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Receptor for Advanced Glycation End Products Activation Injures Primary Sensory Neurons via Oxidative Stress

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The receptor for advanced glycation end products (RAGE) may promote diabetic vascular and renal disease through the activation of intracellular signaling pathways that promote oxidative stress. Oxidative stress is a mediator of hyperglycemia-induced cell injury and a unifying theme for all mechanisms of diabetic complications, but there are few studies on the expression and potential contribution of RAGE in diabetic neuropathy. The current study demonstrates that dorsal root ganglia neurons express functional RAGE and respond to the RAGE ligand S100 with similar downstream signaling, oxidative stress, and cellular injury as other diabetic complication-prone tissues. RAGE-induced phosphatidylinositol-3 kinase activity is associated with formation of reactive oxygen species, caspase-3 activation, and nuclear DNA degradation. These events are prevented by treatment with the antioxidant α-lipoic acid. Our data indicate that therapies aimed at decreasing RAGE ligands, blocking RAGE signaling, or preventing oxidative stress could significantly decrease the development of neuropathy in diabetic patients. (*Endocrinology* 148: 548–558, 2007)

ADVANCED GLYCATION end-products (AGE) are the result of the nonenzymatic addition of glucose or other saccharides to proteins, lipids, and nucleotides (1–3). AGE formation occurs normally over time, but in diabetes, excess glucose accelerates AGE generation (2–4). AGE formation in cells and tissues leads to intra- and extracellular protein cross-linking and protein aggregation (4–8). AGE accumulation in diabetic tissues correlates with age, duration of diabetes, and the degree of glycemic control (9–11).

In addition to producing structural modifications, AGE result in cellular reactions through binding to cell surface receptors (4, 12). There are many of these receptor proteins, and several of these bind, internalize, and destroy AGE-modified substrates (4, 12–14). The receptor for AGE (RAGE) has definite downstream signaling targets and is the main receptor through which AGE signaling is mediated (12, 15–22).

Activation of RAGE is associated with diabetic microvascular complications including specific loss of podocytes in nephropathy and microangiopathy characteristic of retinopathy (23). An environment rich in AGEs also accelerates atherosclerosis via RAGE interaction (24). In mesangial (15) and endothelial (25) cells, RAGE activation results in a burst of reactive oxygen species (ROS). The exact mechanism for this is unknown but is thought to involve nicotinamide adenine dinucleotide phosphate (reduced) [NAD(P)H] oxidase (20). This event alone could contribute to cellular oxidative stress and dysfunction. In addition, RAGE signals via phosphatidylinositol-3 kinase (PI-3K), Ki-Ras, and the MAPKs, Erk1 and Erk2 (15, 26–28). These signaling pathways initiate and sustain the translocation of nuclear factor-κB (NF-κB) from the cytoplasm to the nucleus in a number of cell types including circulating monocytes and endothelial cells (29–31), leading to inflammation and organ damage (32, 33). RAGE knockout and wild-type mice treated with soluble RAGE have markedly decreased streptozotocin-induced diabetic renal and vascular disease (34, 35).

RAGE is expressed in both the central and peripheral nervous systems and is implicated in neurodegenerative disorders including Alzheimer's disease (36), Creutzfeldt-Jakob disease (37), and stroke (38). In peripheral nerves, RAGE is found in nerve bundles associated with the blood vessels (39) and on axons (40). The expression and activation of RAGE in dorsal root ganglia (DRG) neurons, the sensory neurons affected in diabetic neuropathy, have not previously been reported. We recently demonstrated the direct consequences of elevated glucose on the development of oxidative stress in DRG neurons, mitochondrial dysfunction, and cell injury (41–45). Our hypothesis is that uncontrolled diabetes leads to AGE accumulation in complications prone tissue, resulting in RAGE activation, activation of NAD(P)H oxidase, ROS accumulation, and oxidative stress, which in turn induce apoptosis. The current study investigates RAGE expression,

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Abbreviations: Ab-RAGE, RAGE-blocking antibody; AGE, advanced glycation end products; DHE, dihydroethidium; DMSO, dimethylsulfoxide; DRG, dorsal root ganglia; GFP, green fluorescent protein; GSK, glycogen synthase kinase; NAD(P)H, nicotinamide adenine dinucleotide phosphate (reduced); NF-κB, nuclear factor-κB; NGF, nerve growth factor; PI-3K, phosphatidylinositol-3 kinase; RAGE, receptor for AGE; ROS, reactive oxygen species; sRAGE, soluble RAGE; TBARS, thiobarbituric acid reactive substance; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling.

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AGE-mediated RAGE activity and signaling, and RAGE-induced oxidative stress within DRG neurons. We report that DRG neurons express RAGE and that activation of RAGE results in severe oxidative stress and apoptosis in DRG neurons. These results suggest that AGE may play a role in the development of diabetic neuropathy, similar to their role in nephropathy and retinopathy (23, 46, 47).

Materials and Methods

Primary DRG neuron culture

DRG were harvested from embryonic d 15 Sprague Dawley rats, dissociated in 1% trypsin, and then cultured on rat-tail collagen-coated culture plates in growth media (41, 43, 48, 49). All culture reagents were from Invitrogen (Carlsbad, CA) unless stated otherwise. Growth media were prepared using neurobasal medium supplemented with 1 × B-27 additives, 50 ng/ml nerve growth factor (NGF; Harlan Bioscience, Indianapolis, IN), 40 μM fluorodeoxyuridine (Sigma, St. Louis, MO), and 1000 U/ml penicillin/streptomycin/neomycin solution. Initial plating medium contained 2 μM glutamine. DRG neurons were refed after 24 h in fresh media containing all additives except glutamine. On d 2, cells were rinsed and then refed 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 fluorodeoxyuridine, and 1000 U/ml penicillin/streptomycin/neomycin solution). Experiments were performed on DRG neurons on d 3 in culture in the absence of B-27 additives.

Experimental treatments

In these experiments, S100, a well-defined activator of RAGE signaling (33), was used as the RAGE ligand. S100 isolated from bovine brain was obtained from Calbiochem, catalog no. 559284 (EMD Biosciences, San Diego, CA), and was dissolved in neurobasal media and used at the indicated concentrations. Dr. Ann Marie Schmidt (Columbia University, New York, NY) generously provided both soluble RAGE (sRAGE) and RAGE-blocking antibody (Ab-RAGE) (50, 51). Neurobasal media contained 25 mM glucose as standard that is required for the health of neurons in culture (43, 49). To mimic a hyperglycemic insult, 20 mM glucose was applied to the neurobasal culture media or 20 mM *o*-methylglucopyranose as an osmotic control as previously described (41, 43, 49, 52). Signaling inhibitors PD98059, LY294002, and SB203580 were obtained from Calbiochem (San Diego, CA). NAD(P)H oxidase inhibitor apocynin was obtained from Sigma. Inhibitors were dissolved in 10 μl dimethylsulfoxide (DMSO), and then DMSO was diluted to less than 0.1% final concentration.

Fragmentation of nuclear DNA

Per our published protocols terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) staining was used to detect programmed cell death in DRG neurons (43). DRG neurons were fixed in 4% paraformaldehyde before staining. Samples were labeled with digoxigenin-deoxyuridine triphosphate and then stained with horseradish peroxidase-conjugated antidigoxigenin antibody using a kit according to the manufacturer's instructions (Intergen, Gaithersburg, MD).

Caspase-3 activation

Cleavage of procaspase-3 to the active form was detected using the fluorescent substrate DEVD-FMK. Labeling was performed using the reagents and protocol in a kit from Intergen. Labeling was quantitated by counting the percent cleaved caspase-3-positive cells with green fluorescent cell bodies out of the total neuron population, identified by bis-benzamide counterstain per our published protocol (43).

Measurement of superoxide formation using dihydroethidium (DHE)

The cell-permeant DHE (Molecular Probes, Eugene, OR) was used to assess real-time formation of superoxide in DRG neurons. Reduced DHE emits blue fluorescence. Exposure to superoxide causes oxidation to ethidium that emits red fluorescence. Stock DHE was dissolved at a concentration of 10 mg/ml in DMSO, diluted to 150 μM in HEPES-buffered saline solution [10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$] immediately before use, and then added directly to the culture media at a 1:50 dilution, giving a final concentration of 3 μM. The DHE solution was applied to DRG neurons for the final 15 min of an experiment, and then neurons were rinsed once rapidly in HEPES-buffered saline solution 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 excitation, 520 emission) fluorescence was determined with 1 sec integration for each reading.

Protein concentration

For the biochemical assays that followed, activity was normalized to the amount of protein in the sample. The protein concentration was determined by the Bradford-Lowry method using the reagents and protocol supplied by Bio-Rad Laboratories (Hercules, CA). Samples were read in a 96-well plate in an absorbance plate reader (Multiskan; Labsystems).

Thiobarbituric acid reactive substance (TBARS)

Lipid peroxidation was assessed by determining the level of TBARS in cell lysates (53). DRG neurons were lysed in water and then 100 μl sample added to 200 μl ice-cold 10% trichloroacetic acid on ice for 15 min to precipitate protein. During this incubation, standards were prepared using 1,1,3,3-tetramethoxypropane in the range of 0–100 μM. Precipitated samples were centrifuged at 2200 × *g* for 15 min at 4 C. Supernatants were mixed with an equal volume of 0.67% thiobarbituric acid and then boiled for 10 min. Once cooled, the absorbance was read at 532 nm on an absorbance plate reader (Multiskan; Labsystems).

NAD(P)H oxidase

NAD(P)H oxidase activity in DRG neuron lysates was assessed through the chemiluminescent detection of NAD(P)H oxidase-generated superoxide (54, 55). DRG neurons were scraped in 50 mM Tris-MES buffer (pH 7.4) containing 10 μg/ml leupeptin and 10 μg/ml phenylmethylsulfonylfluoride, sonicated, centrifuged at 10,000 × *g* for 5 min and then assayed immediately. On a white 96-well plate, a 30-μl sample was mixed with 45 μl Tris-MES and 100 μl enhanced chemiluminescent reagent (Amersham, Arlington Heights, IL) with either 20 μl additional Tris-MES or 20 μl 1 mg/ml apocynin (final concentration 100 μg/ml) to inhibit NAD(P)H oxidase. The reaction was started by adding 5 μl NAD(P)H solution to a final concentration of 150 μM, and the increase in luminescence was measured (Fluoroskan, Ascent II; LabSystems) every 10 sec for 10 min. The rate of luminescence increase in the presence of apocynin was subtracted from the rate in the absence of apocynin to give a measure of superoxide generation by NAD(P)H oxidase activity in change in luminescence units per minute.

Gene transfer using adenoviral constructs

Adenovirus constructs containing cDNA for myristoylated (constitutively active) Akt, dominant-negative kinase dead K179M mutation of Akt, or green fluorescent protein (GFP) under the cytomegalovirus promoter were prepared, purified, and titered at the University of Iowa Gene Transfer Vector Core per their RAPAd system (http://www.uiowa.edu/~gene/vectors.htm). At 20 h, 95–100% of DRG neurons are infected after applying 1000 plaque-forming units per DRG neuron, determined by counting GFP-positive neurons.

RAGE immunofluorescence

DRG cultures were fixed with 4% paraformaldehyde and rinsed in PBS [0.1 M (pH 7.2)]. The cultures were permeabilized and blocked with 0.1% TX100/PBS containing 2% nonfat dry milk and 1% normal serum to reduce nonspecific adherence of antibodies. DRG cultures were incubated in primary antisera (rabbit anti-RAGE, 1:1000, Chemicon, Temecula, CA) for 24 h at 22 C in a humid chamber. After incubation with

the primary antisera, the DRGs were rinsed and incubated with AlexaFluor 488 (Molecular Probes).

Western immunoblotting

Equal amounts of protein (40 μg) were loaded onto a 12.5% polyacrylamide gel. To verify protein transfer and equal protein loading, nitrocellulose membranes were stained with Ponceau S and then incubated with 5 μg/ml primary antibody in 5% nonfat milk overnight at 4 C, secondary antibody (1:3000) for 1 h at room temperature, visualized with enhanced chemiluminescent reagent (Amersham), and exposed to film (Hyperfilm-ECL; Amersham). Antiphosphoprotein antibodies were obtained from Cell Signaling Technology (Beverly, MA). horseradish peroxidase-conjugated secondary antibodies are obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Blots were stripped by applying a solution containing 2 ml 10% sodium dodecyl sulfate, 0.15 g dithiothreitol, 2 ml 0.5 M Tris (pH 6.8), and 6 ml H_2O and then warmed to 70 C for 20 min. Membranes were then reblocked and probed for loading controls as specified in each experiment.

Statistical analysis

All experimental samples were assayed in duplicate in each protocol. Assays were repeated on three separate occasions, or more where indicated, using different DRG neuron cultures. Statistical significance was determined using ANOVA with 95% confidence intervals.

Results

DRG neurons express RAGE

Primary DRG neurons are well recognized as an *in vitro* model system for examining the mechanisms that underlie glucose-mediated injury and the subsequent development of experimental and clinical diabetic neuropathy (43, 56, 57). The current study used this *in vitro* approach to examine the potential effects of AGE on DRG neurons. We hypothesize that in poorly controlled clinical diabetes, AGE formation results in RAGE activation, RAGE downstream signaling pathway activation, and neuronal injury.

Initially, we examined RAGE protein expression and localization, and effect of the RAGE ligand S100 regulation, on receptor expression and cell survival (Fig. 1). DRG neurons in culture expressed detectable levels of RAGE protein (Fig. 1A). RAGE localization in DRG neurons, as determined by immunocytochemistry and fluorescence microscopy, appeared broadly distributed over the cell body and along neurites with some evidence of punctate sites on both the cell bodies and neurites (Fig. 1A). DRG neurons were exposed to increasing concentrations of S100 because S100 is routinely used to activate RAGE (33) to examine a global cellular effect on survival. After 24 h DRG neurons were fixed and stained for TUNEL (Fig. 1B). The 5 μg/ml dose of S100 significantly increased DRG neuron injury, and this concentration was used for subsequent studies. RAGE expression increased by 30% 6 h after exposure to 5 μg/ml S100 and continued to increase through 9 h (Fig. 1, C and D). The increase in RAGE expression was statistically significant after 9 h ($P < 0.05$). In contrast, exposure to 20 mM added glucose, which produces a similar degree of DRG neuron injury (43, 49), did not alter the expression of RAGE over 9 h (Fig. 1, C and D). Loading the DRG neurons with the antioxidant α-lipoic acid (100 μM) also did not alter the expression of RAGE (not shown).

Ligand binding to RAGE induces intracellular signaling

Although a modest increase in RAGE expression occurred on ligand exposure, we hypothesized that ligand binding would induce a greater effect on RAGE activation and downstream signaling. Activation of RAGE leads to intracellular signaling through two primary signaling pathways: the MAPK pathway as reported in monocytes (29), fibrosarcoma cells (17), intestinal epithelium (16), and vascular smooth muscle cells (51), and the PI-3K pathway, which results in the phosphorylation and activation of Akt, as in renal mesangial cells (15). To determine the effect of ligand binding on RAGE signaling in DRG neurons, cultures were exposed to 5 μg/ml S100 for 0, 15, 30, or 60 min, followed by Western immunoblotting using phospho-specific antibodies (Fig. 2). Blots were then stripped and reprobed for total Akt or total Erk for loading control. Akt phosphorylation increased within 15 min and peaked at 30 min after activation of RAGE with an increase of 30% over basal phosphorylation (Fig. 2A). Phospho-Erk44 and Erk42 also increased within 15 min, but interestingly, total Erk increased over the 60 min time course. The ratio of phospho-Erk to Erk was increased only over basal levels at the 15-min time point (Fig. 2C). Control conditions demonstrated that loading the cells with the antioxidant α-lipoic acid (100 μM) prevented S100-induced signaling, but exposure to H_2O_2 increased signaling intermediate phosphorylation (Fig. 2A). We conclude that RAGE is expressed and functionally active in DRG neurons and produces transient activation of Akt/PI-3K and MAPK. Because the activation of Akt was modest, we performed another series of blots to confirm this finding (Fig. 2B). To decrease basal phospho-Akt, NGF was removed from the culture media and neutralizing NGF antibody (R&D Systems, Minneapolis, MN; catalog no. AF-556-NA) was applied at 1 μg/ml for 3 h before treatment with S100. Lysates from DRG neurons treated with 5 μg/ml S100 for 0, 15, 30, or 60 min were assessed in the same blot for phospho-Akt, total Akt, and the Akt substrate glycogen synthase kinase (GSK)-3. The increase in phospho-Akt was more evident after 15 min S100 treatment in the NGF depleted cultures. Similarly, phospho-GSK-3 increased after 15 min exposure to S100 suggesting functional activation of Akt.

Activation of RAGE produces ROS

Previous studies suggest that a major consequence of RAGE activation is the intracellular generation of ROS (14, 20, 25). To determine whether RAGE activation leads to the formation of ROS in our model, DRG neurons were exposed to the RAGE ligand S100 and then DHE oxidation measured as an indicator of overall ROS production (58) (Fig. 3). DHE oxidation occurred in a dose-dependent manner with significant increases in DHE oxidation detected with concentrations as low as 0.16 μg/ml S100 (Fig. 3A). Initial ROS production was rapid, with 2-fold increases in DHE oxidation within 15 min of S100 application (Fig. 3B). S100-induced ROS formation remained stable up to 3 h and then increased significantly after 4–5 h (Fig. 3B).

To demonstrate that S100-induced ROS formation is RAGE dependent and not produced through nonspecific oxidation, DRG neurons were incubated with sRAGE, which binds S100 extracellularly preventing RAGE-mediated signaling, or an Ab-RAGE, which prevents S100 binding to RAGE at the cell membrane (Fig. 3C). sRAGE decreased

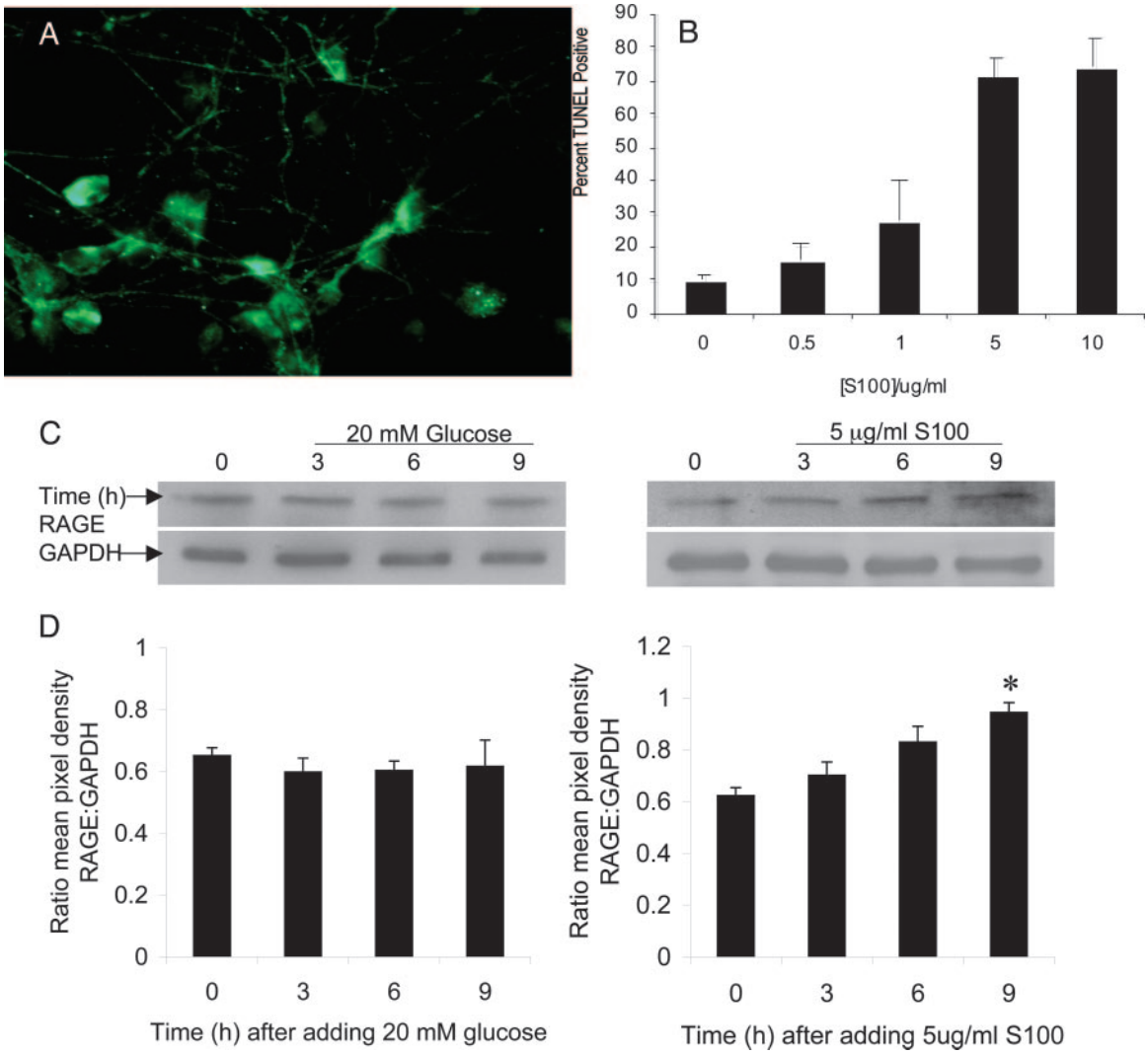

FIG. 1. DRG neurons express RAGE. A, Cultured DRG neurons were fixed and stained using a RAGE antibody, with a fluorescein isothiocyanate-conjugated secondary. The image is obtained using a planar ×40 objective lens. Background fluorescence is subtracted from the image. B, Cultured DRG neurons were exposed to increasing concentrations of S100 for 24 h and then TUNEL stained. Concentrations of 5 μM S100 and higher significantly increased DRG neuron injury. C and D, Cultured DRG neurons were treated with 20 mM added glucose or 5 μg/ml S100 for 0, 3, 6, or 9 h and processed for Western blotting. C, Blots were probed using antibodies against RAGE or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. The figure contains a representative blot from three replicates performed on separate occasions and producing the same pattern of RAGE expression. D, The mean pixel density for each band in the three replicate blots was determined using Scion Image software. The graphs illustrate the mean and SE for all the blots. RAGE bands were normalized against GAPDH in each blot. RAGE expression was significantly increased over control after 9 h S100 treatment. *, $P < 0.05$.

S100-induced DHE oxidation by approximately 70% and the Ab-RAGE prevented greater than 80% of S100-induced DHE oxidation in the DRG neurons (Fig. 3C). The antioxidant α-lipoic acid completely prevented DHE oxidation in DRG neurons. Controls for the Ab-RAGE and sRAGE were included. Nonimmune IgG or an excess of BSA (100 μg/ml) did not prevent S100-induced DHE oxidation.

RAGE activation induces NAD(P)H oxidase activity

NAD(P)H oxidase activity generates ROS, producing cellular injury via oxidative stress (54, 55), and RAGE activation leads to NAD(P)H oxidase activity in vascular endothelial cells (59). Therefore, NAD(P)H oxidase activity was measured in DRG neurons at increasing periods after the application of S100 (Fig. 4). Within 1 h of application of S100 (5 μg/ml), NAD(P)H oxidase activity increased significantly (Fig. 4A; $P < 0.01$). The level of NAD(P)H oxidase activity remained elevated at 3 and 5 h after exposure to S100 (Fig. 4A; $P < 0.05$). S100-induced activation of NAD(P)H oxidase at the 1 h time point was prevented in the presence of sRAGE and Ab-RAGE (Fig. 4B) but not by nonimmune IgG or excess BSA, suggesting that this effect of S100 is also RAGE mediated.

RAGE-induced ROS produce oxidative stress

A consequence of increased ROS in cells is the accumulation of oxidized lipids, proteins, and nucleic acids. The TBARS assay is widely used to detect generalized lipid per-

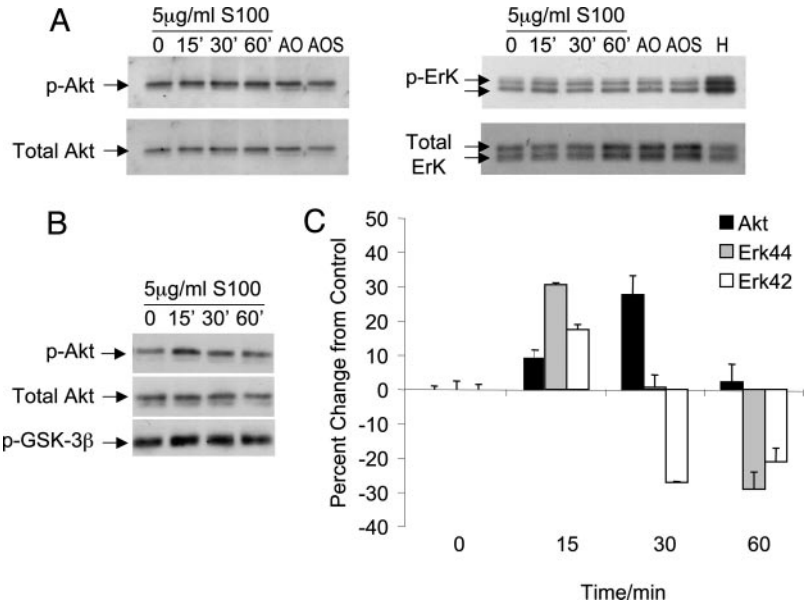

FIG. 2. S100 binding to RAGE stimulates cellular protein phosphorylation pathways. DRG cultures were exposed to 5 μg/ml S100 for 0, 15, 30, or 60 min ± 3 h pretreatment with 100 μM α-lipoic acid (AO) and then lysed for Western blotting (AOS, α-lipoic acid + S100). The blots were probed using antibodies against phosphorylated Akt and Erk, demonstrating the activation of these proteins. Blots were subjected to densitometry then stripped and reprobed for total Akt and Erk as a loading control. A, Representative blots from five replicate experiments performed on separate occasions. B, DRG neuron cultures were first incubated in NGF-free media containing 1 μg/ml NGF neutralizing antibody before exposure to S100. Lysates were prepared for Western blotting as usual and then blots were probed for phospho-Akt, stripped and probed for total Akt, and then reprobed for phospho-GSK-3. C, Mean and SE for each condition from all Akt and Erk blots normalized to the loading controls.

oxidation products that react with thiobarbituric acid including but not limited to malondialdehyde (48). At 1 h after exposure to S100 or S100 plus sRAGE, DRG neurons were lysed and TBARS determined (Fig. 5). The S100 (5 μg/ml) treatment significantly increased the levels of malondialdehyde-like oxidized lipids. In contrast, the presence of sRAGE completely blocked the S100-induced increase in TBARS. The data demonstrate that receptor-mediated effects of S100 cause an increase in ROS resulting in lipid peroxidation and oxidative stress in DRG neurons.

AGE and hyperglycemia induce DRG neuron apoptosis independently via oxidative stress

Our hypothesis is that poorly controlled diabetes leads to AGE accumulation, resulting in RAGE activation, oxidative stress, and neuronal injury and death. To test whether acute hyperglycemia induces apoptosis via RAGE, DRG neurons were treated with 5 μg/ml S100 in the presence or absence of sRAGE or the Ab-RAGE. Exposure to 5 μg/ml S100 induced the activation of caspase-3 in approximately 80% of DRG neurons at 5 h (Fig. 6A). Addition of sRAGE or the Ab-RAGE or α-lipoic acid prevented S100-induced caspase-3 activation (Fig. 6A). Nonimmune IgG or excess BSA, used as control treatments, did not prevent S100-induced activation of caspase-3.

An end point measure of apoptosis is the degradation of nuclear DNA by the TUNEL assay. After 24 h, either 5 μg/ml S100 or 20 mM added glucose-induced DNA degradation in approximately 70% of DRG neurons (Fig. 6B). The effects of high glucose on DRG neuron injury has been extensively studied, and we include the data here for comparison (43, 48, 49). Similar to the activation of caspase-3, S100-induced DRG neuron caspase-3 activation was prevented in the presence of excess sRAGE or Ab-RAGE, but glucose-induced caspase-3 was unaffected by sRAGE or Ab-RAGE. These data imply that acute hyperglycemia induces apoptosis via a mechanism other than RAGE activation. Both hyperglycemia and S100 induce ROS formation and oxidative stress. To test whether DRG neuron injury proceeds through oxidative stress, DRG neurons were pretreated with 100 μM α-lipoic acid before exposure to 5 μg/ml S100 or 20 mM added glucose. Both S100-induced and glucose-induced DNA fragmentation and caspase-3 cleavage were prevented in the presence of α-lipoic acid (Fig. 6). Therefore, both RAGE activation and acute hyperglycemia induce oxidative stress-mediated apoptosis, which may be successfully prevented with antioxidant treatment.

S100-induced DRG neuron injury is mediated via the PI-3K/NAD(P)H oxidase pathway

Prolonged oxidative stress ultimately leads to neuronal injury and apoptosis (41, 43). Evidence of apoptosis in DRG neurons was obtained by assessing DNA degradation using TUNEL stain, which specifically labels the nicked ends of DNA (Fig. 7A). To evaluate the extent of neuronal apoptosis induced by S100 and to determine which signaling pathway mediates S-100-RAGE-induced DRG neuron injury, DRG neurons were treated with 5 μg/ml S100 ± inhibitors of the Erk1/2 MAPK, PI-3K, and p38 MAPK pathways and assessed for apoptosis via TUNEL (Fig. 7A). Addition of the MAPK pathway inhibitor PD98059 (10 μM), which inhibits MAPK kinase-induced Erk activity, or the p38 pathway inhibitor SB203580 (20 μM) did not alter DRG neuron survival either alone or in the presence of S100 injury (Fig. 7A). In contrast, the PI-3K inhibitor LY294002 (10 μM) prevented S100-induced DRG neuron DNA degradation ($P < 0.01$). These data suggest that S100-induced DRG neuron injury may proceed via RAGE-induced PI-3K signaling pathway.

To determine whether PI-3K inhibition prevents DRG neuron injury via blocking S100-induced activation of NAD(P)H oxidase, the NAD(P)H oxidase assay was repeated 1 h after exposure to S100 in cultures that were pretreated ± 10 μM LY294002 (Fig. 7B) or 20 μM SB203580. Pretreatment with LY294002 completely prevented S100-induced activation of NAD(P)H oxidase. These data suggest that PI-3K signaling downstream of RAGE activation leads to NAD(P)H oxidase

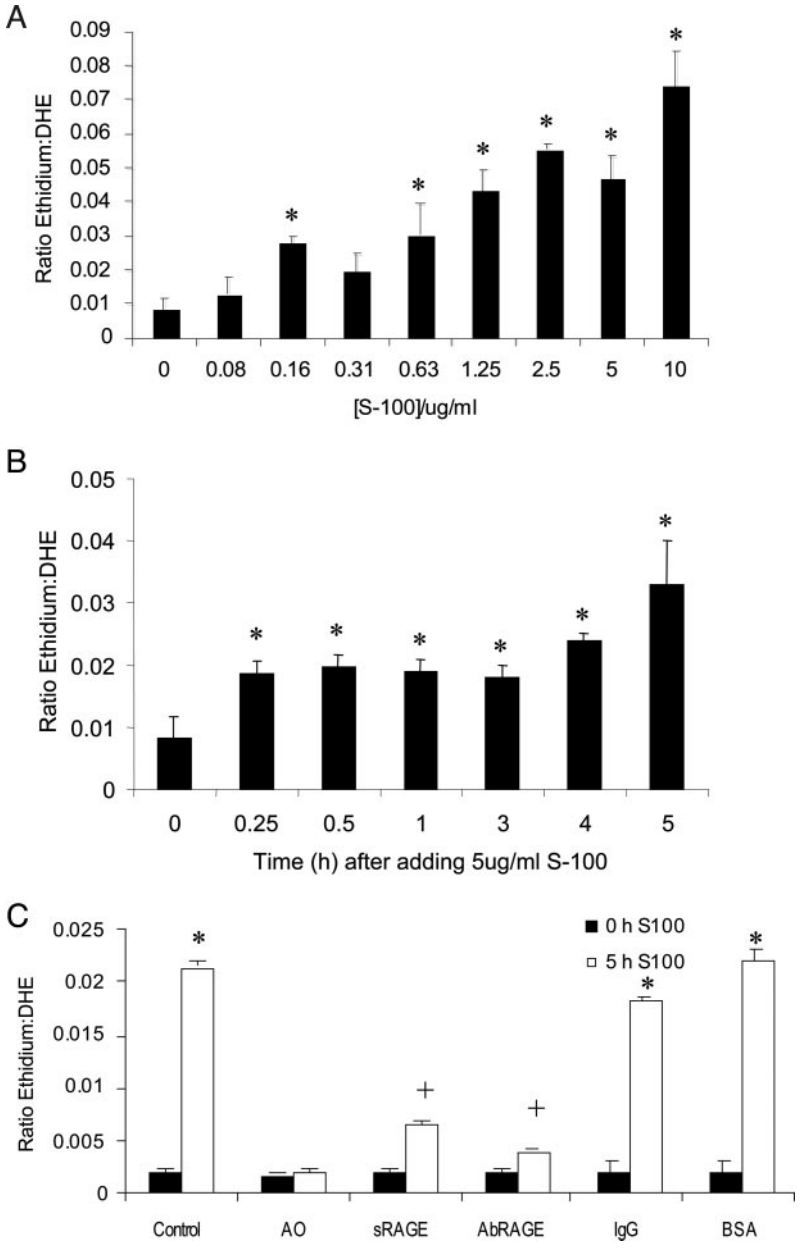

FIG. 3. RAGE signaling leads to the generation of ROS. DRG cultures were treated as described for each panel and loaded with 3 μM DHE for 15 min. The ratio of ethidium (red) to DHE (blue) fluorescence was determined as a measure of oxidation by ROS. In A, increasing doses of S100 were applied for 5 h. In B, 5 μg/ml S100 was applied for periods up to 5 h. In C, 5 μg/ml S100 was applied for 5 h in DRG neurons pretreated with media only, sRAGE (500 μg/ml), Ab-RAGE (1 μg/ml), nonimmune IgG (1 μg/ml), or BSA (20 μg/ml). Annotations indicate DHE oxidation significantly different from untreated control. AO, α-Lipoic acid. *, $P < 0.01$; +, $P < 0.05$.

activation in DRG neurons, as is demonstrated in other cell types (60, 61). This NAD(P)H oxidase activity produces ROS and leads to cellular injury and apoptosis.

A dual role for Akt/PI-3K in DRG neuron survival

We previously demonstrated that activation of Akt/PI-3K is a critical component of IGF-I-mediated DRG neuron protection against hyperglycemia (56). The current data demonstrating Akt-mediated activation of NAD(P)H oxidase and DRG neuron injury seem to contradict these previous findings. To address this contrasting finding, we further examined the role of Akt signaling in our cell system. First, we used adenoviral constructs to overexpress either constitutively active myristoylated Akt or a dominant-negative kinase dead Akt in the DRG neurons and then examine the effect on basal or S100-induced Akt (Fig. 8A). Unlike the result of transient activation of Akt seen with S100-RAGE activity, constitutively active Akt caused a slight decrease in basal NAD(P)H oxidase activity and prevented S100-induced NAD(P)H oxidase activity. Blocking activation of Akt with the kinase dead construct also decreased basal and S100-induced NAD(P)H oxidase activity (Fig. 8A). This suggests that Akt activity does indeed play a role in S100-induced activation of NAD(P)H oxidase. However, we conclude that either activation of Akt alone is insufficient to produce NAD(P)H oxidase activity or long-term expression of activated Akt leads to additional mechanisms that depress NAD(P)H oxidase activity. It is possible that Akt may be permissive in that it is necessary for the activation of NAD(P)H oxidase but not an intermediate in the pathway of NAD(P)H activation downstream of RAGE activation.

As a final exploration of the differential effects of Akt in

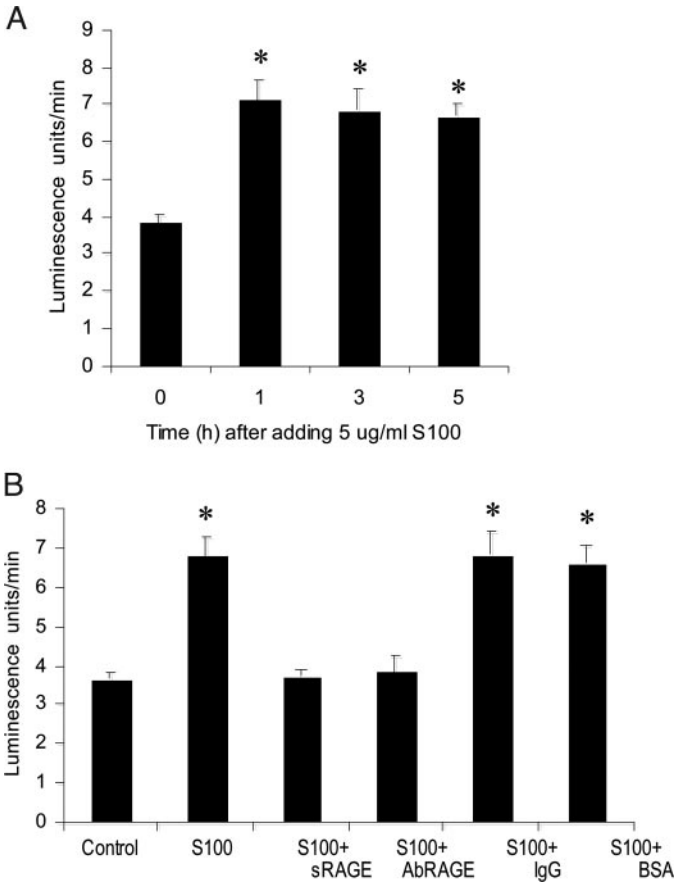

FIG. 4. RAGE signaling rapidly activates NAD(P)H oxidase. The activity of NAD(P)H oxidase was determined in DRG lysates by chemiluminescent detection of NAD(P)H oxidase generation of ROS in samples containing equal amounts of protein lysate. A, DRG neurons were treated for different times with 5 μg/ml S100. In B, the DRG neurons were treated for 1 h with 5 μg/ml S100 or 5 μg/ml S100 plus 500 μg/ml sRAGE, 1 μg/ml Ab-RAGE, 1 mg/ml nonimmune IgG or 20 μg/ml BSA. *, Within 1 h, S100 alone significantly increased NAD(P)H oxidase activity, compared with untreated control cultures ($P < 0.01$). B, At the 1 h time point, sRAGE and Ab-RAGE that block S100 activation of the receptor, but not control nonimmune IgG, BSA blocked the activation of NAD(P)H oxidase.

transient or long-term activation, we examined the effect of IGF-I that promotes Akt activation and survival on NAD(P)H oxidase activity (Fig. 8B). We found that IGF-I actually suppressed basal NAD(P)H oxidase activity and decreased S100-induced NAD(P)H oxidase activity. Thus, the signaling pathways of IGF-I and RAGE are not the same. They share common Akt activation but also display significantly different signaling responses, such as differential activation of Erk and altered downstream cellular effects.

Discussion

Because DRG neurons are susceptible to injury in diabetes (reviewed in Ref. 62) and AGE increase in diabetes (2–4), we propose that signaling through AGE-RAGE may be an additional factor to direct glucose-mediated injury in the pathogenesis of diabetic neuropathy. As a first step, we investigated the effects of RAGE activation on DRG neurons *in vitro*. Our study demonstrates that DRG neurons express functional RAGE that mediates ROS generation and modest transient activation of both MAPK and PI-3K. Downstream of RAGE activation, DRG neurons activate NAD(P)H oxidase with subsequent oxidative stress and neuronal injury. AGE binding to RAGE contributes to diabetic complications, including atherosclerosis and diabetic renal and eye disease (34, 35, 63, 64). We now present evidence that AGE-RAGE interactions promote injury to primary sensory neurons, suggesting that AGE-RAGE contributes to the development of diabetic neuropathy.

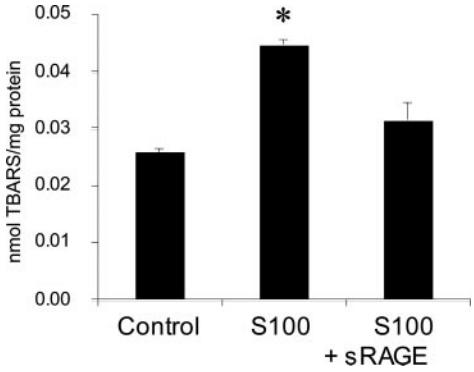

FIG. 5. RAGE signaling produces cellular oxidative stress. DRG cultures were exposed to S100 (5 μg/ml) or S100 + excess sRAGE (500 μg/ml) and then lysed and lipid peroxidation was determined using the assay of TBARS. *, TBARS in S100-treated cultures was significantly higher than untreated controls, $P < 0.01$.

DRG neurons express RAGE and exposure to the RAGE ligand S100 increases RAGE expression. These results in neurons are paralleled in other cell types. The RAGE promoter contains both NF-κB-like and specificity protein-1-like binding sites (30). The NF-κB sites are associated with basal RAGE expression as well as increased RAGE expression in response to lipopolysaccharides (30). Increases in RAGE expression in neuroblastoma cells after exposure to amphoterin are at least partially dependent on specificity protein-1 binding to RAGE promoter elements (65). These data suggest that AGE-RAGE binding produces a continuous cycle of activation, receptor up-regulation, and further activation. Similar feed-forward cycles in RAGE activation and expression occur in vascular endothelial and carcinoma cells (24, 30). In chronic kidney disease, RAGE expression also increases and amplifies monocyte-mediated inflammation (66).

Activation of RAGE in DRG neurons induces transient signaling via both the PI-3K/Akt and MAPK pathways. These pathways are intricately linked to survival, growth, and differentiation in neurons (67–70). Our data, particularly using inhibitors of these signaling pathways, suggest that RAGE activation of the PI-3K pathway is more likely to be involved in S100-induced ROS production and neuronal injury. This is in contrast to our previous work on growth factor rescue from hyperglycemia (56). In these previous experiments, we demonstrated that IGF-I activates the PI-3K pathway and prevents glucose-induced DRG neuron injury (56). The cause for this difference is not defined, but we can highlight certain features that may differ between the two signaling mechanisms that may be significant. First, IGF-I signaling does not activate but decreases NAD(P)H oxidase, yet RAGE signaling significantly enhances NAD(P)H oxi-

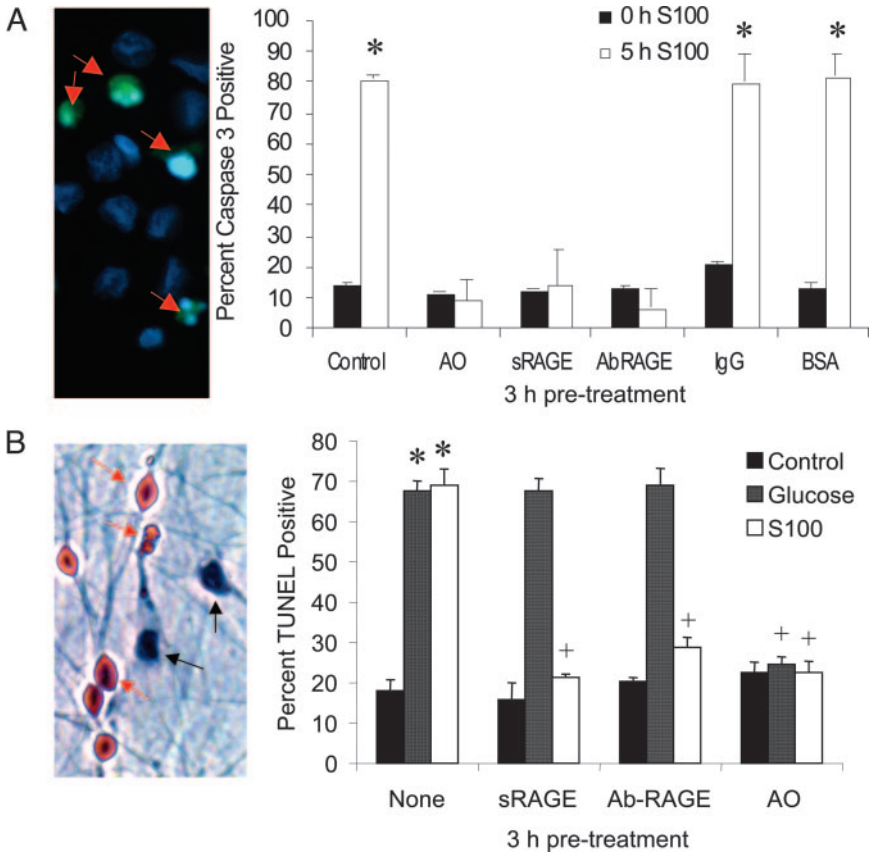

FIG. 6. Activation of RAGE leads to DRG neuron injury via oxidative stress. The development of programmed cell death was assessed in DRG cultures exposed to S100 (5 μg/ml) or hyperglycemia (20 mM added glucose) ± pretreatment with sRAGE (500 μg/ml), α-lipoic acid (AO; 100 μM), Ab-RAGE (1 μg/ml), or controls nonimmune IgG (1 μg/ml) or BSA (20 μg/ml). In A, caspase-3 activation was determined using a fluorescent substrate (CaspaTag) after 6 h exposure to S100. In B, DNA fragmentation was determined using the TUNEL assay 24 h after exposure to S100. *, Glucose or S100 alone significantly increased evidence of DNA degradation by TUNEL. $P < 0.001$. S100-induced death was prevented by pretreatment with sRAGE, Ab-RAGE, or AO. Only AO prevented hyperglycemia-induced injury. + TUNEL labeling significantly decreased, compared with glucose or S100 alone. $P < 0.001$.

dase activity. Whereas both IGF-I receptor and RAGE signal via PI-3K, the effects of RAGE activation are blocked by the antioxidant α-lipoic acid (Fig. 3). This suggests that mild prooxidant activity concurrent with RAGE activation may contribute to PI-3K-mediated NAD(P)H oxidase activation that produces further ROS leading to oxidative stress. It is notable that RAGE but not IGF-I receptor signaling increases Erk (71), so the signaling pathway is not the same, and more differences may be identified as we continue to understand RAGE signaling. RAGE signaling also may impact directly on the mitochondria (72), an effect not observed using IGF-I.

Another point of difference between the protective PI-3K signal of IGF-I and the injurious signal of RAGE is the regulation of receptor expression. DRG neurons demonstrate a feed-forward regulation of RAGE expression and activation similar to other cell types (24, 30). Prolonged exposure to IGF-I generally down-regulates the IGF-I receptor (73). This difference suggests that the duration of signal and the presence of coactivators produces an altered cellular response to Akt activation. Clearly, whereas transient activation of Akt was required to activate NAD(P)H oxidase and lead to injury after RAGE activation, it is other signaling mechanisms or potentially the subcellular localization of the signaling intermediates that produce a differential effect of longer-term Akt [at least 60 min (56)] activation in these same cells downstream of IGF-I activation.

Oxidative stress is another key mediator in the S100-induced injury mechanism. S100 activation of RAGE produces rapid ROS formation, NAD(P)H oxidase induction, and oxidative stress. The antioxidant α-lipoic acid prevents S100-induced DRG neuron injury, similar to its effects on hyperglycemia-induced injury. Oxidative stress is critical in the pathways of hyperglycemia-induced cellular injury (57, 74). Because both AGE- and glucose-induced damage appear mediated by oxidative stress, these data strongly indicate the use of antioxidant therapy to prevent and/or ameliorate diabetic complications. In the current experiments, all of the downstream injury mechanisms of RAGE are prevented by the addition of the α-lipoic acid, so RAGE signaling itself is dependent on a prooxidant mechanism. This type of ROS early second messenger is described for many other receptors including insulin, TNF, and platelet-derived growth factor receptors (75).

The activation of NAD(P)H oxidase is another common feature of glucose-induced and S100-induced DRG neuron injury. Hyperglycemia induces rapid and sustained activation of NAD(P)H oxidase that leads to superoxide formation and DRG neuron injury (48). In the current study, S100 leads to activation of NAD(P)H oxidase within 1 h that persists at 5 h. NAD(P)H oxidase is a major generator of ROS after RAGE activation both *in vivo* and *in vitro* (20). In our DRG culture system, neuronal NAD(P)H oxidase activity is prevented in the presence of the PI-3K inhibitor LY294002 or altered Akt constitutive signaling. Taken together, our data suggest that S100/RAGE interaction produces an oxidant-mediated signal that activates downstream signaling of MAPK and PI-3K. The PI-3K pathway under these conditions may facilitate the activation of NAD(P)H oxidase that produces further robust ROS generation leading to cellular injury. This signaling model is illustrated in Fig. 9.

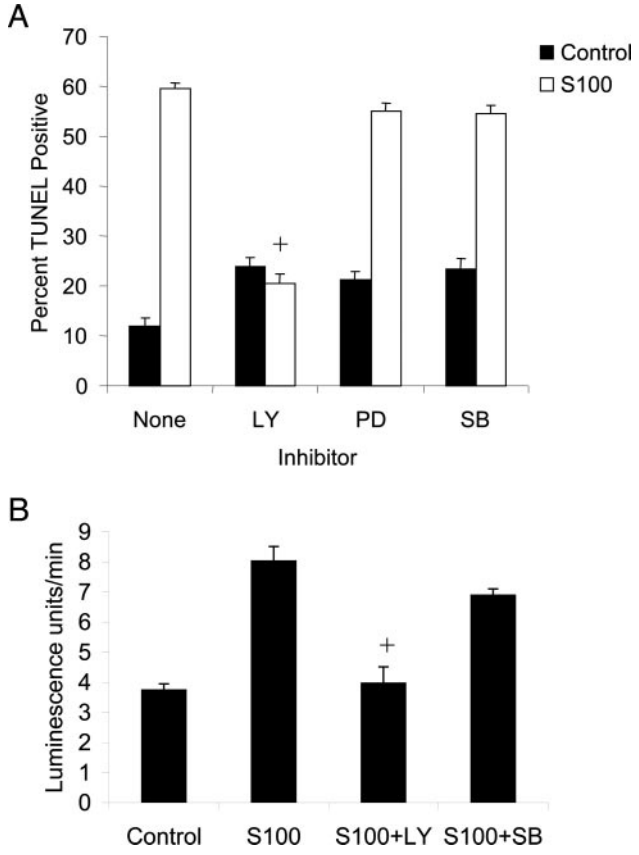

FIG. 7. RAGE-induced DRG neuron injury proceeds via the PI-3K pathway. A, DRG cultures were pretreated for 5 min with vehicle (none), LY294002 (LY; 10 μM), PD98059 (PD; 10 μM), or SB203580 (SB; 20 μM) before the application of 5 μg/ml S100. Cells were fixed after 24 h and stained for DNA fragmentation (TUNEL assay). Annotations indicate a significant decrease in the development of programmed cell death, compared with no pretreatment. +, $P < 0.005$. B, DRG cultures were exposed to 5 μg/ml S100 for 1 h in the presence or absence of pretreated with vehicle LY294002 (LY; 10 μM) before NAD(P)H oxidase activity assay. +, S100-induced NAD(P)H oxidase was significantly prevented by LY29004 ($P < 0.005$).

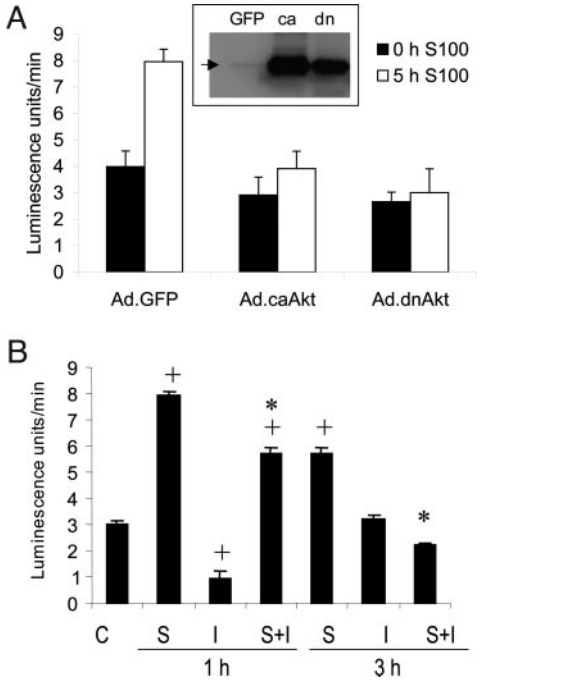

FIG. 8. Altered Akt activation prevents RAGE-mediated NAD(P)H oxidase activity. A, DRG neurons were infected with adenoviruses containing genes coding GFP (Ad.GFP), constitutively active Akt (Ad-.caAkt), or dominant-negative Akt (Ad.dnAkt) for 20 h before exposure to S100 for 0 or 5 h. Cells were lysed for chemiluminescent assay of NAD(P)H oxidase activity. Either constitutively active or dominant-negative Akt slightly decreased basal NAD(P)H oxidase and prevented S100 induction of this enzyme activity. *Inset*, Increase in total Akt by Western blotting after 20 h infection with the three Ad constructs is shown. B, The effect of IGF-I on NAD(P)H oxidase activity was assessed in DRG neurons. After 1 h IGF-I (I) treatment, basal and S100-induced (S) NAD(P)H oxidase activity was significantly decreased. S100-induced NAD(P)H oxidase activity was also prevented after 3 h. +, $P < 0.05$, compared with untreated control (C); *, $P < 0.05$, compared with S100 only.

Activity of NAD(P)H oxidase contributes to neurovascular deficits in diabetic rats through a mechanism involving increased ROS (76). Interestingly, a polymorphism in the NAD(P)H oxidase gene may be predictive of renal disease in type 2 diabetic patients (77). Although NAD(P)H oxidases are a heterogeneous and ubiquitous group of enzymes regulated by many stress pathways (78), recent efforts are devoted to developing inhibitors of these enzymes for the treatment of cardiovascular diseases (79). The current data strongly support the application of these inhibitors to prevent diabetic neuropathy caused by NAD(P)H oxidase that can be activated by AGE-RAGE signaling or by hyperglycemia (48).

Another therapeutic target is RAGE itself. We demonstrate that blocking RAGE *in vitro* effectively prevents DRG neuron injury. This finding is supported by *in vitro* and *in vivo* investigations using the same sRAGE construct as these studies (reviewed in Ref. 63). Interestingly, the plasma levels of sRAGE decrease in diabetic patients and are negatively correlated with development of complications (80). This suggests that sRAGE is lost in patients through interaction and removal of RAGE ligands, and so therapeutic increases in sRAGE would be beneficial. As stated above, Ab-RAGEs in mice prevent diabetic renal and vascular disease (34, 35, 64). In addition to blocking RAGE, alternate ways of preventing AGE effects may be clinically efficacious. There are several potential methods for decreasing AGE in diabetes. Antioxidants decrease AGE formation, and antioxidant therapy may provide additional benefit to the prevention of the downstream consequences of RAGE activation (12). Diet modification to decrease AGE intake and/or increase antioxidant levels through plant-derived flavonoid intake may prove efficacious (12). Finally, the use of agents that decrease cross-link formation, or increase the breakage of cross-links, thus preventing long-term damage of proteins, lipids, and DNA, should offer protection against diabetic complications (12). This study illustrates the importance of considering these therapeutic options for diabetic patients. Whereas oxidative stress is a unifying mechanism of hyperglycemia-induced injury, targeting the distinct mechanisms of the AGE-RAGE axis in addition to glucose regulation and glucose-mediated pathways may provide superior therapeutic strategies against diabetic neuropathy.

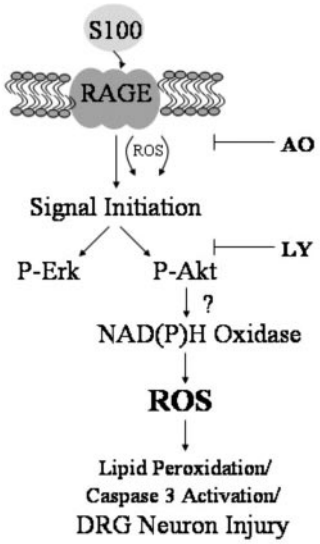

FIG. 9. Model for RAGE signaling leading to DRG neuron injury. Whereas the initial signal initiation is not yet clear, the current studies suggest that this involves ROS signaling or at least an altered redox state because all the consequences of S100 exposure are prevented when DRG neurons are preloaded with the antioxidant α-lipoic acid (AO). Downstream of RAGE activation, both Akt and Erks are transiently activated. Blocking the PI-3K/Akt pathway (LY, LY294002; 10 μM) prevents subsequent NAD(P)H oxidase activation and cell injury, suggesting a link between Akt activity and NAD(P)H oxidase. The parallel activation of Erk may modify the cellular response to Akt activity from protective pathways to injurious activation of NAD(P)H oxidase, or another mechanism may mediate this response.

Acknowledgments

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