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Cell Culture Modeling to Test Therapies Against Hyperglycemia-Mediated Oxidative Stress and Injury

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ABSTRACT

The concept that oxidative stress is a key mediator of nerve injury in diabetes has led us to design therapies that target oxidative stress mechanisms. Using an *in vitro* model of glucose-treated dorsal root ganglion (DRG) neurons in culture, we can examine both free radical generation, using fluorimetric probes for reactive oxygen species, and cell death via the TUNEL assay. The cell culture system is scaled down to a 96-well plate format, and so is well suited to high-throughput screening. In the present study, we test the ability of three drugs, nicotinamide, allopurinol, and α-lipoic acid, alone and in combination to prevent DRG neuron oxidative stress and cell death. This combination of drugs is currently in clinical trial in type 1 diabetic patients. We demonstrate independent effects on oxidative stress and neuronal survival for the three drugs, and neuronal protection using the three drugs in combination. The data strengthen the rationale for the current clinical trial. In addition, we describe an effective tool for rapid preclinical testing of novel therapies against diabetic neuropathy. *Antioxid. Redox Signal.* 7, 1494–1506.

INTRODUCTION

DIABETES OR IMPAIRED GLUCOSE TOLERANCE affects 20 million individuals in the United States, and the number is increasing by 5% per year. Diabetes-specific microvascular complications will eventually affect nearly all individuals with diabetes. Diabetic retinopathy is the most common cause of adult blindness in the United States. Ninety percent of patients have evidence of retinopathy after 15 years of diabetes with ~25,000 new cases of blindness per year (25). Diabetes is the leading cause of renal failure in the United States, accounting for 40% of new cases each year (69). Greater than half of all patients with diabetes develop neuropathy, making diabetic neuropathy the most common cause of nontraumatic amputations and autonomic failure (23, 83). In his or her lifetime, a diabetic patient with neuropathy has a 15% chance to undergo one or more amputations (22).

Except for improved glycemic control, no specific therapies are available to prevent or delay the development or progression of diabetic microvascular complications (68). Animal and *in vitro* experiments implicate a number of enzymatic and nonenzymatic pathways of glucose metabolism in the initiation and progression of complications (72). These include: (a) increased polyol pathway activity leading to sorbitol and fructose accumulation, NAD(P)-redox imbalances, and changes in signal transduction; (b) nonenzymatic glycation of proteins yielding "advanced glycation end-products" (AGEs); (c) activation of protein kinase C (PKC), initiating a cascade of stress responses; (d) increased hexosamine pathway flux; and (e) increased escape of superoxide from the electron transfer chain through mitochondrial stress (6, 10, 72, 81, 86). Although specific inhibitors of each pathway block one or more diabetic microvascular complications, only recently has a link been established among the pathways that provides a unified mechanism of tissue damage. Each pathway is directly or indirectly associated with increased production of reactive oxygen species (ROS) and the development of oxidative stress.

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Our work has focused on one specific microvascular complication, diabetic neuropathy, and supports the concept that glucose-mediated oxidative stress underlies the development of this complication (52, 58, 65, 70, 80). In diabetic rats, there is evidence of mitochondrial dysfunction and ROS accumulation in dorsal root ganglion (DRG) neurons and Schwann cells, followed by caspase cleavage and the ultrastructural hallmarks of apoptosis (18, 31, 32, 64, 66). *In vitro*, DRG neurons exposed to increased concentrations of glucose accumulate ROS with subsequent caspase cleavage and programmed cell death (15, 65, 66, 81). These data parallel recent studies in man, where patients with type 1 diabetes and high serum levels of ROS markers had more severe peripheral and autonomic neuropathy (60). Collectively, these results led us to investigate the potential beneficial effects on neuronal survival of blocking glucose-mediated ROS accumulation. We reasoned that compounds that blocked ROS formation and programmed cell death in glucose-treated neurons could provide new treatment options for patients with diabetic neuropathy. In the current study, we report that the drugs in a current triple therapy clinical trial in man, allopurinol, α-lipoic acid, and nicotinamide, individually rescue DRG neurons from glucose-induced ROS and cell injury, but with different potencies. In addition, these drugs in combination prevent glucose-mediated DRG neuron injury.

MATERIALS AND METHODS

DRG culture model of hyperglycemia

DRG are harvested from embryonic day 15 Sprague–Dawley rats, dissociated in 1% trypsin, then cultured on tissue culture plates in growth medium. Plates are coated with rat tail type 1 collagen (Sigma, St Louis, MO, U.S.A.) prior to applying DRG neurons as follows. 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, U.S.A.) unless stated otherwise. Growth medium is prepared using Neurobasal medium supplemented with 1 × B-27 additives, 50 ng/ml nerve growth factor (NGF; Harlan Bioscience, Indianapolis, IN, U.S.A.), 40 μ*M* fluorodeoxyuridine (FUDR; Sigma), and 1,000 U/ml penicillin/streptomycin/neomycin solution. Initial plating medium contains 2 μ*M* glutamine. DRG neurons are refed after 24 h in fresh medium containing all additives except glutamine. On day 2, cells are rinsed, then refed using treatment medium (Neurobasal medium containing 4 ng/ml selenium, 4 ng/ml hydrocortisone, 0.01 mg/ml transferrin, 3 ng/ml β-estradiol, 50 ng/ml NGF, 40 μ*M* 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 (18, 31, 32, 64–66). A decrease to 5 m*M* glucose leads to increased DRG neuron death, whereas increasing the glucose concentration above 35 m*M* induces hyperglycemic stress, ROS, and cell injury (65). To produce a hyperglycemic insult, 20 m*M* additional glucose (yielding a total 45 m*M* glucose) is added to the medium for the period specified in individual experiments. Medium glucose concentrations do not significantly decrease during the treatment periods as determined by mass spectrometry.

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, U.S.A.).

Cleavage of caspase-3

To detect caspase-3 staining, the CaspaTag technique (Intergen) is used. This kit contains a cell-permeable, fluorescence-tagged caspase-3 substrate that binds irreversibly to the active site of caspase-3. Thus, DRG neurons containing active caspase-3 are rapidly identified by microscopy. Reagents were obtained in the kit form and used according to the manufacturer's instructions. The CaspaTag caspase-3 substrate is applied to the cultures for the final 30 min of an assay incubation period, and then cells are immediately rinsed and fixed. The nuclear chromatin is counterstained with 1 μg/ml bisbenzamide in phosphate-buffered saline. Neurons are considered to have active caspase-3 if staining can be clearly localized to the neuronal cytosol using fluorescence microscopy.

Measurement of ROS

The cell-permeant dihydroethidium (DHE), which is oxidized relatively specifically by superoxide (87), and 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA), which is oxidized primarily by hydrogen peroxide (H_2O_2) (88) (Molecular Probes, Eugene, OR, U.S.A.), are used to assess real-time ROS formation in DRG neurons. Reduced DHE emits blue fluorescence. Exposure to ROS, superoxide in particular, causes oxidation to ethidium that displays red fluorescence. Stock DHE is dissolved at a concentration of 10 mg/ml in dimethyl sulfoxide (DMSO), diluted to 150 μ*M* in 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* $CaCl_2$) immediately prior to use, then added directly to the culture medium at a 1:50 dilution, giving a final concentration of 3 μ*M*. The DHE solution is applied to DRG neurons for the final 15 min of an experiment, and then neurons are rinsed once rapidly in HBSS and immediately placed in a fluorescence plate reader (Fluroskan Ascent II, Labsystems, Helsinki, Finland). For each sample, the ratio of red (518 nm excitation, 605 nm emission) over blue (485 nm excitation, 520 nm emission) fluorescence is determined with 1-s integration for each reading.

Reduced DCFDA is nonfluorescent, but following oxidation, generally by H_2O_2, green fluorescent 2′,7′-dichlorofluorescein (DCF) is generated. Stock DCFDA is dissolved in DMSO initially at 5 mg/ml, then diluted in HBSS to 17 μg/ml. Similar to the DHE assay, the diluted DCFDA is applied to the culture medium at 1:50 dilution 15 min prior to

the end of a treatment period; then after 15 min, the cells are rinsed in HBSS and examined in the fluorescent plate reader. DCF fluorescence is assessed with 485 nm excitation and 520 nm emission with 1-s integration.

Real-time lipid peroxidation

MitoFOX was supplied by Molecular Probes, Inc. This probe is under development and is aimed at the specific detection of mitochondrial lipid peroxidation. It is not clear at present whether the probe is detecting specific lipid peroxidation or general oxidative mechanisms. The probe was dissolved in DMSO to produce a 2 m*M* stock solution, then diluted to 100 μ*M* working solution using HBSS. The probe was added using two different experimental paradigms where DRG neurons were either preloaded with MitoFOX or labeled for the final 15 min of the incubation period. In each case, a final concentration of 250 n*M* MitoFOX was applied to the DRG neurons. At the end of the experimental period, DRG neurons were rinsed in HBSS, then fixed in paraformaldehyde, mounted in Prolong Antifade (Molecular Probes), and examined by fluorescence microscopy.

Statistical analysis

All experimental samples are assayed on three separate occasions with two or three replicates in each assay. For determination of percent positive in assays of cell death, 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, because 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.

RESULTS

A cell culture drug-screening paradigm

Simulation of diabetes-induced DRG neuron stress in cell culture permits the screening for DRG neuron-protective compounds with potential to prevent diabetic neuropathy. Figure 1A illustrates the experimental paradigm used for high-throughput screening with DNA fragmentation as the end-point assay.

Figure 1B shows representative TUNEL staining in untreated control DRG neurons and glucose-treated (20 m*M* added glucose for 24 h) DRG neurons. Currently, the TUNEL assay represents a more quantifiable parameter for the measurement of DRG neuron injury. In particular, the time course of TUNEL staining in the DRG neurons increases with duration of injury up to 8 h following the application of 20 m*M* glucose, but does not decrease with longer exposures (unpublished observations). This is important as other markers, such as caspase-3 staining and MitoFOX labeling, decrease following a peak in staining. A more intense insult may produce an earlier peak in staining, and give a false positive result for a decrease in staining at the selected time point, illustrated in Fig. 1C.

A novel assay to examine peroxidation in mitochondria

An additional technique under appraisal is the detection of mitochondrial lipid peroxidation using a fluorimetric probe, MitoFOX (Molecular Probes). As oxidative stress is an early contributor to glucose-mediated oxidative stress, therapeutics targeted specifically to this mechanism may be rapidly and sensitively assessed through this method. Typical labeling with the probe is illustrated in Fig. 2. In Fig. 2A, untreated control DRG neurons or DRG neurons exposed to 20 m*M* added glucose for 3 h were labeled with 250 n*M* MitoFOX for 15 min. The probe is under development to specifically detect mitochondrial lipid peroxidation, although the true specificity of this probe is not yet known. Markedly greater green fluorescence indicative of mitochondrial lipid peroxidation is seen in DRG neurons exposed to 20 m*M* added glucose compared with DRG neu-

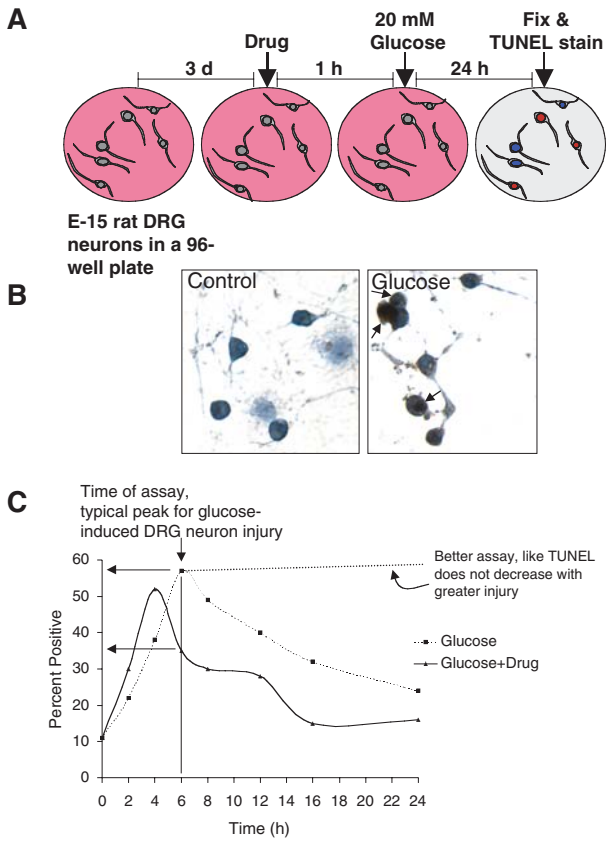

FIG. 1. High-throughput screening in primary DRG neuron cultures. (**A**) Schedule of culture and treatment in the drug screening paradigm. (**B**) Representative images of TUNEL-stained DRG neurons following 24-h exposure to control treatment medium or treatment medium containing 20 m*M* added glucose. Black arrows indicate brown, TUNEL-positive nuclei. Healthy nuclei stain blue with hematoxylin. (**C**) Sample data to illustrate the utility of the TUNEL assay of cell death in this system, as opposed to test systems that rely on a parameter that increases to a peak and then declines with increased stress or injury.

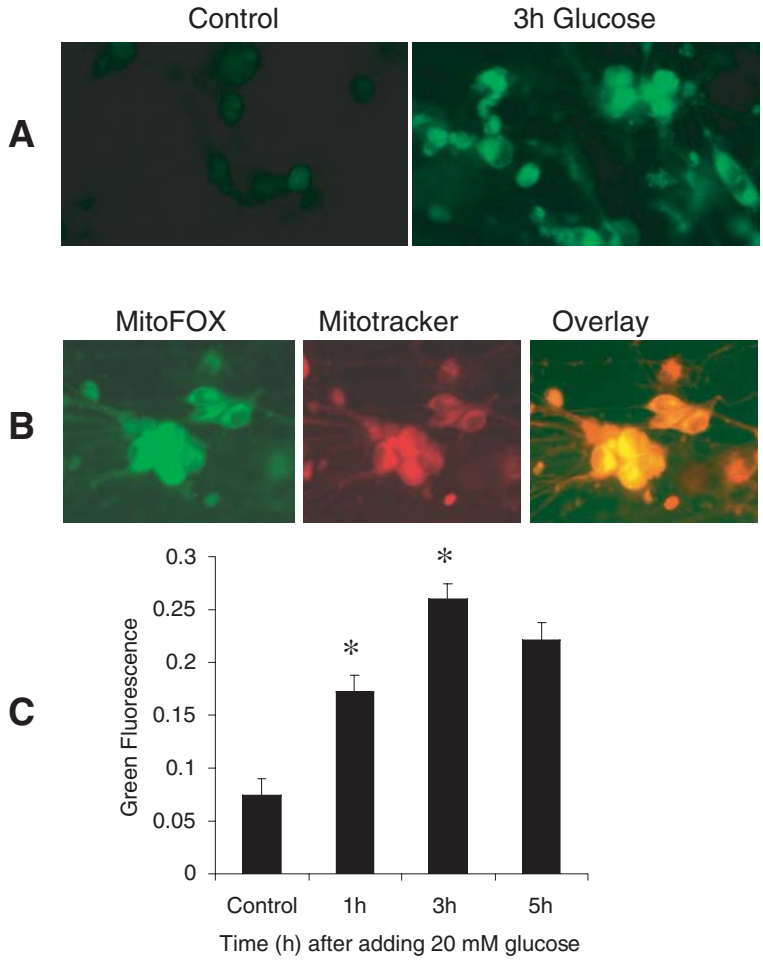

FIG. 2. Assay for lipid peroxidation using MitoFOX. Mitochondrial lipid peroxidation may be rapidly screened using the novel probe MitoFOX. (**A**) DRG neurons in basal (25 m*M*) glucose and neurons exposed to 20 m*M* added (45 m*M* total) glucose for 1 h were loaded with 250 n*M* MitoFOX for 15 min, then photographed with fluorescein isothiocyanate fluorescence filters. (**B**) Images were taken after 1-h exposure to 20 m*M* added glucose. Lipid peroxidation (green) can be observed within 1 h and can be localized to mitochondria using Mitotracker probe (red), with overlap of the two probes shown in yellow in the overlay. (**C**) MitoFOX was added to glucose-treated DRG neurons grown in 24-well plates. The mean fluorescence per well was measured in a fluorescence plate reader (Labsystems Ascent II) at 485 nm excitation and 520 nm emission. The mean and standard errors of the fluorescence for three separate assays are plotted. *Fluorescence increased significantly within 1 h of application of 20 m*M* glucose ($p < 0.05$) and increased again by 3 h.

rons in basal glucose (Fig. 2A). The lipid peroxidation appears to be localized to the mitochondria through double-loading with Mitotracker red at a concentration of 100 n*M* (Molecular Probes) that labels the mitochondria (Fig. 2B). The overlay of red and green fluorescence demonstrates colocalization of the probes, indicating that MitoFOX is most likely accumulating in the mitochondria. Quantitation of green MitoFOX labeling using a fluorescent plate reader (Labsystems Ascent II) demonstrates a significant increase in lipid peroxidation by 1 h following the application of 20 m*M* glucose that continues to increase at 3 h and then begins to decrease by 5 h (Fig. 2C). If the specificity of this probe for lipid peroxidation is confirmed, the sensitivity of this technique suggests this will be a useful tool for determining the mechanisms of oxidative injury.

Antioxidant drugs can decrease glucose-induced DRG neuron injury

These screening protocols were used to assess the efficacy of a novel combination therapy for diabetes complications that is currently in clinical trial (www.umich.edu/~jdrf/). FDA-approved doses of allopurinol, nicotinamide, and α-lipoic acid are being used in a 2-year double-blind placebo-controlled study in type 1 diabetic patients with tests of diabetic neuropathy as the clinical end point. First, these drugs were assessed alone for the ability to prevent glucose-induced DRG neuron death in the high-throughput screening paradigm (Fig. 3). The concentration ranges for these compounds were selected using information available from their use in other systems (27, 44). Nicotinamide demonstrates a narrow

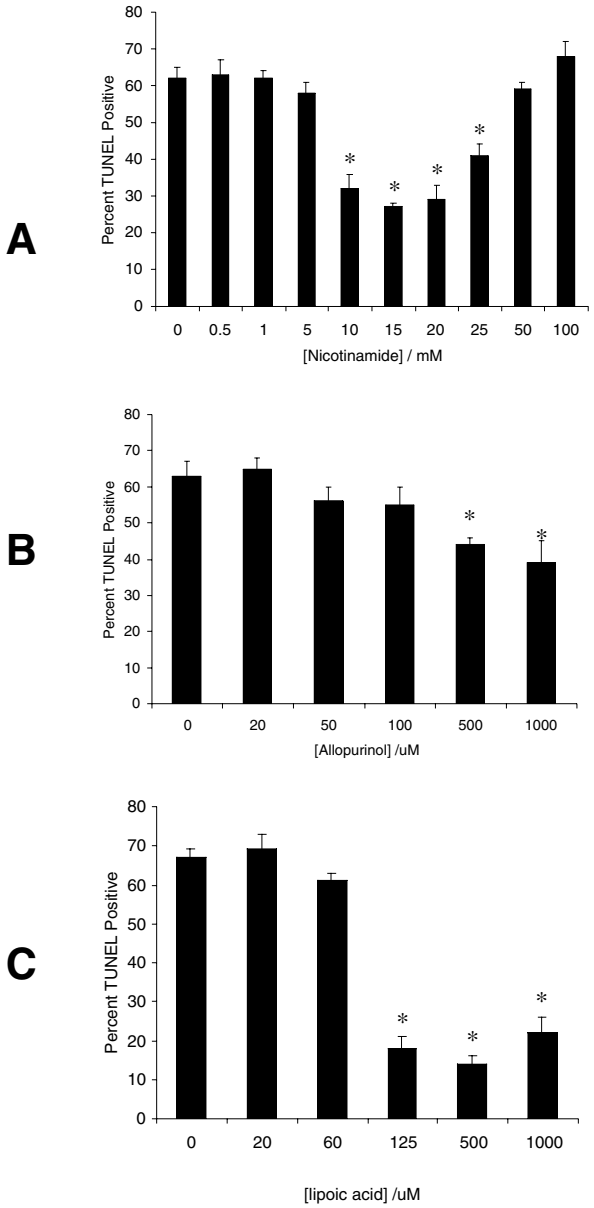

FIG. 3. Effect of individual drug treatments on glucose-induced DRG neuron death. Cell death, as assessed by the TUNEL assay that is indicative but not restricted to programmed cell death pathways, was determined 24 h following exposure to 20 m*M* added glucose with and without increasing concentrations of nicotinamide (**A**), allopurinol (**B**), or a-lipoic acid (**C**). The means and standard errors for three replicate assays are shown. *Drug concentration that significantly decreased DRG neuron injury compared with glucose alone ($p < 0.05$).

range of neuroprotective concentrations from 10 to 25 m*M* (Fig. 3A). In this range, nicotinamide significantly decreases glucose-induced DRG neuron death. In contrast, allopurinol alone produces only a modest decrease in TUNEL labeling at >500 μ*M* concentration (Fig. 3B). α-Lipoic acid is the most potent DRG neuron protective agent. At concentrations of >60 μ*M*, glucose-induced DRG neuron injury is prevented.

Individual drugs differentially alter the generation of ROS

The differential potency of these compounds to protect the DRG neurons may be linked to the ability to prevent oxidative stress. To test this hypothesis, the production of ROS was assessed in another high-throughput assay that utilizes fluorimetric probes. To assess superoxide, DRG neurons were loaded with DHE, and the change in fluorescence as DHE was oxidized to ethidium was measured in a 96-well plate reader (Fluoroskan, Labsystems). Similarly, H_2O_2 may be determined through the oxidation of nonfluorescent DCFDA to green fluorescent DCF in the same plate reader.

Nicotinamide alone decreases basal superoxide over a narrow concentration range from 7.5 to 15 m*M* (Fig. 4A). Lower concentrations of nicotinamide in the range 0.5–1 m*M* modestly decrease glucose-induced DHE oxidation, whereas 12.5 m*M* nicotinamide is the only concentration that significantly decreases basal superoxide as well as decreases glucose-induced superoxide to the level of untreated control DRG neurons. At 20 m*M* nicotinamide and above, DHE oxidation is increased by nicotinamide alone.

Because of the unusual profile of DHE oxidation over the dose–response curve of nicotinamide, the effects of nicotinamide on the oxidation of DCFDA that is attributable to H_2O_2 was also assessed (Fig. 4B). Low concentrations (0.5–5 m*M*) of nicotinamide induce a peak of H_2O_2 that decreases in the neuroprotective range of 10–15 m*M*. At 20 m*M* nicotinamide and higher concentrations, H_2O_2 is again produced by nicotinamide alone. In the presence of glucose, the low concentrations of nicotinamide decrease glucose-induced H_2O_2 in a dose-dependent-manner. At the neuroprotective concentrations, DCFDA oxidation is so low it is undetectable. Above 20 m*M* nicotinamide, the drug no longer prevents and instead exacerbates the production of H_2O_2 in the presence of 20 m*M* added glucose.

Allopurinol also alters the generation of superoxide in DRG neurons (Fig. 4C). Increasing concentrations of allopurinol alone slightly increase superoxide up to the 63 μ*M* concentration. Between 125 and 500 μ*M*, superoxide formation markedly decreases by >50%. Low concentrations of allopurinol do not prevent glucose-induced superoxide formation, but with 16 μ*M* allopurinol and higher, glucose-induced superoxide decreases in a dose-dependent manner up to 500 μ*M* compared with glucose alone.

α-Lipoic acid decreases both basal and glucose-induced superoxide at as low as 10 μ*M* concentration. The decrease in superoxide is greater with increasing concentrations of α-lipoic acid up to 60 μ*M*. Glucose-induced superoxide continues to decrease with up to 1,000 μ*M* α-lipoic acid, when DHE oxidation is slightly lower than basal levels in untreated DRG neurons.

Nicotinamide and α-lipoic acid prevent H_2O_2-induced DRG neuron injury

Because the drugs demonstrate different capacities to alter glucose-induced superoxide and injury in DRG neurons, we further assessed the neuroprotective ability of these compounds against oxidative stress injury. DRG neurons were treated with protective concentrations of nicotinamide (12.5 m*M*),

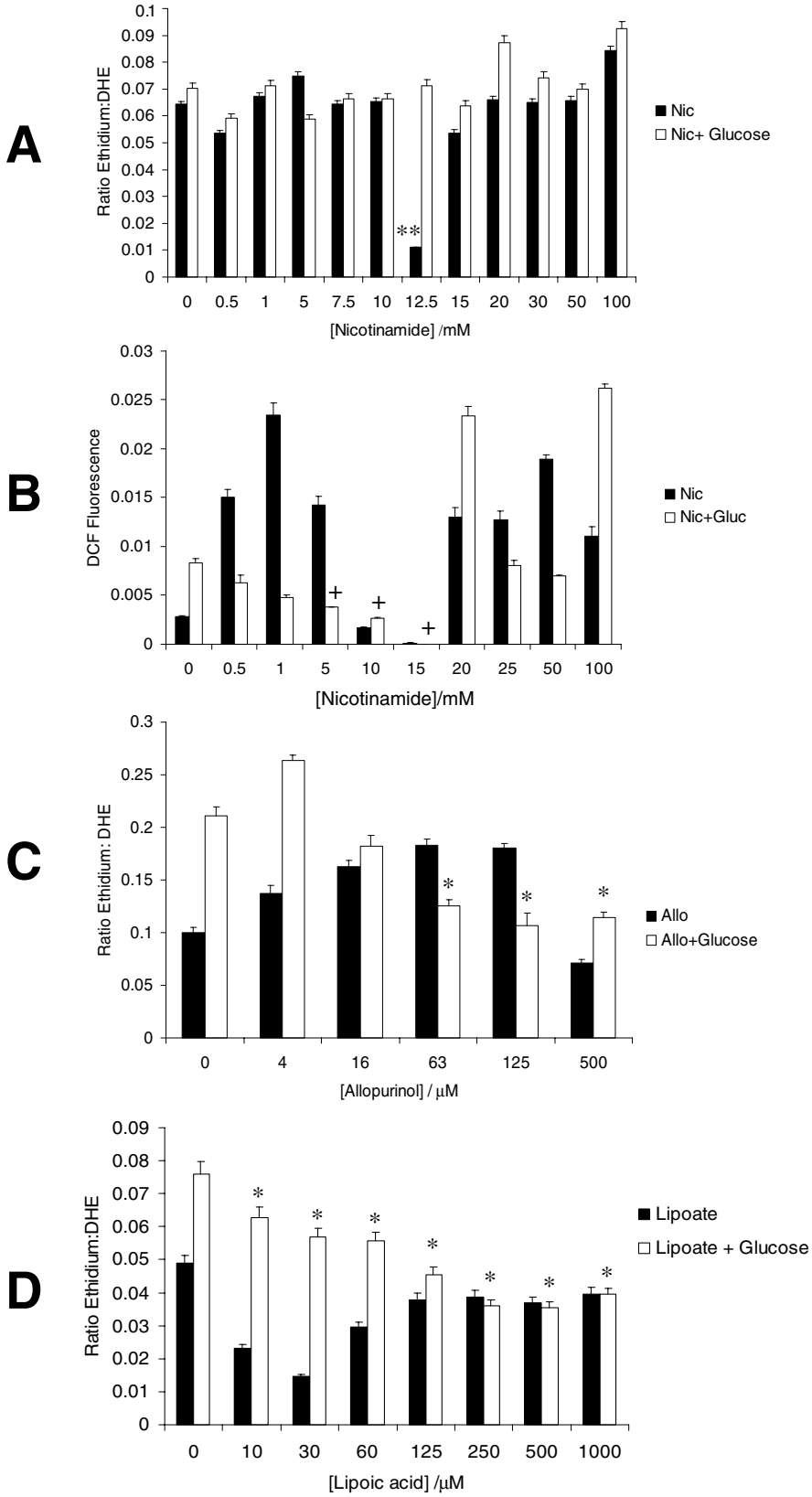

FIG. 4. Effect of individual drug treatments on glucose-induced DRG neuron ROS generation. The fluorimetric probe DHE, which is indicative of superoxide generation, was applied to DRG neurons 5 h following exposure to 20 m*M* added glucose with and without increasing concentrations of each of the test drugs (**A, C,** and **D**). The probe DCFDA was also assessed in a similar manner in a nicotinamide dose curve (**B**). The means and standard errors of the fluorescence for three separate assays are shown in each panel. $^{+}$Nicotinamide in the range 5–15 m*M* decreased glucose-induced DCF oxidation, $p < 0.01$. **12.5 m*M* nicotinamide significantly decreased basal DHE oxidation compared with untreated control cultures. *Significant decrease in ROS formation compared with glucose-exposed control cultures, $p < 0.05$.

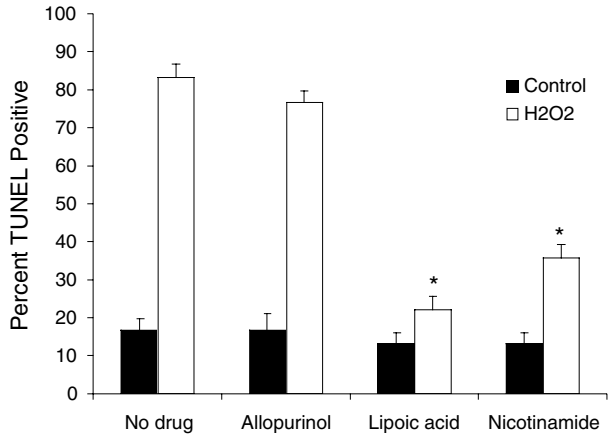

FIG. 5. Effect of individual drugs on H_2O_2-induced DRG neuron injury. Each of the drugs at a concentration that prevented glucose-induced DRG neuron injury was tested for the ability to prevent TUNEL staining induced by 1 μ*M* H_2O_2. The drug concentrations were as follows: nicotinamide, 12.5 m*M*; allopurinol, 1 m*M*; α-lipoic acid, 100 μ*M*. The same treatment schedule as the drug screen illustrated in Fig. 1 was used, with the exception that the α-lipoic acid-treated cultures were rinsed prior to the application of H_2O_2 to remove the catalase that was originally used to treat the α-lipoic acid stock solution. *α-Lipoic acid and nicotinamide significantly prevented H_2O_2-induced DRG neuron injury, $p < 0.01$.

allopurinol (1 m*M*), or α-lipoic acid (100 μ*M*) prior to the application of 1 μ*M* H_2O_2. Similar to the drug-screening protocol, the DRG neurons were then fixed after 24 h and TUNEL-stained. Both nicotinamide and α-lipoic acid prevented H_2O_2-induced DRG neuron injury, whereas allopurinol was not protective (Fig. 5). These data suggest that allopurinol does not effectively scavenge ROS in this experimental system.

The triple therapy drug combination prevents glucose-induced DRG neuron injury in the cell culture screening model

Next, combinations of the drugs were assessed. To perform these studies, stock solutions of the drug combination were treated with 1,000 U/ml catalase for 1 h prior to application to the cells. This removes H_2O_2 that may be generated during the solubilization process (29, 82) and may be directly toxic to the cultured DRG neurons. Because allopurinol demonstrated the lowest ability to protect the DRG neurons, but provided an increase in protection between 500 μ*M* and 1,000 μ*M*, both these concentrations were tested to determine whether allopurinol contributed to the neuronal protection.

Combination of the three drugs provides potent protection against glucose-induced ROS and DRG neuron death (Fig. 6). The drug combination alone does not alter basal superoxide (Fig. 6A) or H_2O_2 (Fig. 6B). One exception is that the combination of 1 m*M* allopurinol with 12.5 m*M* nicotinamide and 100 μ*M* α-lipoic acid modestly decreases basal levels of superoxide. In the presence of glucose, the drug combination decreases, but does not fully prevent the increase in superoxide, with 1 μ*M* allopurinol providing a significant improvement over 0.5 μ*M* allopurinol (Fig. 6A).

The drug combination completely prevents glucose-induced DRG neuron injury, as assessed by both the cleavage of caspase-3 and TUNEL staining (Fig. 6C).

DISCUSSION

Accumulating data suggest that glucose-mediated oxidative stress underlies the development of diabetic neuropathy (52, 58, 65, 70). One rational therapeutic approach to the treatment of diabetic neuropathy is to block oxidative stress (80). The current study tested the feasibility of a therapeutic strategy, which will prevent oxidative damage by three different mechanisms. We propose to attenuate the development of oxidative stress by using the xanthine oxidase inhibitor, allopurinol, quench oxidative stress by the use of the chain-breaking antioxidant α-lipoic acid, and prevent the downstream consequences of oxidative stress using the poly(ADP-ribose) synthase (PARS) inhibitor nicotinamide. Each of these compounds was tested alone and in combination for the ability to prevent oxidative stress and DRG neuron death.

Allopurinol alone modestly increases superoxide formation in a dose-dependent manner and only modestly prevents glucose-induced DRG neuron death at high concentrations. Yet, in the combination therapy, a higher concentration of allopurinol (1,000 μ*M*) provides increased survival compared with a lower (500 μ*M*) concentration. The data suggest that allopurinol does provide neuronal protection, but requires additional antioxidants to observe the effect in a cell culture system. The lack of effect in the experiments using allopurinol alone probably relates to the difficulty of solubilizing the compound and the probable generation of H_2O_2 during solubilization (29). This would explain why we observe an added benefit of allopurinol in the presence of α-lipoic acid.

Taken together, the data do not provide compelling evidence for the use of allopurinol for direct neuronal protection. These data increase our confidence in the drug screening technique to identify compounds that specifically promote DRG neuron survival. Yet allopurinol promotes endothelial function in diabetes (5), and so remains a beneficial component of the triple therapy. Perhaps a similar screen using cultured vascular endothelial cells would demonstrate the protective ability of this compound. Allopurinol is a xanthine oxidase inhibitor. Xanthine oxidase catalyzes conversion of hypoxanthine to xanthine, and xanthine to uric acid and functions as both a scavenger and inhibitor of oxygen free radical production (*i.e.,* superoxide, H_2O_2, and hydroxyl radicals) (24). In human aortic endothelial cells, xanthine oxidase is a major source of free radicals (93), and allopurinol treatment improves endothelial dysfunction (5). Allopurinol has been most extensively evaluated in the prevention of oxidative stress-induced myocardial injury. For example, clinical studies have demonstrated that allopurinol confers neurocardiac protection, improves myocardial function, and decreases arrhythmias following ischemia–reperfusion injury (11, 16, 17, 28, 30, 63, 74). Allopurinol can also preserve high-energy phosphate levels (56) in ischemic myocardium, to decrease muscle creatinine kinase release following cardiac ischemia (12, 42), and reduce infarct size in animal mod-

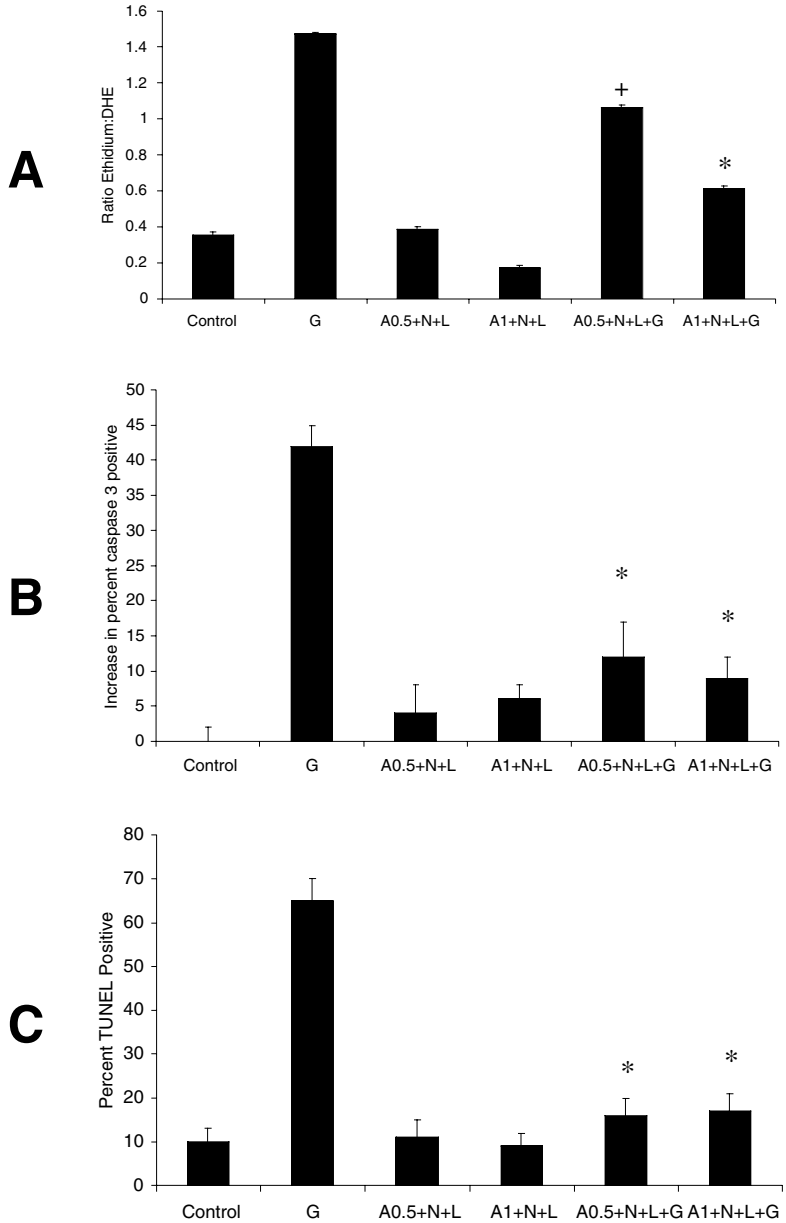

FIG. 6. Effect of the triple drug combination on ROS generation and cell injury. The combinations of allopurinol [0.5 m*M* (A0.5) or 1.0 m*M* (A1)], nicotinamide [12.5 m*M* (N)], and α-lipoic acid [100 μ*M* (L)] were applied to DRG neurons with or without the application of 20 m*M* glucose (G). Generation of superoxide (**A**) was determined after 5 h. Development of caspase-3 activation was determined after 5 h (**B**), or TUNEL staining was determined after 24 h (**C**). In each case, application of the triple combination of drugs did not alter DHE oxidation or DRG neuron injury in the absence of added glucose (compare drug combination with Control). In contrast, the drug combination significantly decreased each of these parameters in the presence of 20 m*M* added glucose: $^{*}p < 0.01$; $^{+}p < 0.05$.

els of myocardial ischemia (1, 3, 47). These effects may be mediated by decreasing oxidative damage. Allopurinol attenuates brain damage in neonatal rats via peri-injury administration, and decreases cerebral cortical free radical production in hypoxic infant piglets (16, 85). Allopurinol prevents accumulation of the lipid peroxidation product malondialdehyde (MDA), improves cerebral perfusion, and maintains cerebral electrical activity in asphyxiated human neonates (16, 79).

A recent clinical trial (8) examined the effects of 300 mg/day allopurinol on endothelial function and oxidative stress in type 2 diabetic patients. Allopurinol or placebo was administered for 1 month and the effects on forearm blood flow responses determined. Allopurinol, but not placebo, increased the endothelium-dependent mean blood flow response to acetylcholine by 30% and decreased systemic levels of MDA. These data are consistent with our hypothesis that oxidative stress is increased in diabetes and that this con-

tributes to endothelial and neuronal dysfunction. In the culture system, allopurinol does not prevent H_2O_2-induced DRG neuron injury, further demonstrating that this compound functions through the prevention of ROS formation, such as through inhibition of xanthine oxidase, rather than through ROS scavenging and antioxidant activity.

α-Lipoic acid potently protects DRG neurons in the cell culture system. This compound decreases glucose-induced free radical generation and cell death in a dose-dependent manner that strongly supports our contention that oxidative stress is a significant mediator of glucose-induced neuronal injury. α-Lipoic acid is an endogenous free radical scavenger (46) and metal chelator that also activates glucose uptake and the pyruvate dehydrogenase complex, and serves as a metabolic substrate for the E3 pyruvate dehydrogenase component. α-Lipoic acid is reduced to dihydrolipoate, which combines free radical (hydroxyl, superoxide, peroxyl, singlet oxygen)-scavenging and metal-chelating properties with the ability to regenerate concentrations of nonenzymatic and enzymatic antioxidants (39, 49). In preclinical studies, α-lipoic acid administration improved nerve function in diabetic rats (9, 46, 71). In streptozotocin-induced diabetes in rats, α-lipoic acid significantly improves or normalizes deficits in digital sensory nerve conduction velocity, endoneurial nutritive nerve blood flow, mitochondrial and cytoplasmic NAD^+/NADH ratios, Na^+,K^+-ATPase activity, levels of total and reduced glutathione, and the activities of superoxide dismutase, catalase, and cytochrome b5 reductase (71). Taurine, an important endogenous metal chelator with antioxidant (4, 49, 55, 76) and anti-PKC effects (38), is also depleted in nerve by streptozotocin-induced diabetes and restored by α-lipoic acid (71). α-Lipoic acid corrects deficits in NGF-dependent neuropeptide-Y and substance-P expression in peripheral nerve and spinal cord in streptozotocin-induced diabetic rats (26).

α-Lipoic acid has been extensively evaluated in man, including seven phase I clinical studies (which included type 1 diabetic patients), three phase II clinical studies in type 2 diabetic subjects, and three phase III clinical studies. In a dose-finding clinical trial [Alpha-Lipoic Acid in Diabetic Neuropathy (ALADIN) Study], treatment with intravenous α-lipoic acid for 3 weeks ameliorated major neuropathic symptoms (*i.e.,* pain and paraesthesias) in type 2 diabetic subjects with polyneuropathy (89). In another study [Deutsche Kardiale Autonome Neuropathie (DEKAN) Study], an improvement of heart rate variability was found after 4 months of oral treatment with α-lipoic acid (800 mg/day) in type 2 diabetic patients with diabetic autonomic neuropathy (90). In the ALADIN III Study (91), 509 subjects with type 2 diabetes and symptomatic diabetic neuropathy were administered sequential treatment with 600 mg/day α-lipoic acid given intravenously for 3 weeks followed by 600 mg three times a day for 6 months. The total neuropathy symptom score was significantly better at early time points in the α-lipoic acid-treated group, and neuropathic deficits were consistently improved throughout the study. Adverse events were not different between groups. Therefore, extensive clinical trial data indicate that α-lipoic acid is effective in ameliorating both neuropathic symptoms and deficits of cardiovascular autonomic function. Moreover, α-lipoic acid is safe and, at the dose of 600 mg three times a day, without significant adverse effects.

Nicotinamide demonstrates potent neuronal protection in the DRG neuron culture system, but over a narrow concentration range. The biphasic nature of nicotinamide-mediated protection suggests that more than one cellular mechanism is activated in DRG neurons by this compound. The best described mechanism of nicotinamide is as an inhibitor of poly(ADP-ribosylation), *i.e.,* the transfer of the ADP-ribose moieties from NAD to nuclear proteins by the enzyme PARS. Activation of PARS has emerged as an important potential mediator of glucose-induced oxidative damage (54). PARS is expressed in all cell types and is activated by DNA single-strand breakage (2), resulting from hydroxyl and superoxide anion radicals and peroxynitrite (14, 43). PARS activation is associated with energy depletion (75) and increased oxidative stress (92). PARS activation contributes to apoptosis (48), and PARS-knockout mice demonstrate protection from apoptosis (67). PARS activation is implicated as a mediator of many pathological conditions associated with oxidative stress, such as myocardial ischemia, cerebrovascular disease, renal ischemia–reperfusion (73), and pancreatic β-cell failure (13, 19, 20, 36, 37, 41, 45, 77, 78). Our present data suggest that at low concentrations nicotinamide increases H_2O_2 generation and modestly prevents superoxide generation. Once a concentration is reached that is known to inhibit PARS, nicotinamide prevents glucose-mediated injury. At higher concentrations, nicotinamide increases oxidative stress and so may override the protective effect of PARS inhibition.

In addition to PARS inhibition, nicotinamide is known to modulate immune cell function and survival via inhibition of ADP-ribosyl transferring enzymes (7, 33, 34, 40). Nicotinamide decreases production of tumor necrosis factor-α and interleukin-12 in cultures of whole blood from prediabetic and diabetic subjects (35). These effects have been proposed to confer protection to pancreatic β cells (57). Indeed, nicotinamide has been used in human clinical trials at high doses for many years, most recently for studying prevention of type 1 diabetes mellitus in high-risk patients through β cell preservation (13, 19, 20, 36, 37, 41, 45, 77, 78). Nicotinamide has been given to subjects with new-onset type 1 diabetes, at doses ranging from 200 mg to 3.5 g per day for 12 months with very low incidences of side effects or toxicity (13, 33, 37, 45, 61, 62, 77, 78, 84), for periods of 4 months to 4 years (19, 20, 33, 36). The European Nicotinamide Diabetes Intervention Trial (ENDIT) is currently monitoring use of nicotinamide (1.2 g/m^2, maximum 3 g/day) in 276 individuals with high risk for developing type 1 diabetes mellitus (33, 57). Taken together, the data suggest that nicotinamide may not scavenge ROS in the cell culture system, but at specific concentrations that activate PARS it is protective. The increase in ROS generation at high concentrations of nicotinamide, seen in this and other systems (92), is detrimental to the DRG neurons in culture and can increase injury in the presence of hyperglycemia.

The concept that increased ROS and oxidative stress occupy a central position in the pathogenesis of diabetic complications led us to identify new therapies aimed at different arms of the oxidative stress pathway (80). Individually, these therapies produce short-term benefits on surrogate measures

of diabetic complications in cell culture and animal models of diabetic complications (50, 51–53, 59). Clinical efficacy in type 1 patients is therapeutically more challenging than animal experiments, in part because hyperglycemia is more chronic and tissue damage more advanced in patients with diabetic clinical complications. Because we and others have now identified multiple metabolic perturbations that act synergistically to produce oxidative stress in complications-prone tissues, we contend that combination therapy targeting the different aspects of oxidative stress will be particularly efficacious (21). The current trial uses nicotinamide, α-lipoic acid, and allopurinol. *In vitro* screening of these compounds supports our choices. These compounds clearly function through different mechanisms to decrease oxidative stress, and together provide synergistic therapeutic benefit. This technique will allow further rapid screening of many more compounds in different combinations in order to efficiently design new clinical trials.

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ABBREVIATIONS

DCF, 2′,7′-dichlorofluorescein; DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; DMSO, dimethyl sulfoxide; DRG, dorsal root ganglion; FUDR, fluorodeoxyuridine; HBSS, HEPES-buffered saline solution; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; NGF, nerve growth factor; PARS, poly(ADP-ribose) synthase; PKC, protein kinase C; ROS, reactive oxygen species; TUNEL, terminal UTP-biotin nick-end labeling.

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