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Peroxynitrite Protects Neurons against Nitric Oxide-mediated Apoptosis

A KEY ROLE FOR GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY IN NEUROPROTECTION*

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Peroxynitrite is thought to be a nitric oxide-derived neurotoxic effector molecule involved in the disruption of key energy-related metabolic targets. To assess the consequences of such interference in cellular glucose metabolism and viability, we studied the possible modulatory role played by peroxynitrite in glucose oxidation in neurons and astrocytes in primary culture. Here, we report that peroxynitrite triggered rapid stimulation of pentose phosphate pathway (PPP) activity and the accumulation of NADPH, an essential cofactor for glutathione regeneration. In contrast to peroxynitrite, nitric oxide elicited NADPH depletion, glutathione oxidation, and apoptotic cell death in neurons, but not in astrocytes. These events were noticeably counteracted by pretreatment of neurons with peroxynitrite. In an attempt to elucidate the mechanism responsible for this PPP stimulation and neuroprotection, we found evidence consistent with both exogenous and endogenous peroxynitrite-mediated activation of glucose-6-phosphate dehydrogenase (G6PD), an enzyme that catalyzes the first rate-limiting step in the PPP. Moreover, functional overexpression of the G6PD gene in stably transformed PC12 cells induced NADPH accumulation and offered remarkable resistance against nitric oxide-mediated apoptosis, whereas G6PD gene-targeted antisense inhibition depleted NADPH levels and exacerbated cellular vulnerability. In light of these results, we suggest that G6PD activation represents a novel role for peroxynitrite in neuroprotection against nitric oxide-mediated apoptosis.

Nitric oxide (·NO) is a short-lived physiological neural messenger involved in diverse biologically relevant functions (see Ref. 1 for a review). In neurons and astrocytes, ·NO is produced through the activation of a constitutive calcium-dependent neuronal nitric-oxide synthase and participates in the signaling pathway, leading to rises in cGMP levels (2–4). In addition, astrocytes have the ability to form ·NO in a calcium-independent fashion that requires prior transcriptional expression of the inducible nitric-oxide synthase isoform by lipopolysaccharide (LPS)[1] and/or certain cytokines (5, 6). Under certain neuropathological situations, however, excessive or inappropriate ·NO biosynthesis (nitrosative stress) is followed by a reaction of ·NO with superoxide ($O_2^{\cdot-}$) to form peroxynitrite ($ONOO^-$), a pro-oxidant molecule thought to execute the neurotoxic ·NO-mediated responses (7–10).

With respect to the neurotoxicity elicited by inappropriate ·NO biosynthesis, the intracellular content of glutathione (GSH) appears to play a key role in dictating cellular vulnerability. Thus, ·NO and peroxynitrite potently oxidize sulfhydryls, including GSH (11, 12). Accordingly, neuronal GSH oxidation has been proposed to be a contributing factor leading to the mitochondrial damage and neurotoxicity associated with nitrosative stress (13–15). Unlike neurons, astrocytes are cells that efficiently maintain GSH in its reduced redox status, even under conditions of excessive endogenous ·NO formation (16, 17). However, the mechanism(s) involved in such different cellular abilities to restore GSH levels is an issue that remains to be elucidated.

Hepatocytes are cells prone to hydrogen peroxide (H_2O_2)-mediated activation of glucose-6-phosphate dehydrogenase (G6PD), an enzyme that catalyzes the first rate-limiting step in the oxidative branch of the pentose phosphate pathway (PPP) (18, 19). Furthermore, stimulation of this pathway in neurons (20) and astrocytes (21) has been proposed to elicit a protective action against H_2O_2 toxicity through the PPP activity-mediated production of NADPH, a cofactor necessary for GSH regeneration from oxidized glutathione (GSSG) (22, 23). A similar protective mechanism has also been reported for Jurkat human T cells, where targeted inactivation of transaldolase, *i.e.* the rate-limiting step in the non-oxidative branch of the PPP, prevents the GSH oxidation and apoptosis caused by a number of pro-oxidant compounds, including ·NO (24).

In keeping with the above-mentioned studies, we reported previously that glucose utilization through the PPP in LPS-stimulated astrocytes offers self-protection against endogenous ·NO-mediated GSH oxidation (25). Because LPS-stimulated astrocytes synthesize $O_2^{\cdot-}$ through inducible nitric-oxide synthase-dependent activity (26), we wondered whether the peroxynitrite thus formed could be an effector molecule directly involved in the modulation of PPP activity. Here, we report that peroxynitrite elicited a rapid stimulation of PPP activity in both neurons and astrocytes through the activation of G6PD. Furthermore, we report strong evidence suggesting that such a

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[1] The abbreviations used are: LPS, lipopolysaccharide; G6PD, glucose-6-phosphate dehydrogenase; PPP, pentose phosphate pathway; DMEM, Dulbecco's modified Eagle's medium; DETA, diethylenetriamine; GFP, green fluorescent protein; PBS, phosphate-buffered saline; GSx, amount of GSH plus 2 times the amount of GSSG.

phenomenon would be involved in the protection of neurons against ·NO-mediated glutathione oxidation and apoptosis.

EXPERIMENTAL PROCEDURES

Materials

Peroxynitrite was synthesized and quantified spectrophotometrically (ϵ_{302} = 1670 M^{-1} cm^{-1}) as previously described (27). Alkaline stock solutions with an approximate $ONOO^-$ concentration of 0.5 M were stable at −70 °C for at least 3–4 months. Nitric oxide was purchased from Al Air Liquide (Madrid, Spain) and extemporarily dissolved in O_2-free water by 20-min bubbling until saturation. The concentration of ·NO solutions was estimated with an ·NO electrode (ISO-NO, World Precision Instruments, Inc.) using the ·NO-saturated solution as a standard (~2 mM ·NO). Dulbecco's modified Eagle's medium (DMEM), LPS, dihydrorhodamine 123, TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl), and hemoglobin were obtained from Sigma. Fetal calf serum was purchased from Roche Diagnostics (Heidelberg, Germany). Type I rat tail collagen was from BD Biosciences. D-[1-^{14}C]Glucose and D-[6-^{14}C]glucose were obtained from ARC Inc. (St. Louis, MO), and [α-^{32}P]dCTP and Hybond® nitrocellulose membranes were from Amersham Biosciences (Buckinghamshire, UK). The peroxynitrite donor SIN-1 (3-morpholinosydnonimine) and the ·NO donor diethylenetriamine (DETA)-NO were purchased from Alexis Corp. (San Diego, CA). 2-Vinylpyridine was from Aldrich (Gillingham-Dorset, UK). Plastic tissue culture dishes were purchased from Nunc (Roskilde, Denmark). Anti-G6PD antiserum was a generous gift from Prof. Matilde V. Ursini (Instituto Internazionale di Genetica e Biofisica, Consiglio Nazionale delle Ricerche, Naples, Italy). Anti-green fluorescent protein (GFP) antibody was obtained from Clontech (Palo Alto, CA), and anti-rabbit secondary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The Vybrant apoptosis assay kit was purchased from Molecular Probes, Inc. (Eugene, OR), and TransFast transfection reagent and the antibiotic G418 were from Promega (Madison, WI). Other substrates, enzymes, and coenzymes were purchased from Sigma, Roche Diagnostics, or Merck (Darmstadt, Germany).

Cell Cultures

Astrocyte-rich primary cultures derived from neonatal 1-day-old Wistar rats were prepared as previously described (28). Cell suspensions were plated in culture medium supplemented with 10% (v/v) fetal calf serum at a density of 1.25 × 10^5 cells/cm^2 in 175-cm^2 flasks. Cells were maintained in a humidified incubator under an atmosphere of 5% CO_2 and 95% air at 37 °C with a change of medium twice a week. After 12–14 days, cells were collected by trypsinization and reseeded in culture medium at a density of 2.5 × 10^5 cells/cm^2 on appropriate plastic dishes. For the experiments, astrocytes were used 24 h after reseeding.

Cerebral cortex neurons in primary culture were prepared from fetal rats at 16–17 days of gestation (29). Dissociated cell suspensions were plated at a density of 2.5 × 10^5 cells/cm^2 on appropriate plastic dishes previously coated with poly-D-lysine (15 μg/ml) in culture medium supplemented with 10% fetal calf serum. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. Forty-eight hours after plating, the culture medium was replaced with DMEM supplemented with 5% horse serum and 20 mM D-glucose. On day 4 of culture, cytosine arabinoside was added (10 μM) to prevent non-neuronal proliferation. For the experiments, neurons were used on day 9.

PC12 cells were kindly provided by Dr. Dionisio Martín-Zanca (Universidad de Salamanca, Salamanca, Spain) and were maintained in DMEM supplemented with 10% fetal calf serum, 6% horse serum, and 20 mM glucose on collagen-coated plastic dishes. For selection and growth of stably transfected cells, this medium was supplemented with G418 (500 μg/ml).

G6PD Plasmid Constructs and PC12 Stable Transfection

Full-length rat G6PD cDNA was subcloned into the *Eco*RI site of the mammalian expression vector pEGFP (Clontech) and sequenced. Constructs with the G6PD gene inserted in the sense (pEGFP-G6PD-sense) or antisense (pEGFP-G6PD-antisense) orientation were selected. PC12 cells were seeded at a density of 1 × 10^5 cells/cm^2 and transfected by lipofection (TransFast) with pEGFP-G6PD-sense, pEGFP-G6PD-antisense, or pEGFP plasmid constructs following the manufacturer's instructions. Forty-eight hours after transfection, cells were passaged and incubated in culture medium containing G418 (500 μg/ml), with a medium change twice a week to allow the selection of stably expressing clones. Isolated colonies were transferred to 12-well plates and expanded. Different clones were subjected to Northern blotting for G6PD mRNA identification as well as G6PD activity determination. Clones showing maximum (sense) or minimum (antisense) G6PD activities were used for the experiments.

Measurement of Glucose Oxidation through the Pentose Phosphate Pathway

The activity of the PPP was measured essentially as described by Hothersall *et al.* (30) based on the determination of the difference in $^{14}CO_2$ production from [1-^{14}C]glucose (decarboxylated by the 6-phosphogluconate dehydrogenase-catalyzed reaction and by the Krebs cycle) and from [6-^{14}C]glucose (decarboxylated only by the Krebs cycle). Astrocytes and neurons grown in 175-cm^2 flasks were collected by trypsinization. Cell pellets were resuspended in O_2-saturated incubation buffer (11 mM sodium phosphate, 122 mM NaCl, 3.1 mM KCl, 0.4 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 1.3 mM $CaCl_2$, pH 7.4). Aliquots (500 μl) of the suspension were placed in Erlenmeyer flasks containing 2 μCi of D-[1-^{14}C]glucose or 8 μCi of D-[6-^{14}C]glucose (5.5 mM D-glucose) in either the absence (control) or presence of SIN-1 at the indicated concentrations. Each Erlenmeyer flask was equipped with a central well containing an Eppendorf tube containing benzethonium hydroxide. The flask atmosphere was flushed with O_2 for 20 s, after which the flask was sealed with a rubber cap and incubated for 5 min at 37 °C in a water bath with shaking. Incubations were stopped by injection into the main well of 0.2 ml of 1.75 M $HClO_4$, although shaking was continued for a further 20 min to facilitate the entrapment of $^{14}CO_2$ by benzethonium hydroxide. The radioactivity trapped by benzethonium hydroxide was measured in scintillation fluid (Universol, ICN Biomedicals Inc., Irvine, CA) by liquid scintillation counting (98% efficiency; LS 6500, Beckman Instruments). Blanks without cells were used in parallel to measure background radioactivity, which was subtracted from the sample values. Preliminary experiments showed that, under these conditions, the $^{14}CO_2$ generated was linear with time, at least up to 30 min, in both control and SIN-1 (up to 1 mM)-treated cells. Protein contents were determined in 10-μl aliquots of the cell suspensions. For the calculations, the specific radioactivity (dpm/mol) of glucose was used, and the results are expressed as pmol of glucose transformed into CO_2/min/mg of protein.

Metabolite Determinations

Total and Oxidized Glutathione—For glutathione determinations, cells grown in 8-cm^2 wells were washed with ice-cold phosphate-buffered saline (PBS) and immediately collected by scraping off with 1% (w/v) sulfosalicylic acid. Cell lysates were centrifuged at 13,000 × *g* for 5 min at 4 °C, and the supernatants were used for glutathione determinations on the same day as previously described (25, 31, 32). Total glutathione contents (GSx) were determined in comparison with GSSG standards (0–50 μM) treated in the same way as the samples. GSSG was quantified after derivatization of GSH with 2-vinylpyridine using similarly treated GSSG standards (0–5 μM). Results are expressed as the oxidized glutathione status ((GSSG/GSx) × 100) (33).

Nicotinamide-adenine Dinucleotides—Cells seeded on 4-cm^2 plates were washed with ice-cold PBS, and 250 μl of 0.5 M KOH in 50% (v/v) ethanol was immediately added, as described by Stocchi *et al.* (34) for the stable extraction of both reduced and oxidized nicotinamide-adenine dinucleotides. Aliquots (200 μl) of the cell lysates were transferred to Eppendorf tubes, neutralized to a pH of 7.8 with 200 μl of 0.5 M triethanolamine and 0.5 M potassium phosphate, and centrifuged at 13,000 × *g* for 2 min at 4 °C. Fifty-microliter aliquots of the supernatant were immediately used for NADPH plus NADH determination by chemiluminescence as previously described (35). For NADPH determinations, NADH was previously oxidized by incubating the samples with 0.5 milliunits/μl lactate dehydrogenase and 1 mM pyruvate (36). For $NADP^+$ determinations, $NADP^+$ was previously reduced by incubating the samples with 5 milliunits/ml G6PD, 5 mM glucose 6-phosphate, and 5 mM $MgSO_4$ (36). For NAD^+ determinations, NAD^+ was previously reduced by incubating the samples with 5 milliunits/μl alcohol dehydrogenase and 172 mM ethanol (36). NADPH, NADH, $NADP^+$, and NAD^+ concentrations were calculated by extrapolation of the sample values to their respective appropriate standard curves obtained with pure standards (Sigma).

Glucose 6-Phosphate and 6-Phosphogluconate—Cells were washed with ice-cold PBS and deproteinized with 0.6 M $HClO_4$. Cells were scraped off the plastic dishes and centrifuged at 20,000 × *g* for 10 min at 4 °C. After neutralization of the supernatants with 5 M K_2CO_3, the neutralized extracts were lyophilized and resuspended in 250 μl of water. Glucose 6-phosphate (37) and 6-phosphogluconate (38) concentrations were determined in these samples as described.

G6PD Activity Determinations

Cells were resuspended in 500 μl of 0.1 M potassium phosphate buffer, pH 7.0, to give a final protein concentration of ~1 mg/ml and homogenized with a Ultraturrax T-8 homogenizer (Ika, Staufen, Germany), and G6PD activity was determined spectrophotometrically as previously described (39). Enzyme activity is expressed as nmol of glucose 6-phosphate transformed per min/mg of protein.

Northern Blotting

Northern blot analysis was carried out on total RNA samples isolated from the cells by the guanidinium isothiocyanate method as previously described (40). The samples were electrophoresed (15 μg of RNA/line) on a formaldehyde-containing 1% (w/v) agarose gel. After transfer to a GeneScreen Plus membrane (PerkinElmer Life Sciences) and cross-linking with ultraviolet irradiation (UV Stratalinker Model 2400, Genetic Research Instruments, Essex, UK), membranes were hybridized for 18 h at 65 °C in the presence of the appropriate random-primed [α-^{32}P]dCTP-radiolabeled cDNA probes and exposed to Eastman Kodak XAR-5 film. As cDNA probes, we used either the 2.2-kb cDNA encoding the full-length rat G6PD gene (a generous gift from Dr. Ye-Shih Ho, Wayne State University) or a 0.7-kb cDNA fragment of the rat cyclophilin gene (generously donated by Dr. Dionisio Martín-Zanca). Cyclophilin mRNA was used as a control of the amount of total RNA loaded in each lane.

Western Blotting

Cells were scraped off the plastic dishes with lysis buffer (12.5 mM Na_2HPO_4, 116 mM NaCl, 0.5 M EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 100 μM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 100 μM phenylmethylsulfonyl fluoride, 1 mM phenanthroline, 10 μg/ml pepstatin A, 100 μM *N*-tosyl-L-phenylalanine chloromethyl ketone, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml soybean trypsin inhibitor, pH 7), and aliquots containing 100 μg of protein from each sample, extemporarily determined following the method of Bradford (41) using ovalbumin as a standard and the BenchMark™ prestained protein ladder (Invitrogen), were electrophoresed on 10% acrylamide gel (MiniProtean®, Bio-Rad) and transferred to a Hybond® nitrocellulose membrane. Membranes were blocked with 5% (w/v) low-fat milk/TBST buffer (20 mM Tris, 500 mM NaCl, and 0.1% (w/v) Tween 20, pH 7.5) for 1 h and then incubated in the presence of the appropriate antibody (either anti-G6PD at 1:1000 dilution or anti-GFP at 1:250 dilution) at 4 °C overnight. After washing, membranes were further incubated in 3% (w/v) low-fat milk/TBST buffer for 45 min at room temperature in the presence of horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody at 1:40,000 dilution and immediately incubated with the luminol chemiluminescence reagent (Santa Cruz Biotechnology) for 1 min before being exposed to Hyperfilm™ chemiluminescence film for 3 min (anti-G6PD antibody) or 25 min (anti-GFP antibody).

G6PD Release Experiments

Astrocytes or neurons were removed from the flasks by mild trypsinization and resuspended at a density of 20×10^6 cells/ml in prewarmed (37 °C) buffered Hanks' solution (5.26 mM KCl, 0.43 mM KH_2PO_4, 132.4 mM NaCl, 4.09 mM $NaHCO_3$, 0.33 mM Na_2HPO_4, 20 mM glucose, 2 mM $CaCl_2$, and 20 mM HEPES, pH 7.4) containing streptolysin O (1 kilounit/ml) (42) and the protease inhibitor mixture (see above). Cells were incubated in either the absence (control) or presence of SIN-1 (1 mM) at 37 °C in a water bath with shaking for 30 min (astrocytes) or 5 min (neurons). When appropriate, some incubations were performed in the presence of a degraded SIN-1 solution (72 h at 37 °C). After the incubation period, aliquots of the cell suspension were centrifuged at 500 × *g*, and both the supernatants and pellets were subjected to anti-G6PD antibody Western blotting as described above. Films were scanned; the intensity of the bands was quantified; and the values were subtracted from the background intensity values using an image analyzer system (NIH Image), kindly supplied by Wayne Rasband (National Institutes of Health).

Formation of Endogenous Peroxynitrite

To estimate the endogenous formation of peroxynitrite from astrocytes (43), cells were treated with LPS (1 μg/ml) for 18 h in either the absence or presence of the peroxynitrite scavenger methionine (10 mM) (44). Peroxynitrite generation was shown by the ability of these cells to oxidize dihydrorhodamine 123 as previously described (45). Thus, cells were incubated with dihydrorhodamine 123 (1 μg/ml) in buffered Hanks' solution for 1 h at 37 °C, and rhodamine 123 fluorescence microphotographs were taken with an inverted microscope using a fluorescein filter (excitation filter, 480–490 nm; and emission filter, 510–560 nm).

Flow Cytometric Analysis of Apoptosis

Early apoptotic cell death was determined after staining with Alexa Fluor 488-conjugated annexin V and propidium iodide using a commercially available kit (Molecular Probes, Inc.) following the manufacturer's instructions. Data acquisition (~10,000 cells) was carried out in a FACSCalibur flow cytometer (BD Biosciences) equipped with a 15-milliwatt argon ion laser tuned at 488 nm using CellQuest software (BD Biosciences). The analyzer threshold was adjusted on the FSC channel to exclude noise and most of the subcellular debris. Annexin V-stained cells that were propidium iodide-negative (annexin V^+/propidium $iodide^-$) were considered to be apoptotic.

Protein Determinations

Proteins were determined in the cell lysates by the method of Lowry *et al.* (46) or Bradford (41) using bovine serum albumin as a standard.

Statistical Analysis

Results are expressed as the means ± S.E. for the number of culture preparations indicated in the figure legends. Statistical significance was evaluated by one-way analysis, followed by the least significant difference multiple range test. In all cases, $p < 0.05$ was considered significant.

RESULTS

Peroxynitrite Triggers a Rapid and Persistent Increase in PPP Activity and NADPH Concentrations in Astrocytes and Neurons—To address the potential involvement of peroxynitrite in the regulation of PPP activity, the differences in the rates of oxidation of D-[1-^{14}C]glucose and D-[6-^{14}C]glucose to $^{14}CO_2$ (30) during a 5-min incubation period were assessed in astrocytes and neurons in primary culture. Because of the extreme instability of authentic peroxynitrite solutions at physiological pH (half-life of ~1.7 s), we used SIN-1 as the peroxynitrite donor (47, 48). Preliminary experiments using the ·NO-sensitive electrode (ISO-NO) (data not shown) suggested an approximate peroxynitrite formation from SIN-1 (1 mM) of ~20 μM, as previously reported (48). To ensure immediate maximum peroxynitrite release from SIN-1, all SIN-1-containing solutions were always preincubated in DMEM at 37 °C for 20 min before addition to the cells. As shown in Fig. 1*A*, the PPP activity found in control astrocytes was ~2.6-fold higher than that found in control neurons. Incubation of both cell types with SIN-1 dose-dependently increased the PPP activity to values ranging between ~2- or ~1.5-fold at 0.25 mM SIN-1 and up to ~3.5- or ~2.5-fold at 1 mM SIN-1 in astrocytes or neurons, respectively (Fig. 1*A*). In all cases, the observed enhancement of PPP activity by peroxynitrite treatment was due to increased [1-^{14}C]glucose oxidation, whereas [6-^{14}C]glucose oxidation remained unaltered (data not shown).

To investigate whether the observed activation of PPP activity by peroxynitrite was associated with NADPH generation, the intracellular concentrations of this dinucleotide were assessed by chemiluminescence after exposure of the cells to SIN-1. As shown in Fig. 1*B*, NADPH concentrations were ~4-fold higher in control astrocytes than in control neurons. Incubation of the cells with SIN-1 for 10 min dose-dependently increased NADPH concentrations by ~1.8- and ~4.4-fold at 1 mM SIN-1 in astrocytes and neurons, respectively (Fig. 1*B*). To test whether peroxynitrite was the molecule responsible for the observed SIN-1-mediated increase in NADPH concentrations, a solution of SIN-1 (1 mM) was preincubated in DMEM at 37 °C for 72 h to ensure complete degradation of SIN-1 before addition to neurons. Under these conditions, incubation of the neurons at 37 °C for 5 min with degraded solutions of SIN-1 showed no effects on NADPH levels compared with control (untreated) cells, whereas "active" SIN-1 triggered an ~4-fold increase (Fig. 1*C*). To further confirm that peroxynitrite forma-

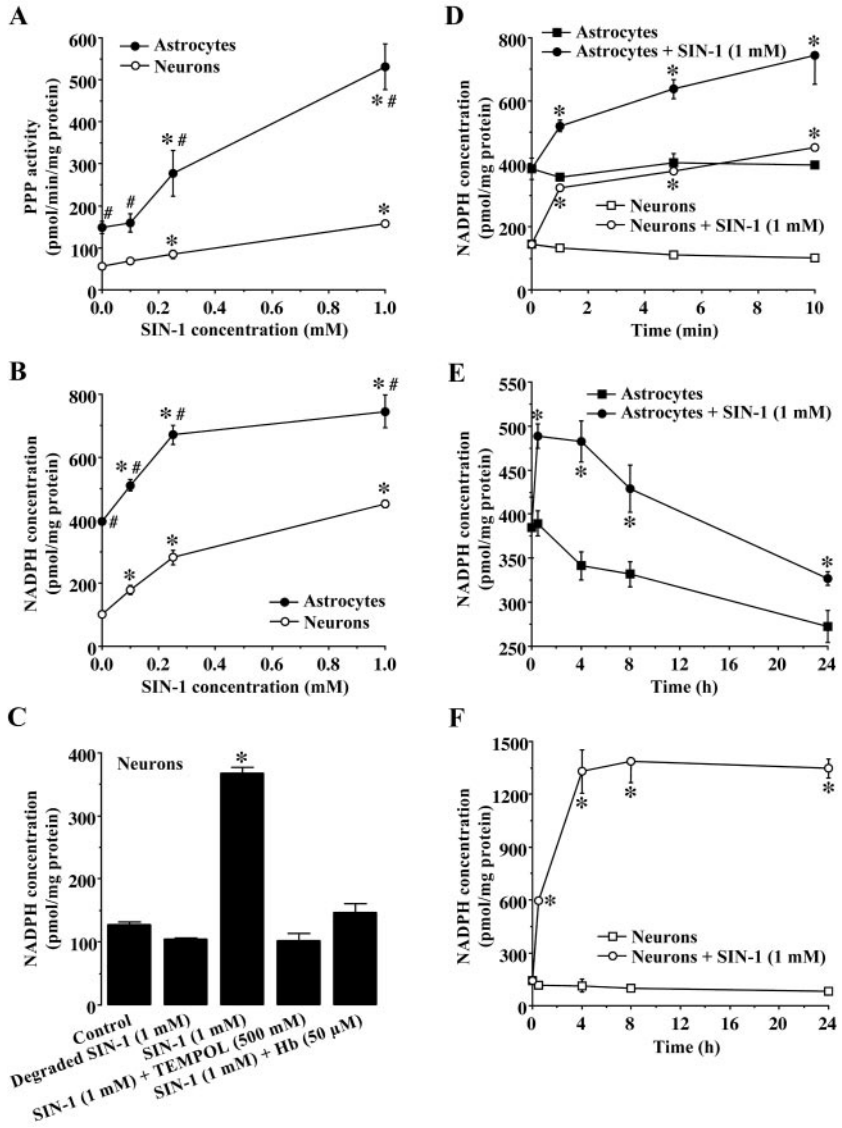

FIG. 1. **Peroxynitrite triggers a rapid and persistent increase in PPP activity and NADPH concentrations in astrocytes and neurons.** *A*, for the measurement of PPP activity, cell suspensions (15×10^6 cells/ml) were incubated in the presence of appropriate concentrations of SIN-1 in O_2-saturated incubation buffer, pH 7.4, containing either 2 μCi of D-[1-^{14}C]glucose or 8 μCi of D-[6-^{14}C]glucose (5.5 mM D-glucose) at 37 °C for 5 min. The $^{14}CO_2$ released by the cells was trapped and quantified for the estimation of PPP activity as described under "Experimental Procedures." *B–F*, for the determination of NADPH concentrations, cells were incubated in DMEM in the absence (control) or presence of SIN-1 at the indicated concentrations and with TEMPOL or hemoglobin when appropriate (*C*) for 1 min to 24 h (10 min in *B* and 5 min in *C*) and digested in KOH/ethanol for NADPH determination by chemiluminescence as described under "Experimental Procedures." Data are the means ± S.E. obtained from three to four different experiments. *, $p < 0.05$ compared with the corresponding control (0 mM SIN-1) values; #, $p < 0.05$ compared with the corresponding values found in neurons.

tion from SIN-1 was responsible for the observed effects on NADPH concentrations, we used TEMPOL (500 mM) or hemoglobin (50 μM) to scavenge $O_2^{\cdot-}$ (49) or $^{\cdot}NO$ (2), respectively. As shown in Fig. 1*C*, the presence of either compound fully prevented SIN-1 (1 mM)-mediated increases in neuronal NADPH concentrations. These results contrast with those obtained by Kirsch and de Groot (44), who found peroxynitrite reaction with reduced nicotinamide nucleotides *in vitro*. Even though we have no explanation for this discrepancy, it may be possible that such a putative reaction (44) would be interfered with by the presence of unknown factors occurring in our biological systems.

Measurements of NADPH levels for short incubation periods revealed that the increase in the concentrations of this dinucleotide by SIN-1 was very rapid (within 1 min) in both cell types (Fig. 1*D*). Furthermore, incubation of the cells in the presence of SIN-1 for longer times revealed that the increase in NADPH concentrations observed at 10 min was transient in astrocytes, but not in neurons (Fig. 1, *B–D*). Thus, after 30 min of incubation, the SIN-1-mediated rise in NADPH concentrations was progressively attenuated, with a modest, although statistically significant, ~1.2-fold value being reached at 24 h (Fig. 1*E*). In contrast to astrocytes, neuronal NADPH concentrations progressively increased after the first minute of incubation with SIN-1, reaching up to ~5.2-fold at 30 min and ~11-fold at 4 h of incubation (Fig. 1*F*). Furthermore, neuronal NADPH concentrations were maintained at high levels along during the incubation time in the presence of SIN-1, for at least up to 24 h (Fig. 1*F*).

Peroxynitrite Triggers Activation of the G6PD-catalyzed Reaction—To investigate the mechanism through which peroxynitrite triggered the rapid activation of PPP activity and NADPH concentrations, we determined the levels of nicotinamide-adenine dinucleotides in peroxynitrite-treated astrocytes. As shown in Fig. 2*A*, the increase in NADPH concentrations observed after 30 min of incubation with SIN-1 (1 mM) was accompanied by a parallel decrease in $NADP^+$ levels, suggesting that at least some of the NADPH produced would be obtained from $NADP^+$ reduction. However, the decreased amount of $NADP^+$ (~50 pmol/mg of protein) *per se* could not account for the increased amount of NADPH (~130 pmol/mg of protein) (Fig. 2*A*). NAD^+ concentrations were noticeably decreased by ~100 pmol/mg of protein, and NADH levels by ~40 pmol/mg of protein following peroxynitrite treatment (Fig. 2*A*). Furthermore, SIN-1 treatment (1 mM, 30 min) elicited a decrease in glucose 6-phosphate concentrations (by 40%) and an increase in 6-phosphogluconate concentrations (by ~5-fold) (Fig. 2*B*), strongly suggesting that peroxynitrite activates the G6PD-catalyzed reaction, *i.e.* the first rate-limiting step in the PPP (22). To investigate the possible activation of G6PD by

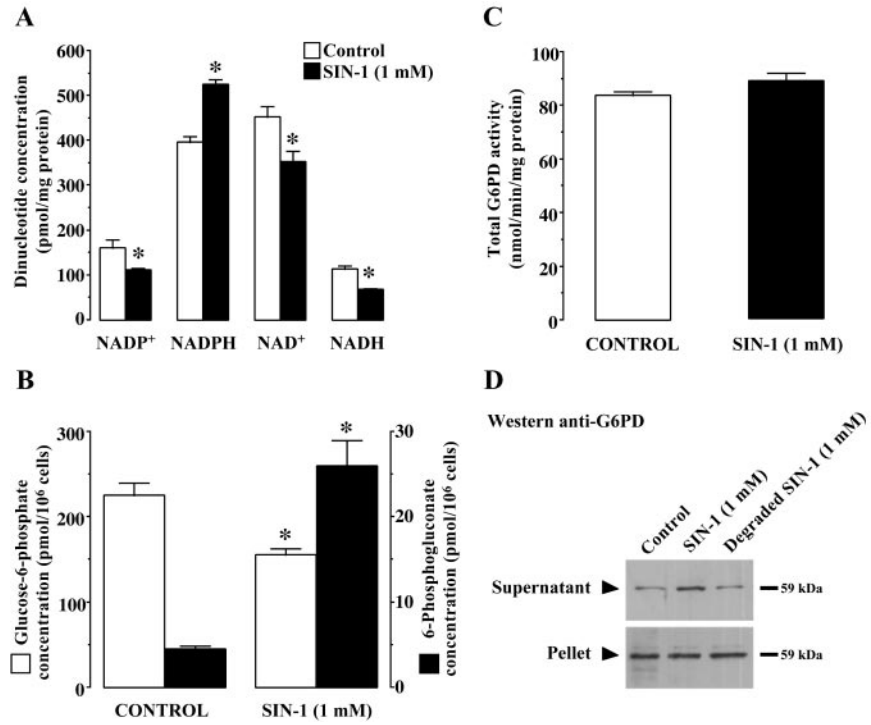

FIG. 2. **Peroxynitrite triggers activation of the G6PD-catalyzed reaction.** Astrocytes were incubated at 37 °C for 30 min in either the absence (control) or presence of SIN-1 (1 mM) as well as, where indicated, a degraded solution of SIN-1. *A*, for the determination of NAD concentrations, cells were digested in KOH/ethanol and used for the NADPH, NADH, $NADP^+$, and NAD^+ chemiluminescence assays as described under "Experimental Procedures." *B*, glucose 6-phosphate and 6-phosphogluconate concentrations were determined in the neutralized perchloric acid cell extracts. *C*, G6PD activity was determined spectrophotometrically in the cell homogenates. *D*, for the assessment of G6PD release, cell suspensions (20×10^6 cells/ml) were incubated in Hanks' solution containing streptolysin O (1 kilounit/ml) plus SIN-1 (1 mM) at 37 °C for 30 min, and aliquots of these suspensions were centrifuged before anti-G6PD antibody Western blot analyses of both the supernatant and pellets. Data are the means ± S.E. obtained from three different experiments. *, $p < 0.05$ compared with the corresponding control values.

peroxynitrite directly, the activity of G6PD was determined spectrophotometrically in the homogenates obtained from control (untreated) or SIN-1-treated astrocytes. The results showed that, despite the biochemical evidence for G6PD activation (Fig. 2*B*), total G6PD was unmodified by peroxynitrite treatment (Fig. 2*C*). In this context, previous observations by Stanton *et al.* (42) have demonstrated that inactive, structural intracellular element-bound G6PD would be prone to rapid activation through the release of G6PD into the cytosolic fraction by treatment of renal cortical cells with growth factors. Accordingly, we decided to investigate whether peroxynitrite-mediated G6PD activation could be due to possible enzyme release. Thus, astrocytes were collected, and the cell suspension was permeabilized by mild treatment with streptolysin O (42) and exposed or not (control) to active SIN-1 (1 mM) or degraded SIN-1. After 30 min, the cell suspensions were centrifuged, and both the cytosolic supernatants and pellets were separately subjected to Western blotting using anti-G6PD antibody. The results showed that, whereas most of the G6PD remained in the pellets, a significant amount of it appeared in the supernatant of SIN-1-treated cells compared with control or degraded SIN-1-treated cells (band intensity in the supernatants, 16.6 ± 0.2 and 36.6 ± 0.1 arbitrary units for degraded SIN-1 and SIN-1, respectively) (Fig. 2*D*). These results suggest that peroxynitrite would activate G6PD activity by promoting the release of G6PD to the cytosol from a structural intracellular element.

Evidence for Activation of the G6PD-catalyzed Reaction by Endogenous Peroxynitrite in Astrocytes—To assess whether endogenous peroxynitrite promoted G6PD activation, astrocytes were incubated with LPS (1 μg/ml) for 18 h as previously reported (43). Endogenous peroxynitrite production was evidenced by the ability of LPS-treated cells to oxidize dihydrorhodamine 123 to rhodamine 123 (45). Thus, LPS increased rhodamine 123 fluorescence, an effect that was prevented by co-incubation with the peroxynitrite scavenger methionine (10 mM) (Fig. 3*A*) (44). As shown in Fig. 3*B*, LPS elicited a decrease in glucose 6-phosphate concentrations (by ~3-fold) and an increase in 6-phosphogluconate concentrations (by ~2.5-fold), strongly suggesting G6PD activation. Furthermore, these effects were counteracted by scavenging peroxynitrite with methionine (Fig. 3*B*).

Evidence for G6PD Activation by Peroxynitrite in Neurons—To further confirm that peroxynitrite was responsible for the increase in neuronal NADPH concentrations, we used a solution of authentic peroxynitrite ($ONOO^-$, stock alkaline solutions of ~0.5 M, synthesized in our laboratory). Due to its extreme instability (half-life of ~1.7 s at pH 7.4), neurons were exposed to repeated pulses (10 pulses, one pulse every 30 s) of peroxynitrite (final concentrations of 50 and 100 μM) during the 5-min incubation period. Under these conditions, our results showed that exposure of neurons to authentic peroxynitrite increased NADPH concentrations by ~1.8-fold (50 μM $ONOO^-$) or ~4-fold (100 μM $ONOO^-$) (Fig. 4), whereas a degraded $ONOO^-$ solution (1 mM, 30 min, 37 °C) had no effect on NADPH concentrations (Fig. 4*A*). To investigate whether the peroxynitrite-mediated G6PD enzyme release observed in astrocytes also occurred in neurons, these cells were collected, and the cell suspension was exposed to active SIN-1 (1 mM) or degraded SIN-1. After 5 min, the cell suspensions were centrifuged, and the cytosolic supernatants were subjected to Western blotting using anti-G6PD antibody. The results showed a significant increase in G6PD protein in the supernatant of SIN-1-treated neurons compared with degraded SIN-1-treated cells (band intensity in the supernatants, 20.8 ± 0.1 and 32.6 ± 0.1 arbitrary units for degraded SIN-1 and SIN-1, respectively) (Fig. 4*B*).

Overexpression of the G6PD Gene Increases and Inhibition of G6PD Gene Expression Decreases NADPH Concentrations in PC12 Cells—To assess the essential role played by G6PD activity in generating intracellular NADPH, we used a transgene approach to endogenously modulate G6PD gene expression. Thus, we obtained stable G6PD gene-expressing cells by transfecting PC12 cells with pEGFP mammalian plasmid vectors into which the full-length G6PD gene had been inserted in either the sense (PC12-pEGFP-G6PD-sense) or antisense (PC12-pEGFP-G6PD-antisense) orientation. Transfection of PC12 cells with the vector alone (PC12-pEGFP) was also car-

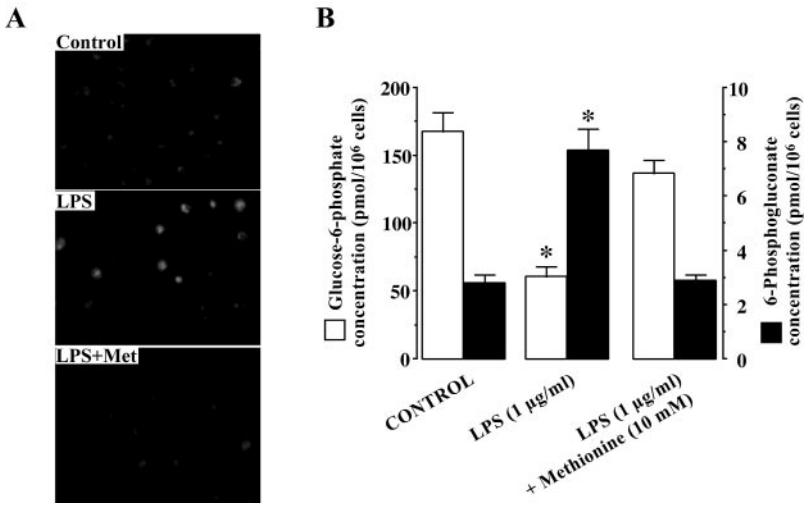

FIG. 3. **Evidence for activation of the G6PD-catalyzed reaction by endogenous peroxynitrite in astrocytes.** Astrocytes were incubated at 37 °C for 18.5 h in either the absence (control) or presence of LPS (1 μg/ml) alone or in combination with methionine (10 mM). *A*, dihydrorhodamine 123 oxidation was examined by fluorescence microscopy as indicated under "Experimental Procedures." *B*, glucose 6-phosphate and 6-phosphogluconate concentrations were determined in the neutralized perchloric acid cell extracts. Data are the means ± S.E. obtained from three different experiments. *, $p < 0.05$ compared with the corresponding control values.

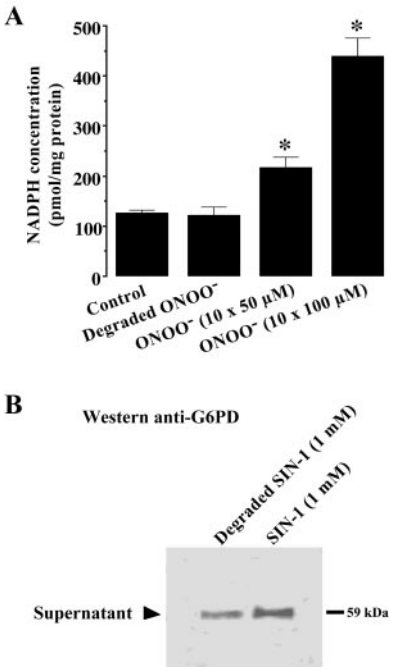

FIG. 4. **Evidence for G6PD activation by peroxynitrite in neurons.** *A*, neurons were incubated for 5 min in the presence of peroxynitrite, which was added to the cells in 10 pulses (one pulse every 30 s) of either 50 or 100 μM (final concentration, in Hanks' solution) from a 0.5 M stock solution. Cells were digested in KOH/ethanol for NADPH determination by chemiluminescence as described under "Experimental Procedures." *B*, for the assessment of G6PD release, cell suspensions (20×10^6 cells/ml) were incubated in Hanks' solution containing SIN-1 (1 mM) at 37 °C for 5 min, and aliquots of these suspensions were centrifuged before anti-G6PD antibody Western blot analyses of the supernatant. Data are the means ± S.E. obtained from three to four different experiments. *, $p < 0.05$ compared with control or degraded $ONOO^-$ values.

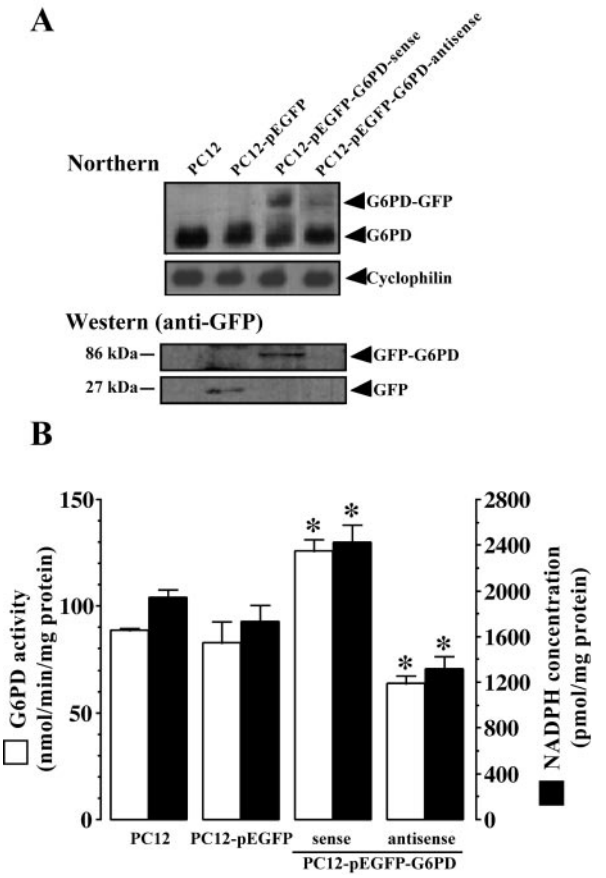

FIG. 5. **Overexpression of G6PD increases and inhibition of G6PD expression decreases NADPH concentrations in PC12 cells.** Cells were stably transfected with pEGFP plasmid vectors into which the G6PD gene had been inserted in either the sense (PC12-pEGFP-G6PD-sense) or antisense (PC12-pEGFP-G6PD-antisense) orientation. As transfection controls, cells were also transfected with pEGFP alone (PC12-pEGFP). *A*, total RNA and protein samples were extracted from these clones and subjected to Northern (15 μg/lane using either a G6PD or cyclophilin cDNA probe) and Western (100 μg/lane using anti-GFP antibody) blot analyses, respectively. *B*, G6PD activity and NADPH concentrations were determined in either cell homogenates (in 0.1 M phosphate buffer, pH 7.0) or alkaline extracts (KOH/ethanol), respectively, as described under "Experimental Procedures." *, $p < 0.05$ compared with the corresponding PC12 and PC12-pEGFP values.

ried out as a control. Stably expressing cells were selected and analyzed for G6PD mRNA levels, G6PD-GFP fusion protein levels, G6PD activity, and intracellular NADPH concentrations. Northern blot analyses showed that control cells (untransfected PC12 and transfected PC12-pEGFP) expressed endogenous G6PD mRNA (Fig. 5*A*). Furthermore, Western blot analyses using anti-GFP antibody revealed an ~27-kDa band in PC12-pEGFP cells that was not present in PC12 cells, confirming successful expression of the GFP protein in the former cells (Fig. 5*A*). Northern blot analyses of sense G6PD gene-overexpressing cells (PC12-pEGFP-G6PD-sense) revealed an additional mRNA band corresponding to the G6PD-GFP mRNA transcript (Fig. 5*A*). Western blot analyses of PC12-pEGFP-G6PD-sense cell protein extracts using anti-GFP antibody disclosed an ~86-kDa band, confirming successful expres-

TABLE I

Effect of nitric oxide on NADPH levels, oxidized glutathione status, and apoptotic cell death in astrocytes and neurons in primary culture

Cells were incubated in the absence (control) or presence of DETA-NO (1 mM; ~2.8 μM ·NO) for 4 h, and NADPH concentrations, total (GSx) and oxidized (GSSG) glutathione concentrations, and apopototic cell death were evaluated as described under "Experimental Procedures." PI, propidium iodide.

	Astrocytes		Neurons	
	Control	DETA-NO	Control	DETA-NO
NADPH (pmol/mg protein)	290 ± 11	279 ± 10	121 ± 9	59 ± 3[a]
(GSSG/GSx) × 100	0.61 ± 0.05	0.41 ± 0.03	2.48 ± 0.21	4.15 ± 0.19[a]
Apoptotic cells (% annexin V^+/PI^-)	2.30 ± 0.01	2.89 ± 0.02	19.31 ± 0.85	33.7 ± 1.60[a]

[a] $p < 0.05$ compared with the corresponding control values.

sion of the G6PD-GFP fusion protein (Fig. 5*A*). Analysis of these protein extracts by Western blotting failed to detect any anti-G6PD immunopositive fusion band, probably due to the interference of the fused GFP moiety in antibody recognition. Northern and Western blot analyses of antisense G6PD gene-overexpressing cells (PC12-pEGFP-G6PD-antisense) revealed a weak G6PD-GFP mRNA band and undetectable GFP or G6PD-GFP protein (Fig. 5*A*), probably due to the increased degradation of newly synthesized G6PD mRNA, as judged by the proposed mechanism for antisense gene-silencing methods (50, 51). Interestingly, both G6PD activity and NADPH concentrations were significantly higher (~1.5-fold) in PC12-pEGFP-G6PD-sense cells compared with control (untransfected) and pEGFP-transfected cells (Fig. 5*B*). In contrast, G6PD activity and NADPH concentrations in PC12-pEGFP-G6PD-antisense cells were significantly lower (by ~24%) (Fig. 5*B*). These results strongly confirm the functional efficiency of G6PD transgene expression in these cells and point to the essential role played by G6PD activity in supporting an enhanced intracellular pool of NADPH.

Peroxynitrite Transiently Prevents Nitric Oxide-mediated Neuronal NADPH Depletion, Glutathione Oxidation, and Apoptosis—Previous results from our laboratory (52) demonstrated that the exposure of neurons to ·NO causes apoptosis, whereas astrocytes remain unaffected. Among other possible mechanisms, this differential intercellular susceptibility appears to involve the higher antioxidant reserve, such as glutathione concentrations, found in astrocytes compared with neurons (17, 53). We were therefore prompted to investigate whether the peroxynitrite-mediated activation of PPP activity and increased NADPH concentrations observed here might serve as a potential neuroprotective mechanism against ·NO-mediated glutathione oxidation and cytotoxicity. Astrocytes and neurons were incubated in the presence of the ·NO donor DETA-NO (1 mM), a compound widely used as a long-term chemical source of ·NO (54). DETA-NO (1 mM) was seen to continuously release ~2.8 μM ·NO for ~24 h in DMEM at 37 °C as measured by an ·NO-sensitive electrode. To ensure immediate maximum ·NO release from DETA-NO, all DETA-NO-containing solutions were always preincubated in DMEM at 37 °C for 20 min before addition to the cells. Exposure of astrocytes to ·NO for 4 h had no detectable effect on NADPH concentrations, the GSSG/GSx ratio, or apoptotic cell death (Table I). In contrast to astrocytes, neurons were highly susceptible. Thus, exposure of neurons to ·NO triggered an ~50% decrease in NADPH concentrations, a 2-fold increase in the GSSG/GSx ratio, and a 1.7-fold increase in the proportion of apoptotic cells (Table I). Neuronal NADPH depletion by DETA-NO (1 mM) treatment occurred very rapidly, as judged by the 30% decrease in NADPH concentrations observed after a 5-min incubation period (Table II). Furthermore, a solution of degraded DETA-NO (incubation of 1 mM DETA-NO in DMEM at 37 °C for 48 h) had no effect, and a bolus of authentic ·NO (10 μM) decreased NADPH concentrations in neurons by 23% after a 5-min incubation period (Table II).

TABLE II

Rapid NADPH depletion by nitric oxide in neurons in primary culture

Neurons were incubated in the absence (control) or presence of either degraded DETA-NO (48 h at 37 °C) or active DETA-NO (1 mM; ~2.8 μM ·NO) for 5 min, and NADPH concentrations were measured as described under "Experimental Procedures."

	NADPH
	pmol/mg protein
Control	126 ± 5
Degraded DETA-NO (1 mM)	126 ± 2
DETA-NO (1 mM)	89 ± 11[a]
Nitric oxide (10 μM)	97 ± 7[a]

[a] $p < 0.05$ compared with control or degraded DETA-NO values.

We next investigated whether the stimulation of PPP activity brought about by peroxynitrite might protect neurons against ·NO-mediated oxidative damage. To perform these experiments, neurons were incubated under four different conditions, (*a*) control (untreated) neurons incubated in DMEM for 9 h; (*b*) neurons preincubated with SIN-1 for 1 h, followed by washing with PBS, and then incubated in DMEM for a further 8 h; (*c*) neurons incubated with DETA-NO for 8 h; and (*d*) neurons preincubated with SIN-1 for 1 h, followed by washing with PBS, and incubated with DETA-NO for a further 8 h. As shown in Fig. 4*A*, incubation of neurons with DETA-NO markedly and persistently decreased neuronal NADPH concentrations over the 8-h incubation period. By contrast, treatment of neurons with SIN-1 for 1 h increased NADPH concentrations (Fig. 6*A*; see also Fig. 1*F*). This effect was transient, as judged by the observed progressive decrease in the concentrations of this dinucleotide after SIN-1 removal. Noticeably, despite this progressive decrease, NADPH concentrations remained at levels that were significantly higher than those observed in control neurons, at least up to the end of the 8-h incubation period (Fig. 6*A*). Interestingly, preincubation of neurons with SIN-1 for 1 h fully or partially prevented the DETA-NO-mediated decrease in NADPH concentrations, at least during the following 8-h incubation period (Fig. 6*A*).

To assess the glutathione redox status in neurons subjected to an identical treatment, we determined oxidized (GSSG) and total (GSx) glutathione concentrations in these cells, expressing the values as the GSSG/GSx ratio (oxidized glutathione status) (33). As shown in Fig. 6*B*, the neuronal oxidized glutathione status remained unaltered over the 8-h incubation period following SIN-1 pretreatment, although it increased after 24 h (control, 5.2 ± 0.5; and SIN-1, 4.8 ± 0.2; $n = 3$). SIN-1 pretreatment noticeably prevented the long-term oxidation (8 h) of glutathione observed in control cells. In contrast to SIN-1, the exposure of neurons to DETA-NO significantly increased the oxidized glutathione status from 1 h up to 8 h of incubation (Fig. 6*B*). Also, pretreatment of neurons with SIN-1 for 1 h partially prevented DETA-NO-mediated glutathione oxidation during the incubation period between 1 and 4 h, but failed to protect glutathione oxidation after 8 h (Fig. 6*B*). It should be mentioned that the changes in the GSSG/GSx ratio observed

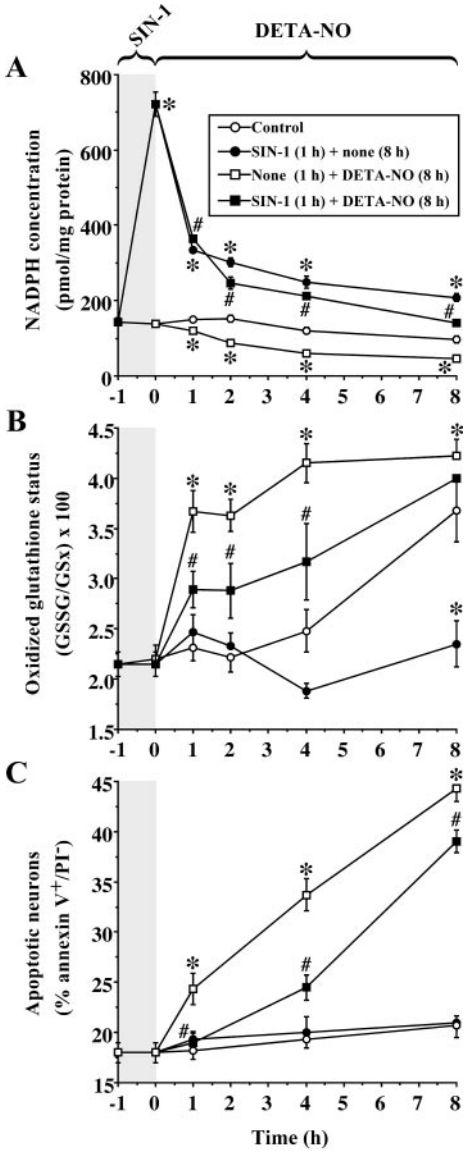

FIG. 6. **Peroxynitrite transiently prevents nitric oxide-mediated neuronal NADPH depletion, glutathione oxidation, and apoptosis.** Neurons were preincubated in the absence (control) or presence of SIN-1 (1 mM) at 37 °C for 1 h in DMEM. After removal of the SIN-1 solutions, the cells were washed with PBS and then further incubated in either the absence (*SIN-1 + none*) or presence (*SIN-1 + DETA-NO*) of DETA-NO (1 mM) in DMEM at 37 °C for 8 h. One group of cells was not preincubated with SIN-1 and was incubated only in the presence of DETA-NO (1 mM) (*none + DETA-NO*) for 8 h. NADPH concentrations (*A*), total (GSx) and oxidized (GSSG) glutathione concentrations (*B*), and the proportion of apoptotic cells (% of annexin V^+/propidium $iodide^-$ (*PI^-*)) (*C*) were determined as described under "Experimental Procedures." Data are the means ± S.E. obtained from three to four different experiments. *, $p < 0.05$ compared with the corresponding control values; #, $p < 0.05$ compared with the corresponding *none + DETA-NO* values.

were due to changes in GSSG (not GSx) concentrations (values for control (2 h), SIN-1 (1 h) plus none (1 h), DETA-NO (1 h), and SIN-1 (1 h) plus DETA-NO (1 h) were 0.30 ± 0.03, 0.30 ± 0.02, 0.45 ± 0.02 ($p < 0.05$ *versus* control), and 0.34 ± 0.02 nmol/mg of protein for GSSG and 13.1 ± 0.6, 12.9 ± 0.3, 12.9 ± 0.6, and 13.1 ± 1.0 nmol/mg of protein for GSx, respectively).

To investigate whether the peroxynitrite-mediated protection of cellular oxidation dictated cellular survival, we determined the proportion of apoptotic cells, *i.e.* propidium iodide-negative neurons showing a positive immunoreaction to annexin V (% of annexin V^+/propidium $iodide^-$ cells), by flow cytometry. As shown in Fig. 6*C*, preincubation of neurons with SIN-1 for 1 h had no effect on the proportion of apoptotic neurons compared with control cells, at least up to 8 h of incubation. In contrast, incubation of neurons with DETA-NO triggered cellular apoptosis in a time-dependent fashion (Fig. 6*C*). However, a 1-h pretreatment of neurons with SIN-1 fully (>1 h) or partially (4–8 h) prevented DETA-NO-mediated neurotoxicity (Fig. 6*C*). Because a considerable body of earlier work (*e.g.* Refs. 8, 9, and 17) has consistently reported the long-term neurotoxicity (~24 h) of peroxynitrite, we also investigated this possibility. Indeed, both a 1-h pretreatment of neurons with SIN-1 (1 mM), followed by a 23-h incubation in DMEM, and a continuous exposure (24 h) of neurons to SIN-1 (1 mM) significantly increased apoptotic cell death (control at 24 h, 19.0 ± 0.6%; a 1-h incubation with SIN-1, followed by a 23-h incubation in DMEM, 50.6 ± 5.1%; and a 24-h incubation with SIN-1, 57.6 ± 0.7%). Finally, to further confirm that $ONOO^-$ was the species responsible for the protection shown by the neurons against ·NO-mediated apoptosis, the cells were treated with $ONOO^-$ (10 pulses of 50 μM $ONOO^-$ for 5 min) before exposure to DETA-NO (1 mM, 30 min). As shown in Table III, the ~1.4-fold increase in apoptotic neurons observed after 30 min of incubation with DETA-NO was prevented by pretreatment with $ONOO^-$.

TABLE III
Peroxynitrite pretreatment prevents nitric oxide-mediated neuronal apoptotic death

Neurons were preincubated with $ONOO^-$ (10 pulses of 50 μM for 5 min) or with a degraded solution of $ONOO^-$ (0.5 mM; control) at 37 °C for 5 min in buffered Hanks' solution. After removal of the buffer, the cells were washed with PBS and then further incubated in the presence of DETA-NO (1 mM) at 37 °C in DMEM for 30 min. The proportion of apoptotic cells (% of annexin V^+/propidium $iodide^-$ (PI^-)) was determined as described under "Experimental Procedures." Data are the means ± S.E. from three different experiments.

	Apoptotic cells (% annexin V^+/PI^-) after 30 min
Control (degraded solution of 0.5 mM $ONOO^-$)	16.9 ± 0.1
DETA-NO (1 mM)	23.0 ± 2.4[a]
$ONOO^-$ (10 pulses of 50 μM), followed by DETA-NO (1 mM)	10.6 ± 1.0[b]

[a] $p < 0.05$ compared with control values.
[b] $p < 0.05$ compared with DETA-NO values.

Overexpression of the G6PD Gene Protects and Inhibition of G6PD Gene Expression Enhances Nitric Oxide-mediated Apoptotic Death in PC12 Cells—In view of the biochemical evidence suggesting a key role for G6PD activity in peroxynitrite-mediated protection against ·NO, we investigated whether the endogenous modulation of G6PD transgene expression might be responsible for ·NO-mediated neurotoxicity. Accordingly, PC12-pEGFP cells were incubated in either the absence (control) or presence of DETA-NO (0.5 mM; ~1.4 μM ·NO) for 60 min. At 10-min intervals, aliquots from the cell suspension were analyzed for annexin V^+/propidium $iodide^-$ cells by flow cytometry. As shown in Fig. 7*A*, DETA-NO caused a rapid (20 min) and time-dependent increase (by ~3-fold at 60 min) in the proportion of apoptotic cells. To compare the differential susceptibility of G6PD modulation to ·NO-mediated apoptosis, these cells were incubated in the presence of DETA-NO (0.5 mM) for 30 and 60 min and subjected to annexin V^+/propidium $iodide^-$ assessment. As shown in Fig. 7*B*, the proportion of apoptotic PC12 cells increased by ~1.9-fold after 30 min and by ~1.5-fold after 60 min of incubation in the presence of DETA-NO. Intriguingly, cells transfected with the vector alone (PC12-pEGFP) showed higher susceptibility to DETA-NO (~2.5- and ~3-fold increase in apoptotic cells after 30 and 60 min, respec-

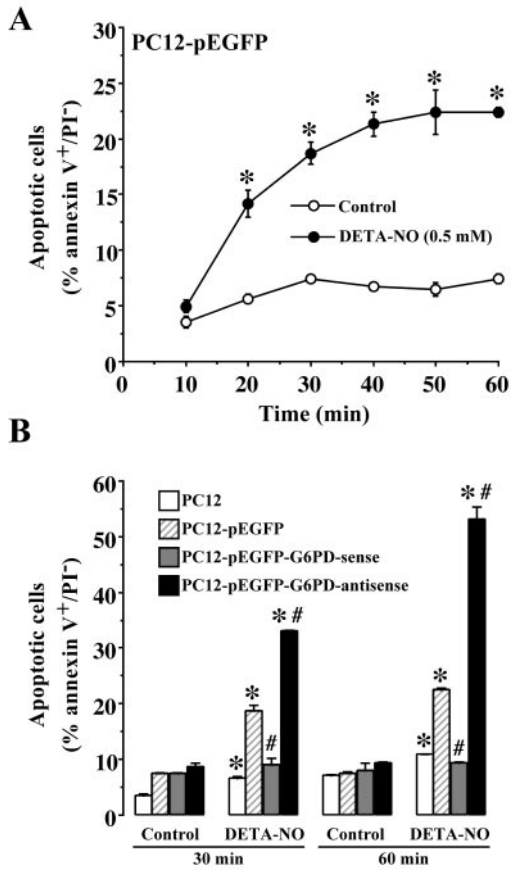

FIG. 7. **Overexpression of G6PD protects and inhibition of G6PD enhances nitric oxide-mediated apoptotic death in PC12 cells.** PC12 cells or PC12 cells stably expressing the vector alone (PC12-pEGFP) or pEGFP mammalian plasmid vectors with the G6PD gene inserted in either the sense (PC12-pEGFP-G6PD-sense) or antisense (PC12-pEGFP-G6PD-antisense) orientation were incubated in the absence (control) or presence of DETA-NO (0.5 mM) in Hanks' solution at 37 °C for the time periods indicated. The proportion of apoptotic cells (% of annexin V^+/propidium $iodide^-$ (PI^-)) was determined by flow cytometry as described under "Experimental Procedures." Data are the means ± S.E. obtained from three to four different experiments. *, $p < 0.05$ compared with the corresponding control values; #, $p < 0.05$ compared with PC12-pEGFP cells.

tively). However, cells overexpressing the sense G6PD gene (PC12-pEGFP-G6PD-sense) showed a remarkable resistance to DETA-NO-mediated apoptotic cell death (Fig. 7*B*). In contrast, cells overexpressing the antisense G6PD gene (PC12-pEGFP-G6PD-antisense) showed marked enhanced susceptibility to DETA-NO-mediated apoptotic cell death (~3.8- and ~5.6-fold increase in apoptotic cells after 30 and 60 min, respectively) (Fig. 7*B*). Intriguingly, expression of the vector alone (PC12-pEGFP) slightly increased the proportion of apoptosis (Fig. 7*B*).

DISCUSSION

The correlation between rapid activation of PPP activity and NADPH accumulation described here upon peroxynitrite treatment confirms the well known NADPH-generating function of the PPP (22). Due to its relatively low concentrations, $NADP^+$ availability alone could not account for NADPH accumulation. However, the decrease in NAD^+ concentrations brought about by peroxynitrite suggests that NAD^+ phosphorylation may provide sufficient $NADP^+$ for ready conversion into NADPH through G6PD activity (55–57). Accordingly, our data are compatible with the notion that peroxynitrite would stimulate G6PD activity, as demonstrated by the increase in the 6-phosphogluconate/glucose 6-phosphate ratio by either exogenous or endogenous peroxynitrite. This hypothesis was further examined in PC12 cells stably expressing plasmid constructs into which the G6PD gene had been inserted in either the sense or antisense orientation. As judged by the good correlation between the G6PD activity and NADPH concentrations observed in these cells, our results confirm the idea that the modulation of G6PD activity does dictate endogenous NADPH concentrations (58, 59).

Previous studies carried out on several cell types have already suggested the essential role of PPP and/or G6PD activity in cellular protection against H_2O_2-induced glutathione oxidation (18–21, 23). Furthermore, our previous work carried out in LPS-activated astrocytes (25) suggested a critical role for G6PD induction in astrocyte autoprotection. However, the rapid activation of G6PD activity by peroxynitrite observed here rules out any possible transcriptional effect. Because peroxynitrite failed to alter total G6PD activity, we investigated whether any changes in subcellular enzyme localization might be responsible for the rapid activation. In this context, previous work focused on studying the regulation of G6PD activity by growth factors in renal cells (42) has elegantly demonstrated that a rapid stimulation of G6PD activity occurs through enzyme release from a structural intracellular element. Our data from Western blot analyses demonstrated that peroxynitrite triggers G6PD release from both astrocytes and neurons, suggesting that such a mechanism would be a feasible explanation for the observed peroxynitrite-mediated G6PD activation.

Astrocytes have efficient self-protective systems, such as active glycolytic (52, 60) and *de novo* glutathione-synthesizing (13, 33, 53, 61) pathways or higher superoxide dismutase expression (62), which account for the resistance shown by glial cells to a wide range of insults, including ·NO exposure (this work). In contrast to astrocytes, neurons are vulnerable cells that, upon exposure to either endogenous (9, 63) or exogenous (52) excess ·NO, rapidly (within 1 h) undergo apoptotic death (this study). Pretreatment of neurons with peroxynitrite fully prevented apoptosis shortly (1 h) after ·NO treatment, although this protection progressively decreased thereafter (to ~60% protection after 4 h and to ~25% after 8 h). Furthermore, these changes in peroxynitrite-mediated neuroprotection showed a good time course correlation with the observed changes in the glutathione redox status. Because glutathione oxidation has been implicated in ·NO-mediated neuronal apoptosis (13, 63–65), it is tempting to speculate that the neuroprotection exerted by peroxynitrite would be associated with its ability to activate PPP activity and to generate NADPH. Thus, during the period of maximum protection (*i.e.* from 1 to 4 h after peroxynitrite treatment), neuronal NADPH levels were ~200–350 pmol/mg of protein. Assuming a cell volume of ~4 μl/mg of protein (66), the concentrations of NADPH would be ~50–100 μM, *i.e.* within the published glutathione reductase k_m values for NADPH (~8–61 μM for bovine brain; reviewed in Ref. 67). Accordingly, it is likely that the maintenance of the reduced status of GSH after peroxynitrite treatment would occur at the expense of increased NADPH availability to serve as the cofactor for glutathione reductase activity.

Because peroxynitrite-mediated neuroprotection against ·NO would be associated with its ability to stimulate G6PD activity, we were prompted to investigate the possible role played by the modulation of G6PD gene expression in ·NO-mediated apoptosis. Our results showed that PC12 cells expressing the sense G6PD gene offered remarkable resistance to ·NO-mediated apoptosis, whereas cells expressing the antisense G6PD gene showed a considerably higher susceptibility. These results confirm the notion that G6PD activity would play an essential role in preventing oxidative/nitrosative cellular damage (23, 58, 59) and suggest a key role for G6PD in neuro-

protection. However, it should be noted that, besides G6PD, other well known NADPH-generating systems have also been proposed to contribute to the protection against oxidative damage, such as cytosolic $NADP^+$-dependent isocitrate dehydrogenase (68), the PPP non-oxidative branch rate-limiting enzyme transaldolase (24), and $NADP^+$-dependent malic enzyme (22). Whether peroxynitrite would be involved in the modulation of such antioxidant systems is unknown and deserves further research.

The transient neuroprotective role for peroxynitrite described here is in apparent contradiction with the widely held assumption that peroxynitrite would be the ·NO-derived neurotoxic effector molecule (7–9, 17). Indeed, peroxynitrite interferes with key energy-related targets, such as aconitase (69), poly(ADP-ribose) synthetase (70–74), and mitochondrial respiratory chain complexes (16, 17, 75, 76). Because neurons are strong energy-demanding cells, these phenomena would affect neuronal survival. In our hands, peroxynitrite failed to cause neuronal apoptotic death up to 8 h. However, it was highly cytotoxic when the incubation time was extended up to 24 h, in good agreement with previous reports (9, 77). Therefore, the relationship between peroxynitrite-mediated interference with key energy-related metabolic targets and the time course of the observed neurotoxicity is an issue that should be revisited. It is also interesting to note that Clancy *et al.* (12) previously found that exposure of human neutrophils to ·NO is associated with O_2^- removal, enhanced [1-^{14}C]glucose oxidation, and GSH regeneration. Furthermore, an increasing body of evidence now suggests that ·NO might play a protective role against O_2^--mediated neurotoxicity (78–81) and H_2O_2-mediated cytotoxicity (82, 83). In view of the spontaneous peroxynitrite formation through the reaction of ·NO with O_2^- (7) and in the light of our own results, it is tempting to speculate that peroxynitrite would mediate these ·NO-derived protective responses. Taken together, our results may provide a clue for understanding the existing controversy concerning the role of ·NO in cell death/survival decisions.

In conclusion, we have show here that peroxynitrite plays a novel neuroprotective role against ·NO-mediated apoptotic cell death. The mechanism responsible for this phenomenon would involve G6PD release from a structural intracellular element, leading to enzyme activation, followed by NADPH generation through PPP activity. The G6PD transgene approach confirmed the critical neuroprotective role played by this enzyme against ·NO-mediated apoptosis. Furthermore, given the essential role of NADPH as a cofactor for glutathione regeneration, our results may contribute to the elucidation of mechanisms through which neurons would be able to protect themselves against ·NO-mediated glutathione oxidation and apoptosis.

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