Karbowski and Youle, 2003). Perturbation of the fusion/fission balance in favor of fission, or treatment with pro-apoptotic agents, induces mitochondrial fission and apoptosis (e.g., apoptotic fission) (Szabadkai et al.,

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2004; Lee et al., 2004; Frank et al., 2001). Apoptotic fission is preceded by BH3-only protein induction, Drp1 localization to mitochondria, Drp1:Bax co-localization at sites where mitochondria divide, inhibition of mitochondrial fusion and cytochrome *c* release (Jagasia et al., 2005; Karbowski et al., 2002, 2004). Interestingly, loss-of-function mutations in fusion proteins cause Charcot–Marie–Tooth neuropathy type 2A and dominant optic neuropathy (Zuchner et al., 2004; Alexander et al., 2000). Disrupted mitochondrial fusion may similarly contribute to DN.

In the current study, we examined hyperglycemia-induced mitochondrial events. High glucose promotes Bim localization to mitochondria, followed by Bax activation and cytochrome *c* release. These events precede the appearance of mitochondrial aggregates, typical of mitochondria that have undergone apoptotic fission. Mitochondrial number decreases after increased Drp1 expression, redistribution of Drp1 to mitochondria and Drp1:Bax binding. IGF-I protects DRG neurons by preventing Bax conformational change and cytochrome *c* release, which reduces fission-like mitochondrial aggregates. These data implicate Bim, Bax and Drp1 in mitochondrial damage after high glucose treatment. Additionally, Drp1 expression is increased in diabetic rat DRG, suggesting that mitochondrial fission contributes to the pathogenesis of DN.

Materials and methods

Cell culture

DRG neurons were isolated from E15 Sprague-Dawley rat embryos and cultured in NB+ media as described previously (Leinninger et al., 2004a). NB+ contains 25 mM glucose, which is optimal for DRG neuron survival (Russell et al., 1999), and is referred to as "control" media. DRG neurons require at least 45 mM glucose to induce apoptosis (Russell et al., 2002), thus, high glucose treatment media consist of control media plus 20 mM additional glucose. 45 mM glucose treatment (1.8-fold above control) is similar to the ≥1.4-fold increase in blood glucose concentration in a person diagnosed as diabetic (Mayfield, 1998). Thus, the relative concentration used in these studies is consistent with other models of hyperglycemia and human diabetes. For some experiments, 10 nM IGF-I (Cephalon) and 20 μM LY294002 (Calbiochem) were added with high glucose media.

Immunocytochemistry

DRG neurons were cultured and treated on collagen-coated glass cover slips, then fixed with 4% paraformaldehyde. Immunocytochemistry was performed as described previously (Leinninger et al., 2004a). Primary antibodies against Cox I (Molecular Probes) and Bim (Cell Signaling) were used at dilutions of 1:50. Antibodies against VDAC (Calbiochem), Bax N20 (Santa Cruz Biotechnology), cytochrome *c* (Pharmingen) and Drp1 (BD Transduction) were used at 1:100 dilutions. For co-labeling experiments, antibodies against Cox I or VDAC were used interchangeably to label mitochondria, depending on the species of the other antibody used in the experiment. Species appropriate AlexaFluor 488 and/or 594 fluorescent antibodies (1:1000, Molecular Probes) were used for secondary detection. Samples were analyzed at room temperature with an Olympus FluoView 500 laser scanning confocal microscope and a 60× water immersion objective (NA = 1.2). Images were acquired and magnified 3 times with FluoView 4.3 software. Images from 3 random fields were collected for each sample. Experiments were repeated in triplicate so that images in photomicrographs are representative of at least 9 separate fields across at least 3 sample sets.

Quantitation of mitochondria

The set of samples used to generate Figs. 4A–C were used for quantitational analysis. Briefly, DRG neurons were analyzed for Cox I by immunocytochemistry as described. Images were collected using a Perkin Elmer Ultraview© spinning disk confocal microscope system outfitted with a 60× oil immersion lens (Nikon, NA = 1.4) and an argon–krypton laser (excitation 500 nm, emission 530 nm), courtesy of Drs. Hao Xu and Martin Philbert (University of Michigan). A z-series (0.1-μm steps) was collected for each of 3 random fields per sample (3–11 neurons per field), spanning through neurons from top to bottom. Images from the center plane of each z-series were selected. Thus, subsequent results reflect an average number of mitochondria per single confocal plane, not a total number throughout the neuron. All further analysis was performed with the Matlab© image processing toolbox. Images were cropped to isolate individual neurons of each field. Cropped images were adjusted to grayscale and subjected to morphological top hat filtering. This operation is very important since it enhances the local contrast, rather than the global contrast, making it easier to identify individual mitochondria. Filtered grayscale images were then converted into black and white binary images. The area, aspect ratio, and number of isolated objects (mitochondria) were measured from each image. Graphed data show the average number of mitochondria per neuron (in the single confocal plane), and error bars represent the standard error of the mean (SEM). Significance of changes between samples was assessed by one-way ANOVA with Tukey's post-test.

Immunoblotting

DRG neurons were cultured in Biocoat collagen-coated tissue culture plates (BD Biosciences). After treatment, cell lysates were collected and analyzed by immunoblotting as described previously (Leinninger et al., 2004a). Equivalent amounts of protein were subjected to 15% SDS-PAGE and transferred to BioTrace PVDF (Pall). Membranes were blocked and probed with antibodies in 5% BSA/Tris-buffered saline pH 8.0 + 0.1% Tween-20 (TBS-T). Each blot was probed with an antibody against GAPDH at a dilution of 1:10,000 (Chemicon) to verify equivalent protein loading between samples. Antibodies against Drp1 (BD Transduction) and phosphorylated Akt (serine 473, Cell Signaling Technology) were used at 1:1000 dilutions.

Immunoprecipitations

Immunoprecipitations were performed with the Catch-and-Release™ Immunoprecipitation System (Upstate Biotechnology) according to manufacturer's instructions. DRG neuron lysates were collected as previously described (Leinninger et al., 2004a) and 250 μg of each lysate was diluted to 500 μl with lysis/wash buffer. Next, 4 μg of Bax 7480 antibody (Santa Cruz Biotechnology) and 10 μl of kit antibody capture affinity ligand were added to each sample. After mixing for 15 min, samples were centrifuged though spin columns (8 min, 4200 rpm). Columns were washed with lysis/

wash buffer and centrifuged 3× (3 min, 5000 rpm). Samples were eluted from columns and mixed with equal volumes of 2× sample buffer. Resulting samples were subjected to 15% SDS-PAGE, transferred, blocked, and probed with Drp1 antibodies as described above. Graphed results represent the average fold changes in binding as normalized to IgG bands ($n = 3$).

Protein isolation from control and diabetic rat DRG

Three-month-old male Sprague-Dawley rats were fasted overnight, then injected with 50 mg/kg streptozotocin (Sigma) and given 10% sucrose water. After 48 h, the animals were checked for the induction of diabetes by measuring tail blood glucoses with a Lifescan ONE TOUCH profile glucometer. Rats were euthanized after 12 weeks of diabetes, at which point DRG were removed (10–15/rat), flash frozen in liquid nitrogen, and stored at −80°C. Protein was isolated from DRG using TRIZOL© Reagent (Gibco) and dissolved in RIPA buffer with 1% SDS. Graphed results represent the average fold changes in binding ($n = 3$).

Densitometry of immunoblots

NIH Image 1.63 for Mac was used to calculate pixel densities from 300 dpi digital scans of immunoblot films. Pixel density was measured in a defined region of each band, and background pixel density was subtracted from each band value. The resulting value was divided by pixel density value of the control sample to determine a relative fold change. Graphed bars are averages of the fold change values. Error bars represent the standard error of the mean (SEM). Significance of fold changes between samples was assessed either by one-way ANOVA with Tukey or Dunnett's post tests or paired *t* test using GraphPad InStat version 3.0a for Macintosh (GraphPad Software). Asterisks denote significant changes.

Results

High glucose causes Bim localization at mitochondria

The BH3-only protein Bim initiates neuronal apoptosis by localizing with mitochondria (Putcha et al., 2001; Shibata et al., 2002). We have previously shown that high glucose increases Bim protein expression in DRG neurons, and that this increase is not attenuated by IGF-I (Leinninger et al., 2004a). We therefore speculated that high glucose promotes, while IGF-I prevents Bim translocalization to mitochondria. To address this, we examined Bim localization to mitochondria (identified by Cox I, a subunit of the mitochondrial oxidative complex IV) following glucose exposure (Fig. 1). DRG neurons treated with control media express a basal level of Bim that is not co-localized with mitochondria (Figs. 1A–A″). Bim expression increases following 1 h (Fig. 1B) and 3 h (Fig. 1C) of high glucose treatment compared to control treated cells (Fig. 1A). IGF-I does not attenuate increased Bim expression, in agreement with our previous results (Fig. 1D) (Leinninger et al., 2004a). After 1 h of high glucose treatment, Bim and Cox I co-localize diffusely in cell bodies (Figs. 1B–B″ arrowheads) and as punctate structures in neurites (Figs. 1B–B″ arrows). These effects are more pronounced at 3 h (Figs. 1C–C″). IGF-I decreases punctate Bim and Cox I staining in neurites but does not prevent diffuse co-localization in cell bodies compared to treatment with glucose alone (Figs. 1D–D″ compared to C–C″). Thus, IGF-I prevents apoptotic changes in most mitochondria but does not completely prevent mitochondrial translocation of apoptotic Bim. Collectively, these results suggest that high glucose increases Bim expression and translocation to mitochondria, and that IGF-I inhibits Bim translocation.

High glucose causes Bax activation and cytochrome c release

The BH3-only protein Bim activates downstream Bax, thereby inducing mitochondrial dysfunction, cytochrome *c* release, caspase activation and apoptosis (Okuno et al., 2004; Kuwana et al., 2005). However, Bax activation requires 3 steps: (1) exposure of an NH_2-terminal epitope of 20 amino acids occluded in inactive Bax, and (2) binding of a BH3-only protein that causes (3) exposure of the C-terminus, which contains the mitochondrial targeting and membrane insertion sequences (Cartron et al., 2004, 2005). The initiation of Bax activation is detected using an antibody against the NH_2-terminal epitope (the Bax N20 antibody) (Makin et al., 2001). We therefore examined the effect of high glucose on Bax activation and localization and subsequent cytochrome *c* release (Fig. 2). DRG neurons were treated with control media (A–A″) or for 3 h with 45 mM glucose alone (B–B″) or with 10 nM IGF-I (C–C″). Neurons shown have intact nuclei (blue), indicating that they have not undergone chromatin condensation and nuclear fragmentation, late signs of apoptosis. DRG neurons treated with control media exhibit punctate cytochrome *c* staining (green) in the cell bodies and neurites, indicative of cytochrome *c* localization in mitochondria (Fig. 2A). These neurons also exhibit active Bax (red) in both the cell bodies and neurites, not co-localized with cytochrome *c* (Figs. 2A–A″). After 3 h of high glucose treatment, most cytochrome *c* staining is diffuse, consistent with its release from mitochondria (Fig. 2B). At this point, active Bax levels are increased above control levels (Fig. 2B′ versus A′) and active Bax co-localizes with cytochrome *c* in both cell bodies and neurites (Figs. 2B–B″, arrows). Addition of IGF-I restores punctate cytochrome *c* staining similar to control and diminishes active Bax staining compared to 3-h glucose treatment alone (Figs. 2C–C″). Although IGF-I prevents co-localization of cytochrome *c* and active Bax in most cells, a minority still exhibit co-localization (Fig. 2C″, arrowhead). Similar results were obtained with glucose and IGF-I treatment for 6 h (data not shown). These results suggest temporal regulation of pro-apoptotic Bcl proteins by high glucose, such that (1) Bim is upregulated and localizes to mitochondria after 1–3 h of exposure, followed by (2) Bax activation and redistribution and (3) mitochondrial release of cytochrome *c*. IGF-I inhibits these events and thereby protects DRG neuron mitochondria.

High glucose induces large, punctiform mitochondria in neurites

The punctate Bim staining and the co-localization of active Bax and cytochrome *c* in DRG neurites suggest that neurite mitochondria are damaged prior to cell body mitochondria. We therefore investigated mitochondrial distribution in DRG neurons after 6 h of high glucose treatment. The 6 h time point was chosen to increase the pool of detectable damaged mitochondria; we expected that mitochondria exhibiting initial mitochondrial membrane permeabilization at 3 h of treatment (refer to Fig. 2) would require more time to undergo full structural degradation, consistent with reports that Bax activation/translocation precedes structural changes (Karbow-

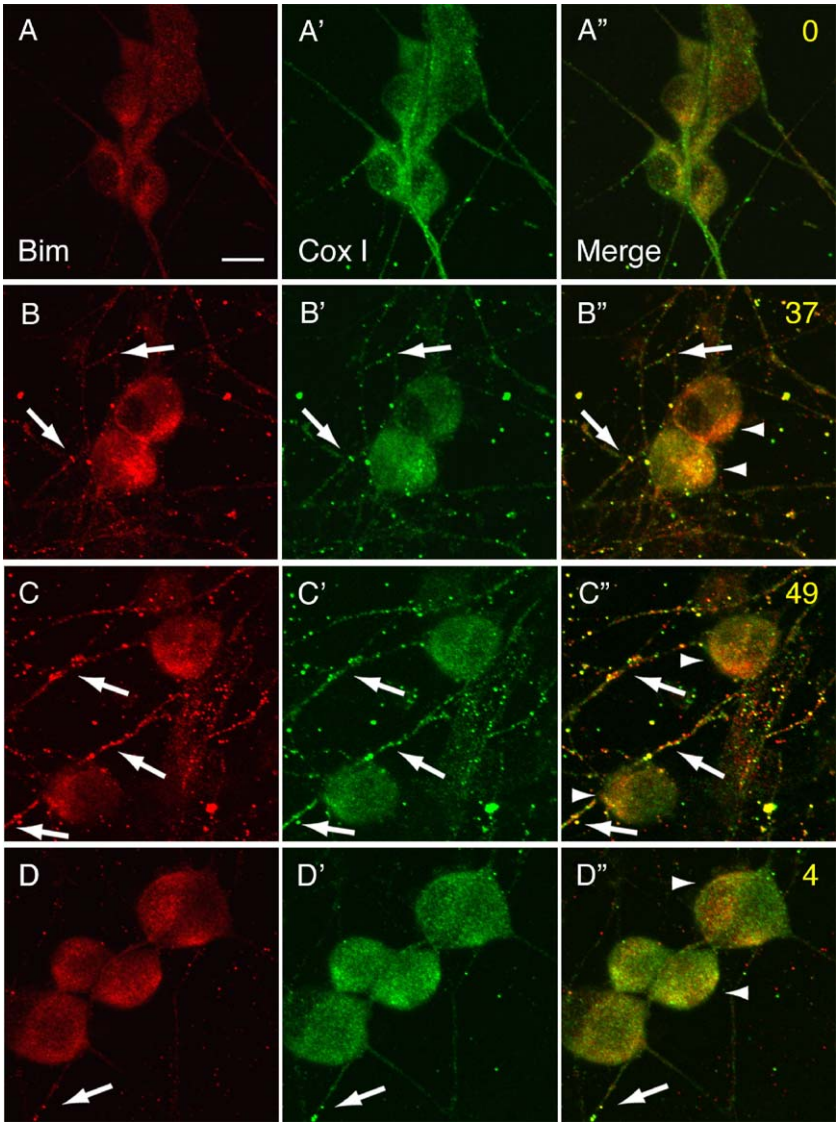

Fig. 1. High glucose causes Bim localization to mitochondria. DRG neurons were analyzed for Bim (red) and Cox I, a mitochondrial protein (green), via confocal microscopy. Co-localized Bim and Cox I (yellow in merged images A″, B″, C″, D″) were quantified by counting yellow puncta from each image (yellow numbers in A″, B″, C″, D″). Neurons treated with control media (A–A″) express Bim that does not co-localize with mitochondria. High glucose (45 mM) increases Bim expression at 1 h (B–B″) and 3 h (C–C″) and promotes co-localization of Bim and Cox I in neurites (arrows) and cell bodies (arrowheads). Co-treatment with high glucose and 10 nM IGF-I does not inhibit Bim protein expression but reduces Bim and Cox I co-localization in neurites (arrow) and cell bodies (arrowheads) compared to treatment with glucose alone (D–D″). Scale bar = 10 μm. Photomicrographs are representative of 3 sample sets.

ski et al., 2004). DRG neurons were treated for 6 h with control media (Figs. 3A–A′), 45 mM glucose (B–B′), and 45 mM glucose with 10 nM IGF-I (C–C′). Confocal micrographs show mitochondria (Cox I, green) merged over a phase image so that mitochondrial distribution can be identified within cellular structures. Images A′, B′ and C′ are 2× digital magnifications of neurites in A, B and C. DRG neurons treated with control media contain many mitochondria (shown as small green puncta) throughout the cell body and neurites (Figs. 3A–A′). DRG neurons treated with high glucose also exhibit many small mitochondria in the cell bodies and neurites, but additionally contain larger "clumps" of mitochondria in the neurites (Figs. 3B–B′, arrows). These large clumps are also found in areas where the cell body narrows into a neurite (Fig. 3B, arrowhead). While the mitochondrial clumping is pronounced after 6 h of high glucose treatment, only a small number of neurons exhibit this effect after 3 h of high glucose treatment (data not shown). This suggests that mitochondrial clumping occurs after Bim and Bax redistribution to mitochondria. Mitochondrial clumps are present, but less pronounced, in DRG neurons treated with high glucose + IGF-I (Figs. 3C–C′, arrows). These clumps resemble the punctate mitochondria of cells that have undergone apoptotic fission (Karbowski et al., 2002; Lee et al., 2004).

High glucose decreases the number of mitochondria in DRG neurons

Our results suggest that high-glucose induced Bim and Bax changes precede phenotypic changes resembling mitochondrial apoptotic fission. Apoptotic fission ultimately results in mito-

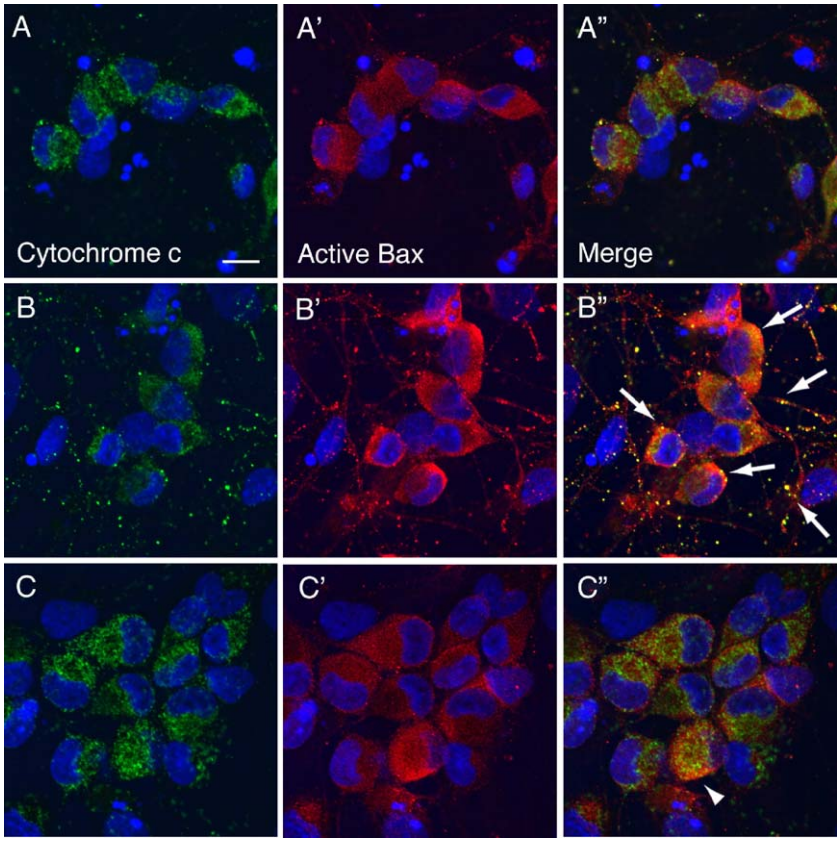

Fig. 2. High glucose causes Bax activation, Bax translocation to mitochondria, and cytochrome *c* loss. DRG neurons were analyzed for cytochrome *c* (green) and conformationally active Bax (red) via confocal microscopy. Co-localization is represented by yellow in merged images. DRG neurons were treated with control media (A–A″), 45 mM glucose for 3 h (B–B″), and 45 mM glucose + 10 nM IGF-I for 3 h (C–C″). High glucose increase Bax activation levels at 3 h, and active Bax co-localizes with punctate cytochrome *c* in intact mitochondria (B–B″, arrows). IGF-I inhibits the increase of Bax activation and sustains punctate cytochrome *c*, suggesting mitochondrial protection (C–C″). However, some active Bax co-localizes with punctate cytochrome *c* in cell bodies in presence of glucose and IGF-I (C″, arrowheads). Scale bar = 10 μm.

chondrial degradation (Szabadkai et al., 2004; Lee et al., 2004; Frank et al., 2001). Mitochondrial number should decrease if mitochondria degenerate; therefore, we quantified the mitochondria from the DRG neurons imaged in Fig. 3D. Mitochondria and neurons were counted from the center plane of a confocal series sampling through the neurons. Thus, subsequent results reflect an average number of mitochondria per neuron in a single confocal plane, not a total number throughout the neuron. Graphed data show the average number of mitochondria per neuron for each sample (Fig. 3D). Control treated DRG neurons contain 53 ± 3 mitochondria. Treatment with high glucose results in 34 ± 2 mitochondria per neuron, a significant decrease compared to control (6G versus C, $***P < 0.001$). However, DRG neurons treated with high glucose + 10 nM IGF-I contain 48 ± 4 mitochondria per neuron, significantly more than high glucose alone (6G + I versus 6G, $**P < 0.01$). These results suggest that high glucose induces mitochondrial degradation, inhibited by IGF-I.

High glucose regulates expression of the fission protein Drp1

Our results show that high glucose decreases the number of mitochondria in DRG neurons. Three possibilities exist to explain these data: (1) mitochondria degrade via autophagy, (2) mitochondria degrade due to typical apoptotic changes, and/or (3) mitochondria undergo apoptotic fission. Mitochondrial autophagy in neurons is distinct from apoptosis and occurs in the absence of cytochrome *c* release, Bax localization to mitochondria and activation of caspases-9 and -3 (Yu et al., 2003; Sperandio et al., 2000). All of these apoptotic events are induced by high glucose treatment in DRG neurons [this manuscript and Leinninger et al., 2004a], suggesting that mitochondrial autophagy does not mediate the decrease in mitochondrial number. In order to implicate apoptotic fission in glucose-induced mitochondrial degradation, we examined expression of the fission protein Drp1 following 3 h (3G) or 6 h (6G) glucose exposure (Figs. 4A, B). Neurons were also treated for 3 h with 45 mM glucose + 10 nM IGF-I and 0.1% DMSO vehicle (3G + I + D) or 20 μM LY294002 (3G + I + LY) to determine whether or not IGF-I modulates Drp1 expression via PI-3K pathway activation. IGF-I activates the PI3K/Akt pathway (which transduces IGF-I mediated neuroprotection), indicated by phosphorylation of Akt (Fig. 4A), and LY294002 inhibits the pathway. Drp1 expression increases above control levels after 3 h of glucose treatment (Fig. 4B, $*P < 0.05$, $n = 3$). However, Drp1 expression is not regulated by IGF-I. Neither IGF-I induced activation of the PI3K/Akt pathway (3G + I + D) nor PI-3K inhibition (3G + I + LY) alters Drp1 expression compared to glucose treatment alone (3G) (Fig. 4B). These data demonstrate that glucose regulates Drp1 expression, but IGF-I does not protect via modulation of Drp1.

Drp1 localizes to mitochondrial clumps after high glucose treatment

Shifting the dynamic balance of mitochondrial fusion/fission events in favor of fission induces mitochondrial fragmentation (Karbowski et al., 2002; Lee et al., 2004). Our results suggest that high glucose may yield Drp1-induced mitochondrial fission, resulting in mitochondria fragmentation, clumping and reduced mitochondrial number. For this to be the case, Drp1 must localize to affected mitochondria (mitochondrial clumps) in DRG neurons. We therefore investigated the localization of Drp1 (green) and mitochondria (red, labeled with an antibody against the mitochondrial voltage-dependent anion channel, VDAC) following glucose exposure (Fig. 5). Control neurons express both Drp1 and VDAC in cell bodies and neurons in a diffuse manner (Figs. 5A–A″). Treatment with high glucose for 3 h promotes punctate Drp1 and VDAC, particularly in neurites (Figs. 5B–B″). The merged image shows modest co-localization of Drp1 and VDAC at 3 h (Fig. 5B, arrows). By 6 h, the punctate pattern of Drp1 and VDAC staining increases, and co-localization is more pronounced (Figs. 5C–C″, arrows). At this point, most neurites in the field exhibit bright puncta of co-localized Drp1 and VDAC, and the VDAC profile is similar to that seen at 6 h with mitochondrial labeling by Cox I (refer to Fig. 3). Co-treatment with high glucose and 10 nM IGF-I for 6 h (6G + I) does not prevent Drp1-containing mitochondrial clumps in neurites (Figs. 5D–D″), suggesting that IGF-I does not control Drp1 localization. Altogether, these results demonstrate that high glucose regulates localization of the fission protein Drp1, which IGF-I does not interrupt.

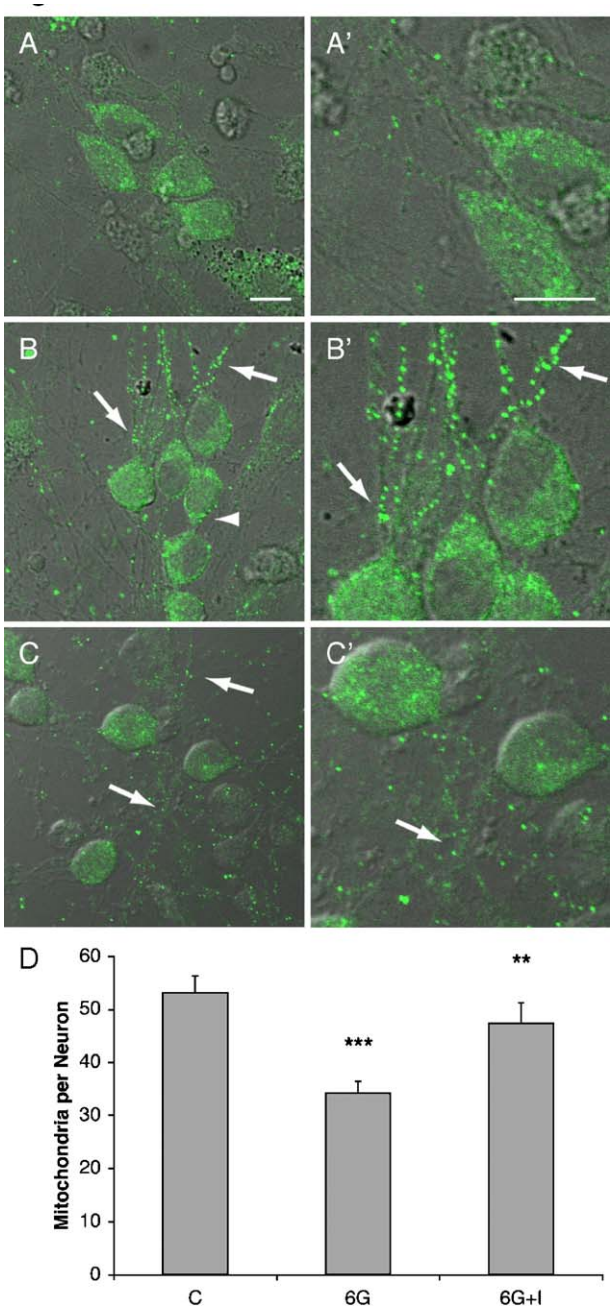

Fig. 3. High glucose induces mitochondrial clumping in neurites and decreases the number of mitochondria in DRG neurons. (A–C) Confocal micrographs of DRG neuron mitochondria (green, immunolabeled for the mitochondrial protein Cox I) overlaid on phase images to discern neuronal structures. Micrographs A′, B′ and C′ are 2× digital magnifications of neurites in images A, B and C. Mitochondria in control-treated neurons are small, numerous and evenly distributed throughout cell bodies and neurites (A–A′). Treatment with high glucose (45 mM) causes the appearance of large mitochondrial clumps (represented by bright puncta, B–B′, arrows) in the neurites. Mitochondrial clumps are also seen where the cell body narrows into the neurite (B, arrowhead). Co-treatment with high glucose + 10 nM IGF-I decreases the number of mitochondrial clumps in neurites but does not completely prevent them (C–C′, arrows). Scale bar = 10 μm. (D) The set of samples used to generate panels A–C were used for quantitational analysis of mitochondria. Three separate fields were analyzed per sample. Briefly, a confocal z-series was collected spanning through the neurons from top to bottom. Images from the center plane of each z-series were selected for quantitation. After quantitation, the total number of mitochondria in each plane was divided by the number of neurons in the plane to determine the average number of mitochondria per neuron (in a single confocal plane). Graphed results represent the average number of mitochondria per neuron in a single neuronal confocal plane, not a total number throughout the neuron. Error bars represent the standard error of the mean (SEM). High glucose-treated neurons (6G) contain significantly fewer mitochondria than control-treated neurons (C) (6G versus C, $***P < 0.001$). Addition of IGF-I (6G + I) significantly increases the number of mitochondria compared to treatment with glucose alone (6G + I versus 6G, $**P < 0.01$). There is no significant difference between the number of mitochondria in control and high glucose-treated neurons (C versus 6G + I). Number of neurons analyzed per condition: $C = 20$, $6G = 29$, $6G + I = 13$.

High glucose promotes Drp1 binding to the pro-apoptotic protein bax

Bax binds to mitochondrial Drp1 and activates scission of mitochondria in Cos-7 and HeLa cells (Karbowski et al., 2002). Our data indicate Bax activation, and mitochondrial localization occurs upstream of the formation of fission-like mitochondrial clumps, suggesting that Bax co-localization with Drp1 may induce mitochondrial fission. We therefore investigated whether Bax binds to Drp1 in glucose-exposed DRG neurons (Fig. 6). DRG neurons were treated with control media (C), 45 mM glucose (3G), or 45 mM glucose + 10 nM IGF-I (3G + I). The Catch and Release system (Upstate Biotechnology) was used to immunoprecipitate

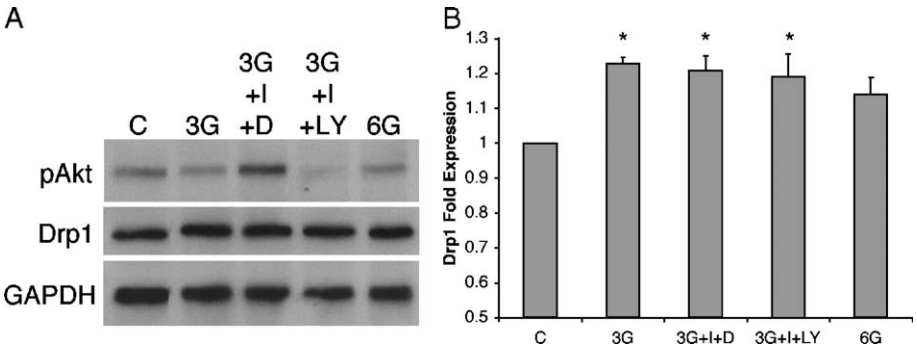

Fig. 4. High glucose regulates expression of the fission protein Drp1. DRG neurons were treated with control media for 6 h or with 45 mM glucose for 3 h (3G) or 6 h (6G). Neurons were also treated for 3 h with 45 mM glucose + 10 nM IGF-I and 0.1% DMSO vehicle (3G + I + D) or 20 μM LY294002 (3G + I + LY). Protein lysates were analyzed via immunoblotting (A) followed by densitometry of fold expression levels (B). IGF-I activates the PI3K/Akt pathway, and this activation is inhibited by the PI3K/Akt pathway inhibitor LY294002. High glucose significantly increases protein expression of the fission protein Drp1 but IGF-I has no affect on Drp1 expression (B, $*P < 0.05$, $n = 3$). Error bars represent the SEM.

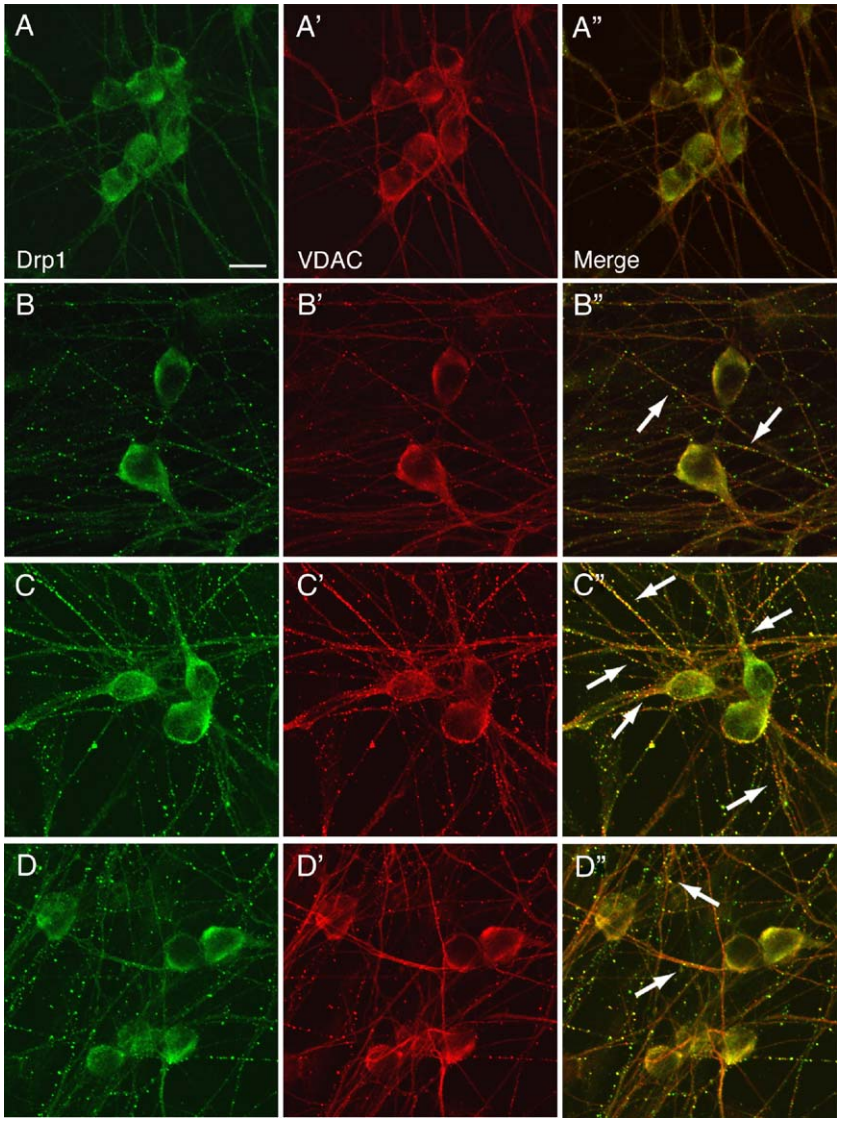

Fig. 5. Drp1 localizes to mitochondrial puncta after high glucose treatment. DRG neurons were analyzed for Drp1 (green) and VDAC (mitochondria, red) via confocal microscopy. Merged images display yellow where Drp1 and VDAC co-localize. Control-treated neurons express both Drp1 and VDAC in cell bodies and neurons in a diffuse manner (A–A″). Treatment with high glucose (45 mM) for 3 h promotes co-localized punctate Drp1 and VDAC in neurites (B–B″, arrows). The co-localization of Drp1 and VDAC is more pronounced after 6 h of high glucose treatment (C–C″). Treatment with high glucose and 10 nM IGF-I for 6 h (D–D″) diminishes mitochondrial clumps in the neurites but does not completely prevent them (*6G + I* versus *6G*, arrows). Scale bar = 10 μm.

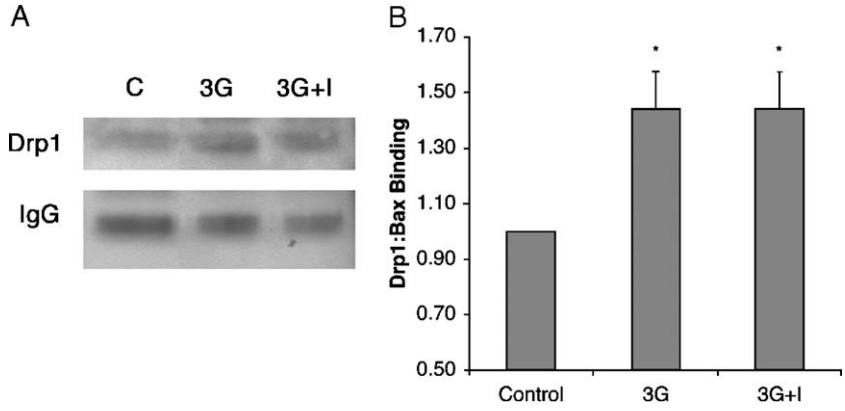

Fig. 6. High glucose promotes Drp1 binding to the pro-apoptotic protein Bax. DRG neurons were treated with control media (*C*), or for 3 h with 45 mM glucose alone (3*G*), or 45 mM glucose + 10 nM IGF-I (3*G* + *I*). Bax was immunoprecipitated from DRG neuronal lysates, followed by immunoblotting for Drp1. Graphed results represent normalized fold expression of Drp1:Bax binding. High glucose increases Bax:Drp1 binding compared to control (*$P < 0.05$, $n = 3$). However, addition of IGF-I does not inhibit Bax:Drp1 binding compared to control (*$P < 0.05$, $n = 3$).

Bax from DRG neuronal lysates, followed by immunoblotting for Drp1 (Fig. 6A). Drp1 bands were normalized to IgG bands, and the fold changes in Drp1:Bax binding are shown in Fig. 6B. High glucose increases Bax:Drp1 binding compared to control (*$P < 0.05$, $n = 3$); IGF-I does not inhibit glucose-induced Bax:Drp1 binding (Fig. 6B).

Drp1 protein expression is increased in diabetic rats

As our data in cultured DRG neurons suggest that high glucose induces mitochondrial damage via Drp1, we were interested whether Drp1 was regulated in an animal model of DN. To investigate this, we examined Drp1 levels in protein lysates collected from the DRG of control and 12-week rats made diabetic by streptozotocin (STZ) injection (Fig. 7). STZ rats are a model of type 1 diabetes that exhibit DN and DRG degeneration (Courteix et al., 1993; Russell et al., 1999; Schmeichel et al., 2003). Mean blood glucose levels are significantly higher in STZ rats compared to control rats, confirming diabetes (control = 72 ± 16 mg/dl versus diabetic = 399 ± 44 mg/dl, $P < 0.05$, $n = 3$). Our results demonstrate a 20% increase in Drp1 expression in diabetic rats compared to control rats (Fig. 8, *$P < 0.05$, $n = 3$). These results suggest that the balance of mitochondrial fusion/fission may be shifted toward fission in DN, similar to other neuropathies.

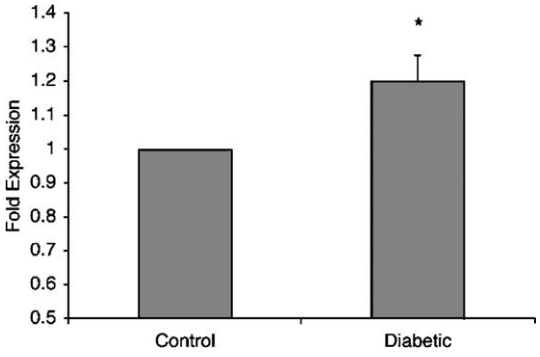

Fig. 7. Drp1 protein expression increases in diabetic rats. Protein isolated from DRG of control and STZ diabetic rats was analyzed for Drp1 via immunoblotting. Graphed results demonstrate a 20% increase in Drp1 expression in diabetic rats compared to control rats (*$P < 0.05$, $n = 3$).

Discussion

Mitochondrial dysfunction is evident in multiple human neurodegenerative disorders, including Alzheimer's dementia, Parkinson disease, and Amyotrophic Lateral Sclerosis (ALS) (Kang and Hamasaki, 2005; Zeevalk et al., 2005; Beal, 2005; Xu et al., 2004). In many of these disorders, oxidative stress promotes mitochondrial dysfunction and degeneration through DNA or protein alteration or through induction of the mitochondrial apoptosis pathway (Le et al., 2005; Kang and Hamasaki, 2005; Zeevalk et al., 2005; Beal, 2005). Our laboratory over the past decade has investigated the link between oxidative stress generated during the diabetic state and neuronal injury, ultimately leading to clinical diabetic neuropathy. Similar to the neurodegenerative disorders listed above, we have reported that in diabetes, high glucose levels promote neuronal injury and death through reactive oxygen species production, mitochondrial damage, and apoptosis (Russell et al., 2002; Vincent and Feldman, 2004; Vincent et al., 2004b, 2005). Furthermore, IGF-I, currently in clinical trials in ALS, also prevents glucose-induced neuronal damage (Leinninger and Feldman, 2005; Leinninger et al., 2004a,b), suggesting that IGF-I may prove efficacious for the treatment of diabetic neuropathy. The current study examines the early events leading to mitochondrial dysfunction in glucose-exposed DRG neurons and which of these events IGF-I prevents. We find that (1) high glucose exposure leads to pro-apoptotic Bcl protein activation, (2) IGF-I prevents translocation of pro-apoptotic Bcl proteins to neuronal mitochondria, and (3) high glucose induces mitochondrial degradation through a process suggestive of apoptotic fission.

High glucose induces caspase activation in DRG neurons after 3 h and apoptosis begins by 6 h (Leinninger et al., 2004a; Vincent et al., 2005). This suggests that mitochondria are injured within the first 6 h of high glucose treatment. Consistent with previous findings, we demonstrate that 3 h of high glucose induces cytochrome *c* release (Russell et al., 2002; Vincent et al., 2004a), and that IGF-I inhibits this release. Translocation of cytochrome *c* from mitochondria to cytosol activates caspases-9 and -3 and is required for mitochondrial-induced apoptosis (Liu et al., 1996). Thus, our results demonstrating that IGF-I inhibits mitochondrial cytochrome *c* release agree with previous data that IGF-I inhibits activation of downstream caspases-9 and -3 and subsequent apoptosis (Leinninger et al., 2004a; Vincent et al., 2004a). Since IGF-I

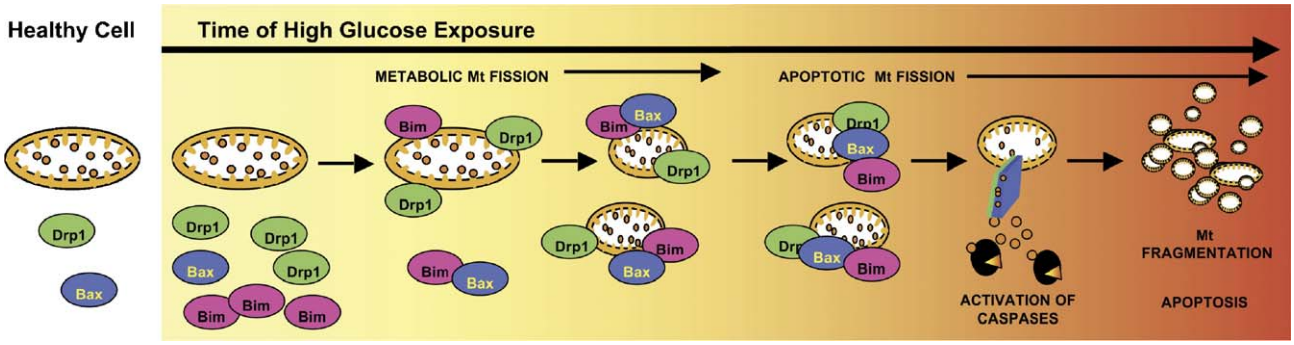

Fig. 8. Proposed model of high-glucose induced injury via Bim, Bax and Drp1. In healthy neurons, Drp1 and Bax are sequestered in the cytosol. Addition of high glucose increases expression of Bim and Drp1, which translocate to mitochondria. At this point, mitochondrial Drp1 can facilitate metabolic fission, creating a larger pool of mitochondria to process increased metabolites. However, prolonged high glucose exposure promotes Bim-mediated activation of Bax and Bim/Bax localization at mitochondria. Association of mitochondrial Bax with Drp1 corrupts metabolic fission and promotes apoptotic fission. This allows Bax-containing channels to form in the mitochondrial membrane, which facilitate cytochrome *c* release into the cytosol. Cytochrome *c* activates caspase-9, which goes on to activate a cascade of downstream caspases, including caspase-3. Accrued caspase activation damages vital proteins and organelles, while apoptotic fission causes mitochondrial dysfunction and fragmentation. Together, these processes ultimately result in neuronal apoptosis.

protection is mediated prior to disruption of mitochondrial membranes, these findings suggest that it interrupts deregulators of mitochondrial membrane stability.

The pro-apoptotic Bcl proteins compromise mitochondrial structure and viability upstream of cytochrome *c* release (Kuwana and Newmeyer, 2003) and therefore seemed likely mediators of DRG neuron mitochondrial damage. Immunolabeling and microscopy were used to demonstrate that high glucose induces mitochondrial translocation of Bim and Bax. Protein redistribution to mitochondria may also be confirmed via lysate fractionation into cytosolic and mitochondrial fractions and subsequent immunoblotting. However, this method requires a large cell input (>9 million cells per sample), which greatly exceeds the number of DRG neurons that are isolated in primary culture (<1 million cells per rat). Therefore, microscopy is the only feasible method of monitoring protein localization in primary DRG neuron cultures. Our results suggest that Bim begins to localize at mitochondria after 1 h of high glucose treatment, prior to the increase of active Bax and its co-localization with mitochondria beginning at 3 h. The presence of mitochondrial Bim is more pronounced after 3 h of high glucose treatment and coincides with Bax localization at mitochondria. These data indicate that Bim may activate Bax in glucose-treated DRG neurons. This is consistent with reports that Bim binds to Bax and induces Bax conformational change leading to apoptosis (Kuwana et al., 2005). Our data suggest that Bim is "activated" by glucose-induced upregulation of Bim protein, similar to the effects of other apoptotic stimuli (Bouillet and Strasser, 2002). Such an increase overwhelms the level of anti-apoptotic Bcl proteins that retain basal levels of Bim in an inactive state and localization. IGF-I does not inhibit DRG neuron apoptosis by suppressing Bim expression, contrary to reports in cerebellar neurons [this report (Leinninger et al., 2004a; Linseman et al., 2002)]. Our data suggest instead that IGF-I protects DRG neurons by preventing Bim localization at mitochondria. Bim is normally associated with microtubules in healthy cells, and IGF-I may stabilize this association (Chen and Zhou, 2004; Puthalakath et al., 1999).

We demonstrate that Bim upregulation and mitochondrial localization occur prior to, and in concert with, Bax activation and mitochondrial localization. Apoptotic stimuli alter Bax conformation, exposing a 20 amino acid epitope in the NH_2-terminus, which precedes Bax translocation to mitochondria and cytochrome *c* release (Makin et al., 2001; Schinzel et al., 2004). High glucose increases conformationally active Bax in DRG neurons, leading to co-localization of active Bax with mitochondrial cytochrome *c* and cytochrome *c* release from mitochondria. IGF-I inhibits the increase of active Bax and its co-localization with intact mitochondria, thereby preventing cytochrome *c* release from glucose-treated DRG neurons. These data collectively suggest that IGF-I inhibits mitochondrial damage at the level of Bax and/or its upstream activator (by inhibiting Bim translocation to mitochondria). Interestingly, viable control and IGF-I-treated DRG neurons express a basal population of active Bax that does not co-localize with cytochrome *c*, indicating that exposure of the NH_2-terminal epitope alone does not force apoptosis. Binding proteins at the Bax NH_2-terminus, such as Ku70, could prevent additional conformational change and mitochondrial targeting (Sawada et al., 2003). Additionally, full Bax activation (exposure of the C-terminus and mitochondrial insertion sequences) and mitochondrial translocation require binding by BH3-only proteins (Cartron et al., 2004). Thus, the pool of active Bax in control-treated DRG neurons acts as a "sensor" for apoptotic stimuli. High glucose may degrade or decrease binding of putative stabilizing proteins or increase expression and mitochondrial localization of upstream BH3-only initiator proteins, producing rapid Bax conformational change and mitochondrial targeting. A glucose-induced increase in Bax with the NH_2-terminus exposed could also overwhelm the normal pool of putative stabilizing NH_2-terminal proteins, thereby permitting further Bax conformational change.

Throughout our microscopic imaging studies, we detected mitochondrial clumps indicative of mitochondrial fission. Balanced mitochondrial fusion/fission is pushed toward fission if there is loss of fusion or gain of fission protein function/expression (Karbowski et al., 2002; Lee et al., 2004). Mutations in fusion proteins are linked to genetic neuropathies (Alexander et al., 2000; Delettre et al., 2000; Zuchner et al., 2004). Fusion proteins are also decreased in the skeletal muscle of type 2 diabetics and in obese Zucker rats (an experimental model of type 2 diabetes), which exhibit mitochondrial density changes suggestive of increased fission events (Bach et al., 2003, 2005). We demonstrate upregulation of the mitochondrial fission protein Drp1 and its redistribution to mitochondria in a time course similar to that of Bim and Bax

activation. The involvement of a mitochondrial fission protein suggests that DRG neurons are subject to injurious mitochondrial remodeling. The mitochondria of normal DRG neurons are numerous, evenly distributed through cell bodies and neurites, and are punctate, not filamentous or interconnected as mitochondria of HeLa or Cos cells (Karbowski et al., 2002). Neurons may require such mitochondrial morphology to evenly distribute the mitochondria along the length of neurites, as in chick DRG neurons, thereby ensuring optimal energy dispersal throughout the cell structure (Miller and Sheetz, 2004). In fact, neurons may be prone to fission because they innately have lower rates of mitochondrial fusion than non-neuronal cells (Karbowski et al., 2004).

Recent studies suggest that increased mitochondrial fission is protective in certain circumstances; this is known as metabolic fission. For instance, HeLa cells are protected from Ca^{2+}-induced mitochondrial damage via fission of the existing mitochondrial network, which increases the number of small mitochondria to handle the Ca^{2+} load (Szabadkai et al., 2004). Hippocampal neurons require active mitochondrial fission for formation, function and maintenance of synapses, processes that place a high metabolic demand on neurons (Li et al., 2004). Drp1 overexpression or metabolic stimulation via creatine similarly increases mitochondrial density and synapse formation, suggesting that metabolic fission is neuroprotective. However, apoptotic stimuli such as staurosporine treatment or Bax overexpression cause cells to undergo apoptotic mitochondrial fission that results in death (Szabadkai et al., 2004; Lee et al., 2004; Karbowski et al., 2002, 2004; Arai et al., 2004; Frank et al., 2001). Thus, fission may be beneficial in healthy neurons, but intersection with the pro-apoptotic cascade (Bax) dysregulates mitochondrial fission, contributing to apoptosis.

Collectively, our studies suggest that high glucose promotes convergence of Drp1-mediated mitochondrial fission with pro-apoptotic Bcl proteins, resulting in mitochondrial injury and apoptosis (Fig. 8). Following high glucose-induced Bim, Bax and Drp1 redistribution to mitochondria, enlarged puncta form in DRG neurons that resemble mitochondria that have undergone apoptotic fission (Karbowski et al., 2002; Lee et al., 2004). Our results from Fig. 3 confirm that DRG neurons treated with high glucose contain fewer mitochondria than control-treated cells. Our data further show that high glucose increases protein expression of the mitochondrial fission protein Drp1 and implicate Drp1-mediated fission in the glucose-induced decrease of mitochondrial number. Drp1 upregulation may be a neuroprotective response to metabolize increased glycolytic substrates via metabolic fission. In support of this idea, increased expression of Drp1 in and of itself does not induce apoptosis (Lee et al., 2004), but Drp1 association with Bax precipitates mitochondrial fission, cytochrome *c* release, caspase activation and apoptosis (Karbowski et al., 2002, 2004; Jagasia et al., 2005). High glucose promotes Drp1 binding to Bax in DRG neurons, and both Drp1 and Bax localize to mitochondrial puncta. We therefore suggest a pathogenetic model in which high glucose induces 2 separate but ultimately convergent responses: (1) an initial upregulation of Drp1 as a protective fission response (metabolic fission) and (2) activation of Bim and Bax, that corrupt mitochondrial fission, resulting in apoptotic fission. IGF-I does not protect DRG neurons from high glucose-induced mitochondrial damage by regulation of Drp1 protein expression/localization, but instead by regulating Bim/Bax activation and localization. Although it was recently shown that BH3-only proteins (like Bim) and multidomain Bcl proteins (like Bax) are critical for apoptotic fission in *C. elegans* (Jagasia et al., 2005), this is the first report to demonstrate neuronal apoptotic fission involving pro-apoptotic Bcl proteins.

In summary, our results in cultured DRG neurons suggest a potential role for apoptotic fission in the pathogenesis of DN. Recent reports link abnormalities in mitochondrial fusion to the development of optic and motor neuropathies (Zuchner et al., 2004; Alexander et al., 2000). Although fission protein expression has not been analyzed in human DN, we demonstrate an increase in Drp1 in the DRG of experimentally diabetic rats that exhibit sensory neuropathy (Courteix et al., 1993). Our results complement findings of reduced fusion protein expression and mitochondrial changes resembling fission in diabetic patients and rodent models of type 2 diabetes (Bach et al., 2003, 2005). The imbalance in fusion/fission proteins detected in all of these models suggests that mitochondrial dysregulation contributes to the pathogenesis of DN and neurodegenerative disease.

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