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News & Views

Can Drug Screening Lead to Candidate Therapies for Testing in Diabetic Neuropathy?

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ABSTRACT

A key mechanism of dorsal root ganglia (DRG) neuron injury in high glucose is mitochondrial overload leading to oxidative stress. We screened selected compounds for the ability to prevent hyperglycemia-induced mitochondrial superoxide in primary sensory DRG neurons. Twenty five out of 1,040 compounds decreased both mitochondrial superoxide and subsequent neuronal injury. These data both validate our screening strategy and indicate further mechanistic evaluation of drug hits and related compounds. Such studies may lead to the design of rational therapeutic approaches for this severe complication of diabetes. *Antioxid. Redox Signal.* 10, 387–393.

DRUG SCREENING AS A TOOL FOR DISCOVERY

DRUG AND SMALL MOLECULE SCREENING is a developing field of discovery science for rapid translational research. A PubMed search on 6/16/07 combining "high-throughput" and "primary" for relevant cell-based assays yielded 608 hits of which 82% were published in the last 5 years. These assays are used for both drug development and diagnostic purposes (3, 29). Whereas compound set screens generate large amounts of data, the translation of screening data to clinical utility requires careful follow-up studies and broad, intensive literature searching.

OXIDATIVE STRESS IN DIABETIC NEUROPATHY

Diabetic neuropathy is a common and increasingly prevalent disorder that is responsible for significant patient morbidity and healthcare burden (17, 26). While many mechanisms contributing to the development of the disease are known, and many drugs have progressed to clinical trials, there is still no effective FDA-approved treatment for diabetic neuropathy. The mechanisms leading to sensory neuron injury in diabetic neuropathy converge upon the development of oxidative stress (1, 23), although decreased neurotrophic support and cellular inflammation also contribute to the disorder (2, 27). Because an array of nerve functions is dysregulated in diabetic neuropathy, testing the efficacy of therapeutic strategies is difficult in clinical and experimental diabetes. Primary testing still requires an *in vitro* system to assess single therapeutic targets alone and in combination. A major contributor to neuronal injury in diabetes is glucose-induced mitochondrial oxidative stress (14, 20, 25). We recently found strong evidence to support this mechanism in dorsal root ganglia (DRG) neurons. Overexpression of the mitochondrial antioxidant enzyme MnSOD prevents glucose-induced DRG neuron injury (24). Recognizing the importance of this pathway, the Juvenile Diabetes Research Foundation (JDRF), in conjunction with National Institute of Health-National Institute of Diabetes and Digestive and Kidney Diseases (NIH-NIDDK), funded a program to screen a panel of 1040 selected compounds for the prevention of mitochondrial oxidative stress in cell types relevant to diabetic neuropathy. A document containing a summary of these assays and the outcomes is available online (http://www.t1diabetes.nih.gov/investigator/Drug-ScreeningSummary-Final.doc).

AN INITIAL SCREEN FOR MITOCHONDRIAL OXIDATIVE STRESS

In response to the JDRF/NIH-NIDDK program, we applied our assay of mitochondrial superoxide (MitoSOX) generation

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in embryonic rat primary DRG neurons (25). Exposure to 20 m*M* added glucose increases MitoSOX oxidation in DRG neurons (Fig. 1A). Mean fluorescence is close to zero in untreated controls, and the fluorescence increases significantly at every time-point from 1 to 5 h. MitoSOX oxidation peaks at 2 h following the application of glucose. To confirm that MitoSOX is measuring mitochondrial ROS, mitochondrial function was increased using creatine (2 m*M*) or decreased by the uncoupling agent FCCP (10 μM) (Fig. 1B). We previously demonstrated that 25 m*M* FCCP produces robust mitochondrial depolarization that decreases ROS in the presence of high glucose (22). Now we find that mild uncoupling decreases MitoSOX oxidation, demonstrating that MitoSOX is indeed oxidized by mitochondrial generation of ROS. The localization of MitoSOX oxidation is illustrated in the representative images in Fig. 1C. Red MitoSOX fluorescence is weak in control DRG neurons, but markedly increased after 1 h glucose exposure. We identified 30 compounds that decreased glucose-induced mitochondrial superoxide at 1 μM concentration at 1 h (Table 1). A dose curve in the range 100 μM to 1.28 n*M* with 5× dilutions was performed for each of the 30 compounds. The lowest concentration of compound that significantly ($p < 0.05$) decreased mitochondrial superoxide compared with glucose alone is reported in Table 1 as the effective concentration.

A SECONDARY OXIDATIVE STRESS ASSAY VALIDATES PRIMARY SCREENING

To confirm that the 30 compounds decreased oxidative stress leading to cellular oxidative modifications, a repeat set of sam-

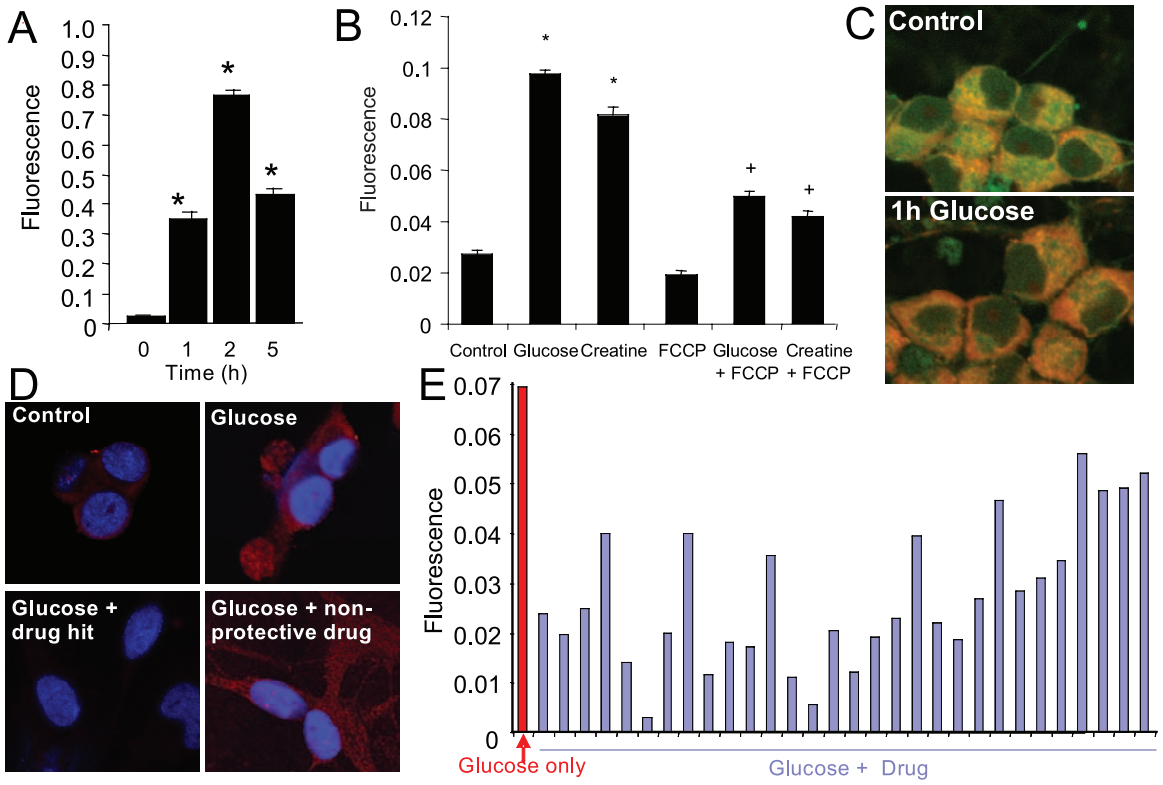

FIG. 1. Assessment of oxidative stress in DRG neurons. (**A**) A time-course of MitoSOX oxidation indicates rapid and sustained mitochondrial superoxide following the application of 20 m*M* glucose. Each time point showed increased mitochondrial superoxide compared to untreated control neurons, $*p < 0.01$. (**B**) To confirm the mitochondrial source of ROS, DRG neurons were exposed to 2 m*M* creatine, 10 μM FCCP, 20 m*M* glucose, or a combination for 1 h. MitoSOX oxidation at 1 h reveals that creatine that increases mitochondrial activity increases MitoSOX oxidation compared to control ($*p < 0.01$). Mild mitochondrial uncoupling using FCCP slightly decreases basal MitoSOX oxidation and significantly decreases glucose-induced and creatine-induced MitoSOX oxidation ($+p < 0.01$ compared to glucose or creatine only). (**C**) Representative MitoSOX oxidation is examined by confocal microscopy. DRG neurons were loaded with MitoSOX (*red*) and Mitotracker (*green*) for 15 min, then fixed and mounted for microscopy. In the overlay, *yellow fluorescence* indicates mitochondrial superoxide. The *red signal* generally is stronger than the *green*, so overwhelms the *yellow overlay color*. (**D**) Representative nitrotyrosine immunohistochemistry is examined by confocal microscopy. *Red* nitrotyrosine labeling increased 5 h after the application of 20 m*M* glucose. A drug that prevented MitoSOX oxidation ("drug hit") also prevented glucose-induced nitrotyrosine. A drug that did not prevent MitoSOX oxidation ("nonprotective drug") did not prevent glucose-induced nitrotyrosine. (**E**) All of the compounds that decreased MitoSOX oxidation were assessed at 2 μM for the ability to decrease nitrotyrosine measured in the fluorescence plate reader. Each *blue bar* represents a single test of one drug. All of the drugs assessed in this secondary screen decreased glucose-induced nitrotyrosine.

TABLE 1. RANKING OF DRG NEURON PROTECTIVE COMPOUNDS

Rank	*Compound*	*Typical application*	*Known mechanisms of action*	*Effective concentration**
10	Cetylpyridinium Cl	Anti-infective		4 μM
10	Phenylmercuric acetate	Antifungal		4 μM
20	Ergocalciferol	Antirachitic vitamin	Vitamin D_2	1.28 nM
20	Benazepril HCl	ACE inhibitor,		20 μM
20	Crustecdysdone	Insect molting hormone		0.8 μM
30	Gossypol	Antispermatigenic, antineoplastic, antiHIV	Causes conf D in BCl2, release cyto *c*	20 μM
30	Levonordefrin	Vasoconstrictor		1.28 nM
30	Clemastine	Antihistamine	Potent inhibitor of K^+ channels	32 nM
30	Carnitine HCl	Antihyperlipoproteinemic	Prevents ox stress and PCD in neurodeg dis	0.8 μM
40	Prothionamide	Antibacterial	Dehydrogenease inhibitor	0.8 μM
40	Betamipron	Sweetener		0.8 μM
40	isoproterenol HCl	Bronchodilator		0.16 μM
40	Roxarsone	Antibacterial		0.8 μM
50	Caffeine	CNS stimulant		0.8 μM
50	Meclizine HCl	Antiemetic		6.4 nM
50	Fenofibrate	Antihyperlipidemic	ALS hit, PPARa agonist, activates MAPK	0.8 μM
50	3-Aminopropanesulfonic acid	Antibacterial	GABA Agonist	0.8 μM
50	Aminohippuric acid	Renal function diagnosis		0.8 μM
50	Morin	P450 & ATPase inhibitor		0.16 μM
60	Bepridil HCl	Antiarrhythmic		4 μM
60	Paclitaxel	Antineoplastic		0.16 μM
60	Gentamicin HCl	Antibacterial		20 μM
60	Minoxidil	Antihypertensive	Activates ATP-dep K^+ channels	0.8 μM
60	Piperazine	Antihelmintic	ALS hit, blocks ion flux, can increase memb potl	6.4 nM
70	SNAP	Muscle relaxant	NO donor	0.8 μM
70	Praziquantel	Antihelmintic		32 nM
70	Cefuorxamine Na	Antibacterial		6.4 nM
70	Carbenoxolone Na	Antiinflammatory, antisecretory		0.16 μM
90	Atenolol	Beta adregnergic blocker		0.8 μM
90	Suprofen	Antiinflammatory	NSAID—inhibits P450	0.8 μM

*Lowest concentration that prevented glucose-induced DRG MitoSOX oxidation

ples treated with drug, then glucose, was prepared. In this and all subsequent experiments, the drugs were applied at 2 μM. A single dose was selected to simplify the assay and maintain the screening format. We anticipated that having identified drug hits, we would more easily observe a response using 2 μM rather than 1 μM. After 5 h glucose treatment, the DRG neurons were fixed, and then immunolabeled for nitrotyrosine. The immunolabel was measured on the fluorescence plate reader (Fig. 1E). All of the drugs that decreased MitoSOX oxidation also decreased nitrotyrosine, compared to glucose alone. A separate set of DRG neurons was cultured on glass coverslips, and the localization and relative fluorescence intensity was examined by confocal microscopy. Representative images of nitrotyrosine labeling in these cultures are shown in Fig. 1D. A

drug that did not prevent MitoSOX oxidation is presented for comparison. This nonprotective drug did not prevent glucose-induced increase in nitrotyrosine (Fig. 1D). Taken together, Fig. 1 demonstrates that MitoSOX is a robust, specific assay for mitochondrial superoxide that is sensitive enough for drug screening. Confirmation that MitoSOX oxidation is related to cellular oxidative modification was obtained using antinitrotyrosine staining.

PREVENTION OF MITOCHONDRIAL OXIDATIVE STRESS IS NEUROPROTECTIVE

Next, the ability to prevent DRG neuron injury was assessed using the TUNEL assay with each of the 30 primary drug hits tested at 2 μM. Compounds were then ranked according to the ability to prevent DRG neuron injury (Table 1). Toxic compounds were given the lowest rank of 10. Compounds that neither increased nor decreased glucose-induced DRG neuron death were scored 20. For every subsequent decrease in TUNEL staining, the rank score was increased by 10. The initial decrease of 10% in TUNEL compared with glucose scored 30. Further decreases of 5% in TUNEL were scored in increments of 10. Thus, at 2 μM, suprofen and atenolol were the most protective at the 2 μM concentration, producing a 40% decrease in glucose-induced TUNEL positivity, which is similar to untreated control. Note that this is not necessarily the maximum protection, since the drugs were screened at a single low concentration (2 μM). The data demonstrate that almost all compounds which prevent mitochondrial oxidative stress also prevent cell death. This supports the significance of mitochondrial superoxide in neuronal hyperglycemic injury as we have previously proposed (14, 20, 25) and further underscores the importance of preventing DRG neuron mitochondrial oxidative injury in diabetes patients.

SUPPORTING EVIDENCE FOR THE ROLE OF MITOCHONDRIAL STRESS IN HYPERGLYCEMIC INJURY

Three compounds prevented mitochondrial oxidative stress but were not protective. These compounds, cetylpyridinium Cl, phenylmercuric acetate, and ergocalciferol, likely directly blocked mitochondrial function. It is important, therefore, that compounds identified in a single screening assay should undergo secondary or related assays prior to translation to experimental animal or clinical trials. Other mild mitochondrial inhibitors such as gossypol and SNAP both decreased oxidative stress and improved DRG neuron survival in our short-term assay. These data further support our hypothesis that glucose-induced overactivation of mitochondria produces oxidative injury and leads to diabetic neuropathy (22, 25). However, rational consideration of the likely mechanism of action excludes them from further exploration as experimental therapeutics.

THE SCREEN IDENTIFIES POTENTIAL NEW THERAPEUTIC AGENTS FOR DIABETIC NEUROPATHY

Reasonable mechanisms of action are inferred from previous studies for all of the other drug "hits" from this screen. Several of them suggest promising avenues for the development of new therapies against hyperglycemic injury. Particular compounds, notably morin (4, 8), carnitine (4), and carbenoxolone (6) directly act as antioxidants, further supporting our contention that preventing oxidative stress is an important adjunct therapy for diabetic neuropathy. We are intrigued by the identification of both anti-inflammatory compounds and compounds that alter ion channel function. Inflammatory mechanisms are receiving increasing interest in the development of diabetic neuropathy. Largely, studies suggest that inflammation secondary to reduced blood flow exacerbates diabetic neuropathy (13, 27). The drug screen suggests an additional beneficial effect of anti-inflammatory agents by directly impacting upon the survival of DRG neurons. In a related screen using a model of amyotrophic lateral sclerosis with motor neurons exposed to glutamate, many different ion channel inhibitors prevented programmed cell death (19). Taken with the current data, we infer that ion channel inhibition in neurons can broadly prevent toxin-induced injury. Probably the neuronal insults cause loss of ion channel regulation and the inhibitors permit conservation of energy status in the face of toxin-induced stress.

FENOFIBRATE AS AN EXPERIMENTAL THERAPEUTIC

The most interesting compound is fenofibrate. This compound is a PPARα agonist used to decrease hyperlipidemia (18, 28). In mice with hereditary fatty liver disease, fenofibrate decreased liver triglycerides and improved antioxidant status (5). For this reason, fenofibrate is used in type 2 diabetic patients. Several recent studies demonstrated additional benefits from using this compound. Plasma inflammatory mediators are elevated in patients with impaired glucose tolerance, but short-term therapy with fenofibrate decreased both cytokines and C-reactive protein (11). In a genetic model of type 2 diabetes, the db/db mouse, fenofibrate improved all the signs and symptoms of both diabetes and diabetic nephropathy with decreased hyperglycemia and hyperinsulinemia and decreased urinary albumin excretion (12). Furthermore, fenofibrate prevented excess collagen I deposition by cultured mesangial cells (12). A recent review highlighted the pleiotropic benefits against type 2 diabetes and resulting nephropathy (18), and another suggested that fibrates protect against diabetic cardiovascular disease as well as nephropathy and retinopathy (28). We now present preliminary evidence that fibrates may directly prevent neuronal injury in hyperglycemia in addition to the effects demonstrated by others. This drug demands further experimental testing and we are initiating a trial of fenofibrate in a mouse model of type 1 diabetes. Generally, fibrates are well tolerated and safe in combination therapies (28), suggesting that this could be a powerful component in a cocktail targeting the multiple mechanisms leading to diabetes complications.

DATA VALIDATION IN ADULT DRG NEURONS

Adult DRG neurons are a better accepted model for assessment of the mechanisms of diabetic neuropathy for two major reasons: [a] they are mature, unlike embryonic cultures, and diabetic neuropathy occurs in adult patients; [b] they may be cultured in lower basal concentrations of glucose, so a hyperglycemic insult is more representative of the *in vivo* environment. Therefore, we cultured adult rat DRG neurons in 96-well plates to confirm our positive results. First, we performed the MitoSOX oxidation analysis. The glucose-induced increase in MitoSOX fluorescence at 1 h in adult DRG neurons was ~10-fold lower compared to embryonic DRG neurons (Fig. 2A). This may be related to a lower cell density on the plate, determined visually, or may be a result of lower basal glucose. Nevertheless, the increase was highly significant and reproducible. Fenofibrate (2 μM) treatment was combined with exposure to 20 mM added glucose (total 25.7 mM glucose) for 1 h. Fenofibrate was added at the same time (Fen-Co) or as a 3 h pretreatment (Fen-Pre) relative to 20 mM added glucose. Both co-treatment and pretreatment with fenofibrate significantly decreased glucose-induced mitochondrial superoxide generation. The 3 h pretreatment provided greater protection, suggesting that DRG neurons respond to fenofibrate with a mechanism that decreases susceptibility to high glucose, such as new gene expression or post-translational modification.

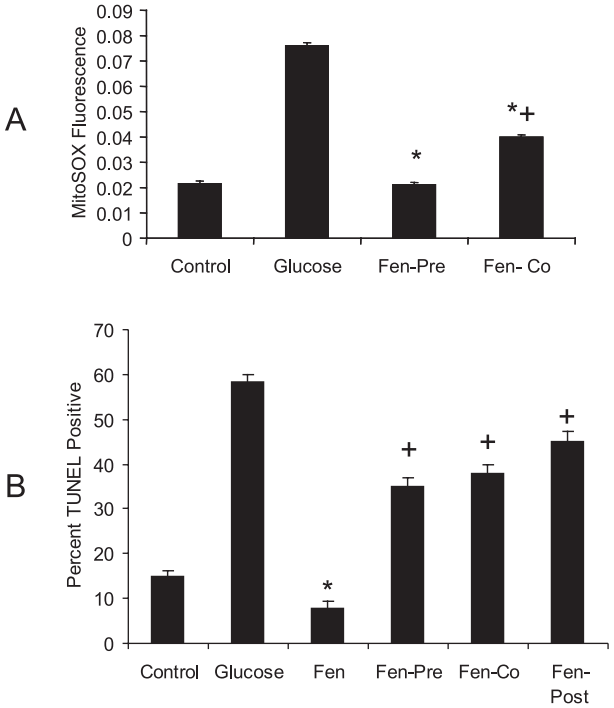

FIG. 2. Validation of results using adult DRG neurons. (**A**) MitoSOX oxidation was assessed after 1 h exposure to 20 mM glucose. Fenofibrate (2 μM) was applied 3 h prior to (Fen-Pre), or at the same time as (Fen-Co) the glucose. *$p < 0.01$ compared with glucose only; +$p < 0.05$ compared to pretreatment. (**B**) DRG neuron injury was assessed by TUNEL 24 h after exposure to 20 mM glucose and/or fenofibrate. An additional post-treatment paradigm (Fen-Post) was included where fenofibrate was applied 1 h subsequent to glucose. *$p < 0.05$ compared to untreated control; +$p < 0.01$ compared to glucose only treatment.

Next, we assessed adult DRG neuron injury using the TUNEL assay (Fig. 2B). In this experiment, fenofibrate (2 μM) was applied alone (Fen), as a 3 h pretreatment (Fen-Pre), a co-treatment (Fen-Co), or 1 h subsequent to 20 mM added glucose (Fen-Post). DRG neuron injury following exposure to 20 mM added glucose (total 25.7 mM glucose) was similar to the injury seen in embryonic DRG neurons. This result strengthens our case for the use of embryonic DRG in our screening strategies despite the high basal glucose. We previously maintained that fluctuations in glycemia injures neurons and is an important consideration for clinical management of diabetes (20). All fenofibrate treatment paradigms significantly decreased glucose-induced DRG neuron injury, with the greatest protection with earlier application of the drug. The protection observed with a 1 h post-treatment strongly suggests that fenofibrate functions as a direct antioxidant in this system.

IMPLICATIONS OF THE STUDY

Drug screening using primary DRG neuron cultures led us to begin further *in vitro* and *in vivo* assessment of fenofibrate as an experimental therapeutic for diabetic neuropathy. In addition, carnitine (9) and bepridil have received recent attention in experimental diabetes. Several of these compounds may merit further study. We are convinced that certain of these compounds, potentially in combination with conventional antioxidant therapy (25), will produce innovative clinical approaches to prevent or treat diabetic neuropathy. These data validate the use of MitoSOX as a drug screening strategy including and the use of embryonic DRG neurons that can be obtained in sufficient numbers and purity for the screening protocol.

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ABBREVIATIONS

DRG, dorsal root ganglia; FUDR, fluoro-2-deoxyuridine; NGF, nerve growth factor; TUNEL, terminal deoxy-UTP nick-end labeling.

APPENDIX

Embryonic DRG cultures for drug screening

Timed pregnant rats are purchased from Charles River Laboratories. All rodent care and use is approved and regulated by the Unit for Lab-

oratory Animal Medicine at the University of Michigan. DRG are harvested from embryonic day 15 Sprague–Dawley rats, dissociated in 1% trypsin, then cultured on tissue culture plates in growth media. Black-walled 96-well plates (Falcon Labware, BD Biosciences, San Jose, CA) are coated with rat tail type 1 collagen (Sigma, St Louis, MO) prior to applying DRG neurons. Immediately prior to use, collagen is dissolved in sterile 1 *M* acetic acid to 10 mg/ml, then diluted in sterile distilled water to 1 mg/ml, spread in a thin layer on the culture plate, and allowed to air dry in a laminar flow hood. All culture reagents are from Gibco (Grand Island, NY) unless stated otherwise. Growth media is prepared using Neurobasal medium supplemented with 1 × B-27 additives, 50 ng/ml NGF (Harlan Bioscience, Indianapolis, IN), 40 μM FUDR (Sigma), and 1,000 U/ml penicillin/streptomycin/neomycin solution. Initial plating medium contains 2 μM glutamine. Fifteen embryos are used to seed 1× 96-well plate. DRG neurons are re-fed after 24 h in fresh media containing all additives except glutamine. On day 2, cells are rinsed, then re-fed using treatment media (Neurobasal media containing 4 ng/ml selenium, 4 ng/ml hydrocortisone, 0.01 mg/ml transferrin, 3 ng/ml β-estradiol, 50 ng/ml NGF, 40 μM FUDR, and 1,000 U/ml penicillin/streptomycin/neomycin. Experiments are performed on DRG neurons on the third day in culture in the absence of B-27 additives. Neurobasal medium contains a basal 25 m*M* glucose that is essential for DRG neuron culture (7, 14–16). A decrease of 5 m*M* glucose leads to increased DRG neuron death, while increasing the glucose concentration above 35 m*M* induces hyperglycemic stress, ROS, and cell injury (14). To produce a hyperglycemic insult, 20 m*M* additional glucose (yielding a total 45 m*M* glucose) is added to the media for the period specified in individual experiments. This glucose load is rather higher than may be achieved except in extreme cases of uncontrolled diabetes. However, the basal 25 m*M* glucose is required for these cultures *in vitro*. We and others have demonstrated that it is acute changes in glucose load that produce injury, more than sustained elevated glucose (10, 20), making this a useful model for drug screening (25). The glucose concentrations used do not produce increased stress and injury due to osmotic effects, as demonstrated in previous studies (20, 25).

Adult DRG neuron cultures

DRG are collected from the euthanized adult female rat and dissociated in 0.2% collagenase for 30 min, followed by 1% trypsin for 15 min. Cells are resuspended in adult neuron growth media, then seeded on collagen-coated plates as described for embryonic DRG. All subsequent experimental procedures are the same as for embryonic cultures except for the substitution of adult growth media (DMEM:F-10, 50:50, 1 × B27 additives, 40 μM FUDR, and 1,000 U/ml penicillin/streptomycin/neomycin. In this media the basal glucose concentration is 5.7 m*M*. To produce a hyperglycemic insult, 20 m*M* additional glucose (yielding a total 25.7 m*M* glucose) is added to the media for the period specified in individual experiments.

Measurement of reactive oxygen species

MitoSOX (Molecular Probes, Eugene, OR) is a cell-permeable probe that accumulates specifically in mitochondria and becomes fluorescent following oxidation by superoxide. MitoSOX is dissolved in DMSO immediately prior to use, then applied to DRG neurons at a final concentration of 4 μM with DMSO diluted to <0.1%. After 15 min, media is removed and replaced with 100 μL Hepes-buffered saline solution (HBSS: 10 m*M* HEPES, pH 7.4, 150 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2$, 1.8 m*M* $CaCl_2$); then red fluorescence is read at 485 nm excitation and 590 nm emission (Fluoroskan Ascent II plate reader, LabSystems, Helsinki).

Fragmentation of genomic DNA

TdT mediated dUTP-biotin nick end labeling (TUNEL) staining is used to detect programmed cell death in DRG neurons. DRG neurons are fixed in 4% paraformaldehyde prior to staining. Samples are labeled with digoxygenin-dUTP and then stained with horseradish peroxidase-conjugated anti-digoxygenin antibody using a kit according to the manufacturer's instructions (Intergen, Gaithersburg, MD). We previously demonstrated that the TUNEL assay is a reliable and reproducible indicator of neuronal injury (19, 21, 25).

Nitrotyrosine immunohistochemistry

DRG neurons were treated as described, then fixed in 4% paraformaldehyde in 0.1 *M* phosphate buffered saline solution (PBS) for 5 min. Primary antibody was anti-nitrotyrosine 244 (Upstate Biologicals, Lake Placid, NY) at 10 μg/ml final concentration in 0.1 *M* PBS containing 1% Triton X100 and 5% goat serum for 16 h at 4°C. Cells were washed for 3× 10 min in PBS, then goat anti-rabbit-AlexaFluor 594 secondary antibody (Molecular Probes) was applied for 1 h at room temperature. Secondary was diluted 1:1,000 in 0.1 *M* PBS containing 1% Triton X100 and 5% goat serum. After 3x 10 min washes in PBS, fluorescence was assessed by reading on the fluorescent plate reader at 485 ex, 590 em, or coverslipped and examined on a Nikon Diaphot 200 inverted fluorescent microscope (Nikon, Melville, NY). Digital images were captured with a Hamamatsu ORCA ER camera (Hamamatsu, Bridgewater, NJ).

Statistical analysis

All experimental samples are assayed on three separate occasions with two–three replicates in each assay. For determination of percent positive in TUNEL assay, at least 10 fields, each containing at least 10 neurons, are counted per replicate assay. The total numbers of cells in each field are compared between experimental and control conditions to confirm that the numbers are equivalent. This is important to consider, since if dying cells detach from the dish, the data are skewed. The mean and standard error for the three repeats are calculated. Statistical significance is determined using ANOVA with 95% confidence intervals.

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