Poly(ADP-ribose) glycohydrolase mediates oxidative and excitotoxic neuronal death

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Excessive activation of poly(ADP-ribose) polymerase 1 (PARP1) leads to NAD^+ depletion and cell death during ischemia and other conditions that generate extensive DNA damage. When activated by DNA strand breaks, PARP1 uses NAD^+ as substrate to form ADP-ribose polymers on specific acceptor proteins. These polymers are in turn rapidly degraded by poly(ADP-ribose) glycohydrolase (PARG), a ubiquitously expressed exo- and endoglycohydrolase. In this study, we examined the role of PARG in the PARP1-mediated cell death pathway. Mouse neuron and astrocyte cultures were exposed to hydrogen peroxide, *N*-methyl-D-aspartate (NMDA), or the DNA alkylating agent, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG). Cell death in each condition was markedly reduced by the PARP1 inhibitor benzamide and equally reduced by the PARG inhibitors gallotannin and nobotanin B. The PARP1 inhibitor benzamide and the PARG inhibitor gallotannin both prevented the NAD^+ depletion that otherwise results from PARP1 activation by MNNG or H_2O_2. However, these agents had opposite effects on protein poly(ADP-ribosyl)ation. Immunostaining for poly(ADP-ribose) on Western blots and neuron cultures showed benzamide to decrease and gallotannin to increase poly(ADP-ribose) accumulation during MNNG exposure. These results suggest that PARG inhibitors do not inhibit PARP1 directly, but instead prevent PARP1-mediated cell death by slowing the turnover of poly(ADP-ribose) and thus slowing NAD^+ consumption. PARG appears to be a necessary component of the PARP-mediated cell death pathway, and PARG inhibitors may have promise as neuroprotective agents.

poly(ADP-ribose) polymerase | NAD^+ | nobotanin B | gallotannin | *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine

Poly(ADP-ribose) polymerase (PARP) can contribute to both DNA repair and cell death (1, 2). Poly(ADP-ribose) polymerase 1 (PARP1) is the most abundant and best characterized member of the PARP family (3, 4). PARP1 appears to function in the detection and intracellular signaling of DNA damage, because PARP1 is activated by DNA strand breaks or kinks (1). PARP1 transfers ADP-ribose moieties from NAD^+ to specific acceptor proteins to form complex, branched chains with lengths of up to 200 residues. Known acceptor proteins include many proteins that function in DNA repair and cell cycle regulation, such as histones, DNA polymerases, DNA ligases, p53, and Fos (1, 5, 6). PARP1 itself is also an acceptor protein, and PARP1 is strongly inhibited when extensively poly(ADP-ribosyl)ated (1, 7).

Despite its function in DNA repair, overactivation of PARP has long been recognized to induce cell death under some conditions (8). PARP inhibitors and PARP1 gene disruption can reduce cell death resulting from oxidative stress (9), radiation (10), nitric oxide, peroxynitrite (11–13), and other agents that damage DNA (13, 14). Oxidative stress contributes to cell death in cerebral ischemia (15, 16), and genetic or pharmacological inhibition of PARP1 reduces ischemic cell death (13, 17). PARP1 inactivation can also prevent neuronal death induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (18) and other oxidants (2, 13).

The mechanism by which PARP1 activation leads to cell death appears linked to the rapid utilization of NAD^+ during formation of poly(ADP-ribose) (PAR) (2, 19). PAR has a fast turnover rate, with a half-life approaching 1 min (1, 20), due to rapid degradation by the endo-exoglycosidase poly(ADP-ribose) glycohydrolase (PARG) (1, 20). In contrast to PARP, little is known about the role of PARG in cell function. There are, however, at least two mechanisms by which PARG could influence PARP-mediated cell death. First, PARG inhibition could slow the turnover of PAR and thereby limit NAD^+ depletion. Second, PARG inhibition could prevent the removal of PAR from PARP1. Because PARP1 is inhibited by extensive poly(ADP-ribosyl)ation, PARG inhibitors could thereby indirectly inhibit PARP1 activity. Prior work has shown that the PARG inhibitor gallotannin can markedly reduce death of astrocytes after oxidative stress (21). In this report, we present evidence supporting a crucial role for PARG activity in PARP-mediated neuronal and astrocyte death.

Materials and Methods

Cell Cultures. Astrocyte and neuron-astrocyte cocultures were prepared by using Swiss–Webster mice (Bantin & Kingman, Fremont, CA) as described (22, 23). The astrocyte cultures were used for experiments at 20–30 days *in vitro* or as a plating surface for neurons at 14–18 days *in vitro*. The neuron/astrocyte cocultures were used when the neurons reached 14–18 days *in vitro*.

Experimental Procedures. Experiments were initiated by replacing the culture medium with a balanced salt solution (BSS). The BSS contained (in mM) KCl, 3.1; NaCl, 134; $CaCl_2$, 1.2; $MgSO_4$, 1.2; KH_2PO_4, 0.25; $NaHCO_3$, 15.7; and glucose, 2. The pH was adjusted to 7.2 while the solution was equilibrated with 5% CO_2 at 37°C. Osmolarity was verified at 290–310 mOsm with a Wescor vapor pressure osmometer (Logan, UT). Drug stocks were prepared in BSS immediately before use and adjusted to pH 7.2. Cultures were preincubated in a 37°C, 5% CO_2 incubator in BSS with or without PARP or PARG inhibitors for 30–60 min. Hydrogen peroxide (H_2O_2), *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) or *N*-methyl-D-aspartate (NMDA) were then added, and the cultures were incubated for an additional 5–60 min. Exposures were terminated by exchanging the BSS with Eagle's MEM containing 0.1% BSA.

Cell Death Determinations. Neurons were distinguished from the underlying astrocyte layer by their phase-bright, process-bearing morphology, as confirmed by immunostaining for the neuron-specific nuclear protein, NeuN (24). Dead neurons were identified 20–24 h after drug exposures by propidium iodide (PI) fluorescence. PI was added at 0.42 mg/ml to each well, and both the PI-fluorescing dead neurons and nonfluorescing live neurons

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Abbreviations: NMDA, *N*-methyl-D-aspartate; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; NAC, *N*-acetyl cysteine; PAR, poly(ADP-ribose); PARG, poly(ADP-ribose) glycohydrolase; PARP, poly(ADP-ribose) polymerase.

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were counted in five randomly picked optical fields. At least 500 neurons were counted in each well, and results from each well were expressed as % neuronal death. Neuronal death was also quantified by measurements of lactate dehydrogenase (LDH) activity in the incubation medium (25) in a subset of the studies. For studies performed in monotype astrocyte cultures, astrocyte survival was quantified by measuring the LDH activity of cell lysates 24 h after drug exposures (22).

NAD^+ Assay. The recycling assay described by Szabo and colleagues (26) was used with minor modifications. Cells were extracted in 0.5 N $HClO_4$, neutralized with 3 M KOH/125 mM Gly-Gly buffer (pH 7.4), and centrifuged at $10,000 \times g$ for 5 min. Supernatants were mixed with a reaction medium containing 0.1 mM 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), 0.9 mM phenazine methosulfate, 13 units/ml alcohol dehydrogenase (Boehringer Mannheim), 100 mM nicotinamide, and 5.7% ethanol in 61 mM Gly-Gly buffer (pH 7.4). The $A_{560\ nm}$ was determined immediately and after 10 min, and results were calibrated with NAD^+ standards. Results were normalized to protein content as determined by the bicinchonic acid (BCA) method (27) using BSA standards.

Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) Activity. Cells were lysed in buffer containing 1 mM EDTA, 5 mM $MgSO_4$, 2% Triton X-100, and 100 mM Tris·HCl (final pH, 7.6). Cell lysates were mixed with equal volumes of assay buffer consisting of 2.5 mM ATP and 2.5 mM glycerate-3-phosphate. The $A_{340\ nm}$ was monitored for 5 min after addition of 0.2 mM NADH and 3 units/ml phosphoglycerate kinase. Slopes were calibrated against samples with known GAPDH content and expressed as micromoles of NAD^+ formed per minute per microgram of protein (28).

Poly(ADP-ribose) Western Blots. Cultures were lysed in buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 2× CØMPLETE protease inhibitor mixture (Roche Molecular Biochemicals), and 50 mM Tris·HCl, pH 7.5. Sonicated samples were mixed with loading buffer (5% β-mercaptoethanol/10% glycerol/2% SDS/0.01% bromophenol blue/62.5 mM Tris·HCl, pH 6.8) and heated to 65°C for 15 min. Aliquots were applied to a 7.5% resolving polyacrylamide SDS gel (10 μg protein per lane) for electrophoresis, then transferred to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia). The membranes were incubated in blocking buffer containing 5% blotting grade nonfat dry milk and 0.1% Tween-20 in 0.1 M sodium phosphate (pH 7.2), followed by incubation with mouse anti-PAR monoclonal antibody 4335-MC (Trevigen, Gaithersburg, MD) diluted 1:5000. After washing, the membranes were incubated in a 1:5000 dilution of peroxidase-labeled anti-mouse IgG antibody (Vector Laboratories) and bound antibody was visualized by chemiluminescence. For each experiment, adjacent blots were prepared from sister culture wells treated with MNNG only, MNNG plus benzamide, and MNNG plus gallotannin. To quantify PAR formation, relative optical density (ROD) was measured in the region between 112 kDA and 210 kDa of each lane and normalized to the ROD of this region in the corresponding "MNNG only" blots. The normalized values were pooled for statistical analysis.

Poly(ADP-ribose) Immunostaining. Immunostaining was performed according to the method of Burkle *et al.* (29) with modifications. Cells were fixed in ice-cold 10% trichloroacetic acid for 10 min, dehydrated by successive 5 min washes in 70%, 90%, and 100% ethanol at −20°C, and air dried. The cells were incubated overnight at 4°C with monoclonal anti-PAR antibody diluted 1:2000, then incubated with biotinylated horse anti-mouse IgG (Vector Laboratories) diluted 1:200 for 1 h at room temperature. Excess antibody was removed and the bound antibody was visualized by using the ABC streptavidin detection system (Vector Laboratories) and 3,3′-diaminobenzidene.

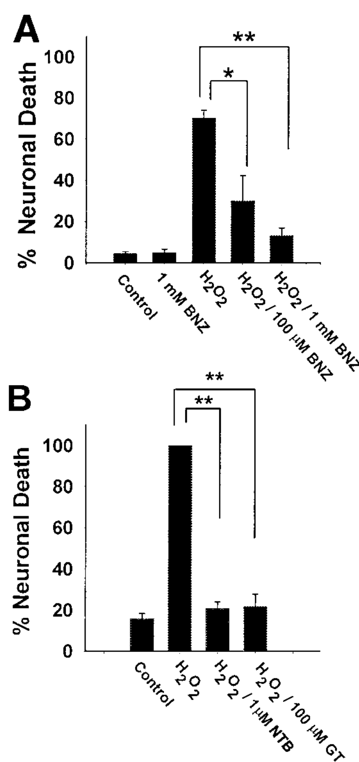

Fig. 1. Effects of PARP and PARG inhibitors on H_2O_2-induced neuronal death. (*A*) The PARP inhibitor benzamide reduced neuronal death induced by H_2O_2 (200 μM) by more than 80%. (*B*) The PARG inhibitors nobotanin B and gallotannin prevented neuronal death induced by 1-hr H_2O_2 (100 μM) exposure. Data are mean ± SE; **, $P < 0.01$; $n = 3$–7 culture wells per experimental condition. Results are representative of two independent experiments. BNZ, benzamide; NTB, nobotanin B; GT, gallotannin.

Statistical Analyses. Densitometry data from Western blots are presented as medians ±95% confidence intervals with statistical comparisons by the Mann–Whitney *U* test. All other data are presented as means ±SE, with analysis of variance (ANOVA) followed by the Student–Newman–Keuls post hoc test. A *P* value less than 0.05 was defined as statistically significant.

Results

Neuronal death occurred in a dose-dependent manner after incubations with both the oxidant H_2O_2 and the glutamate receptor agonist NMDA. The dose-response curve for H_2O_2 was very steep, such that the LD_{50} varied by up to 2-fold from experiment to experiment. In all studies, however, the PARP and PARG inhibitors had large effects on neuronal survival. As shown in Fig. 1*A*, H_2O_2-induced neuronal death was reduced by 80% in the presence of the PARP inhibitor benzamide, suggesting that the H_2O_2-induced neuronal death is largely mediated by PARP activation. To investigate the role of PARG in oxidative neuronal death, two PARG inhibitors, nobotanin B and gallotannin (30, 31), were used in place of benzamide during H_2O_2 incubations. One μM nobotanin B or 100 μM gallotannin reduced H_2O_2-induced neuronal death to a degree comparable to that achieved with 1 mM benzamide (Fig. 1*B*). Additional studies with higher H_2O_2 concentrations showed these to be the maximally effective concentrations of gallotannin, nobotanin B,

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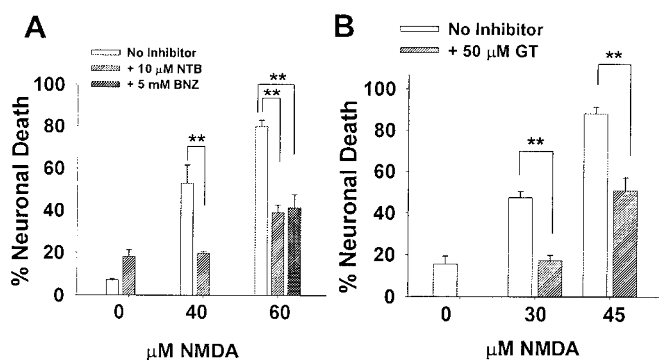

Fig. 2. Effects of PARP and PARG inhibitors on NMDA-induced neuronal death. (*A*) The PARP inhibitor benzamide and the PARG inhibitor NTB both reduced NMDA-induced neuronal death. Higher concentrations of these agents had no further effect (not shown). (*B*) The PARG inhibitor GT similarly reduced NMDA-induced neuronal death. Data are means ± SE; **, $P < 0.01$; $n = 4$–7 culture wells per experimental condition. Results are representative of three independent experiments. BNZ, benzamide; NTB, nobotanin B; GT, gallotannin; NMDA, *N*-methyl-D-aspartate.

and benzamide (data not shown). Gallotannin above 100 μM and nobotanin B above 10 μM began to have neurotoxic effects.

The PARG inhibitors were also used to test whether PARG, like PARP, contributes to excitotoxic neuronal death. Ten micromoles of nobotanin B reduced NMDA-induced neuronal death by about 50%, an effect comparable to that achieved with the supramaximal concentration (5 mM) of benzamide (Fig. 2*A*). A similar protection was achieved with 50 μM gallotannin, a less potent but commercially available PARG inhibitor (Fig. 2*B*). The effect of gallotannin on NMDA-induced neuronal death is illustrated in the photomicrographs shown in Fig. 3. Additional studies showed that the PARG inhibitors also reduced glutamate-induced neuronal death (data not shown).

Astrocytes in monotype cultures similarly showed reduced oxidative death in the presence of the PARG inhibitors. As shown in Fig. 4 *A* and *B*, 2 μM nobotanin B and 10 μM gallotannin each reduced H_2O_2-induced astrocyte death by more than 50%. Because peroxynitrite is an important PARP activator *in vivo* (32), we assessed the effects of gallotannin on astrocyte death induced by 3-morpholinosydnonimine (SIN-1,

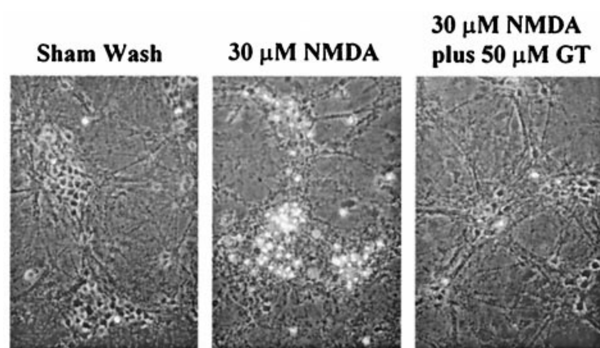

Fig. 3. Photomicrographs of cortical astrocyte/neuron cocultures treated with NMDA, ± gallotannin, 24 h after drug treatments. Photographs are taken with combined phase contrast and epifluorescence to show propidium iodide staining of dead cells. Neurons have phase bright cell bodies resting above the astrocyte layer. Dead cells are identified by bright propidium iodide fluorescence. Cultures treated with sham washes show only few, scattered dead neurons (*Left*). Cultures treated with 30 μM NMDA show death of almost all neurons (*Center*). Cultures treated with NMDA plus 50 μGT show markedly fewer propidium iodide stained (dead) neurons. GT, gallotannin; NMDA, *N*-methyl-D-aspartate.

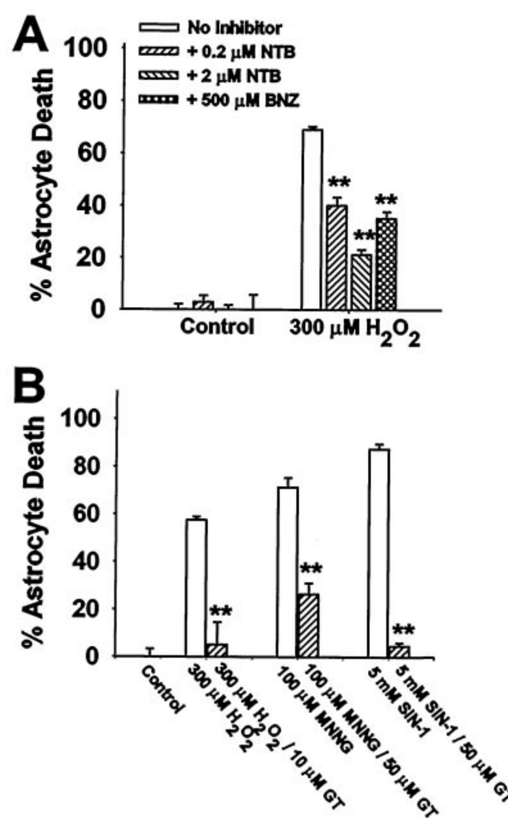

Fig. 4. PARP-mediated death of astrocytes is also attenuated by PARG inhibitors. (*A*) The PARP inhibitor benzamide and the PARG inhibitor NTB both reduced astrocyte death after exposure to 300 μM H_2O_2. Higher concentrations of these agents had no further neuroprotective effect (not shown). (*B*) The PARG inhibitor GT also reduced astrocyte death induced by PARP-activating agents; the oxidant H_2O_2, the DNA alkylating agent MNNG, and the peroxynitrite generator SIN-1. Data are means ± SE; **, $P < 0.01$; $n = 3$–9 culture wells per experimental condition. BNZ, benzamide; NTB, nobotanin B; GT, gallotannin; MNNG, *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine; SIN-1, 3-morpholinosydnonimine.

Calbiochem), which generates peroxynitrite by releasing both nitric oxide and superoxide in aqueous solution (33). We also tested the effect of gallotannin on astrocyte death induced by the DNA alkylating agent MNNG, which is widely used as a more selective DNA-damaging agent and activator of PARP (8, 14). As shown in Fig. 4*B*, gallotannin markedly decreased astrocyte death induced by either SIN-1 or MNNG, further supporting the idea that PARG inhibitors can block PARP-mediated cell death.

We considered the possibility that the cytoprotective effect of gallotannin might be due to a free radical scavenging effect, rather than to PARG inhibition. To test this possibility, gallotannin was compared with the potent and well characterized free radical scavengers, *N-tert*-butyl phenylnitrone and *N*-acetyl cysteine (34, 35). As shown in Fig. 5*A*, 10 μM gallotannin reduced H_2O_2-induced astrocyte death more effectively than 100 times higher concentrations of either of the free radical scavengers. Moreover, 10 μM gallotannin had no effect on H_2O_2-induced inactivation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is a sensitive indicator of intracellular oxidative stress (ref. 36; Fig. 5*B*).

One mechanism by which PARG inhibitors could prevent PARP-mediated cell death is by slowing the normally rapid rate of PAR turnover. This possibility was tested by determining whether gallotannin could prevent PARP-mediated NAD^+ de-

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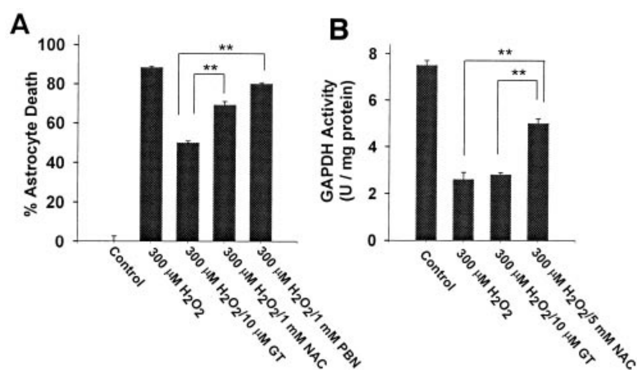

Fig. 5. Cytoprotective effects of gallotannin (GT) are not attributable to actions as a free radical scavenger. (*A*) The reduction in H_2O_2-induced astrocyte death afforded by 10 μM GT is significantly greater than that produced by 100-fold higher concentrations of the free radical scavengers *N*-acetyl cysteine (NAC) or N-*tert* butyl phenylnitrone (PBN). (*B*) GT has no effect on H_2O_2-induced inactivation of GAPDH activity in the astrocyte cultures under the same conditions that GT had large effects on astrocyte survival. The 5 mM NAC condition provides a positive control for this experiment. Data are means ± SE; **, $P < 0.01$; $n = 5$.

pletion. Astrocyte monocultures were used for these studies because the presence of multiple cell types in the neuron/astrocyte cocultures complicates the interpretation of biochemical measures. Total NAD^+ content in the astrocyte cultures under control conditions was 2.58 ± 0.39 nmol/mg protein ($n = 6$). Incubation with 100 μM H_2O_2 reduced NAD^+ levels to roughly 40% of controls (Fig. 6*A*), consistent with widespread PARP activation (1, 2, 8), and co-incubation with the PARP inhibitor benzamide prevented the NAD^+ depletion. As expected, the PARG inhibitor gallotannin (50 μM) also prevented NAD^+ depletion under these conditions. Similar results were observed when using MNNG to more selectively activate PARP. MNNG caused a depletion in NAD^+ that was attenuated by both benzamide and gallotannin (Fig. 6*B*).

To further test whether PARG inhibition slows PAR turnover, Western blots using anti-PAR antibody were performed on the cell lysates of astrocytes exposed to MNNG. As shown in Fig. 7, MNNG produced an increase in protein poly(ADP-ribosyl)ation at early time points (0–10 min incubation), as evidenced by a diffuse increase in PAR immunoreactivity on proteins of many molecular weights. This increase in PAR immunoreactivity was reduced by 5 mM benzamide, but enhanced by 50 μM gallotannin; the median relative optical density (ROD) of the high molecular weight region was 0.11 (95% confidence interval 0.04–0.24) in benzamide-treated samples and 1.63 (95% confidence interval 1.10–1.89) in gallotannin-treated samples, after normalization to the corresponding bands from cultures treated with MNNG alone ($n = 6$). The increase in PAR observed in the presence of gallotannin is consistent with the expected effects of a PARG inhibitor, because PARG inhibition would be expected to slow the rate at which PAR is removed from acceptor proteins at early time points. (Behavior at later time points may be complicated by the competing effect of slowed PAR production as a result of increased PAR on PARP1 itself.) Similar results were observed in studies using H_2O_2 to activate PARP1 (data not shown).

Fig. 8 shows the immunostaining of PAR in neurons treated with MNNG. Consistent with the patterns in the Western blots, MNNG caused an increase in nuclear PAR immunoreactivity that was prevented by the PARP inhibitor benzamide but accentuated by the PARG inhibitor gallotannin. MNNG under these conditions produced only minimally increased staining in

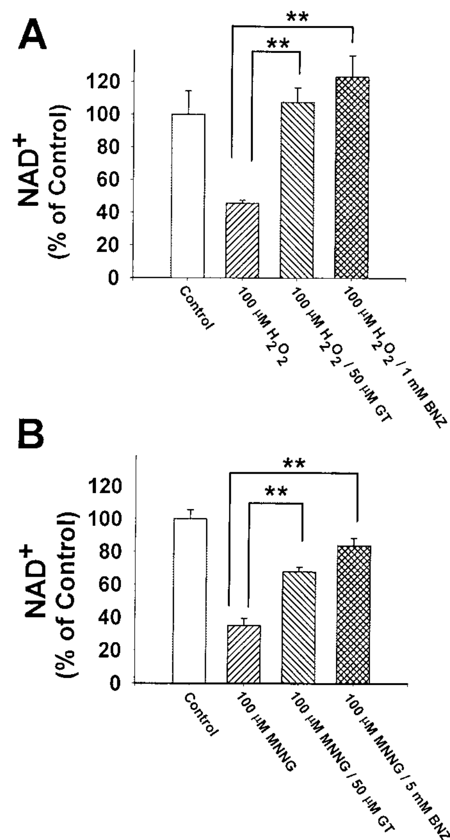

Fig. 6. Both the PARP inhibitor BNZ and the PARG inhibitor GT attenuate NAD^+ depletion in astrocytes exposed to H_2O_2 (*A*) or MNNG (*B*). Data are means ± SE; **, $P < 0.01$; $n = 3$ cultures wells per experimental condition. Results are representative of three independent experiments.

the astrocytes. The PAR immunostaining that appears to be in the neuronal cell cytoplasm has been reported (29) and is of unknown significance.

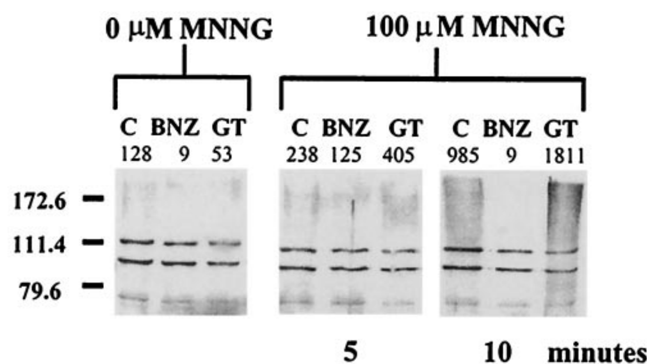

Fig. 7. Effects of BNZ and GT on MNNG-induced poly(ADP-ribose) formation. Western blots were prepared from astrocyte cultures treated with sham washes or 100 μM MNNG for 5 or 10 min. Cultures in each condition were also treated with 5 mM benzamide (BNZ), 50 μM gallotannin (GT), or neither (C). Poly(ADP-ribose) immunostaining shows two prominent bands present at 110 kDa and 89 kDa that probably represent poly(ADP-ribose) on PARP itself. Diffuse staining on proteins of other molecular weights is seen with increasing MNNG incubation periods. This increase in staining is slowed or attenuated in the presence of BNZ, but it is increased in the GT-treated cultures. Numbers in parentheses above each lane indicate the relative optical density of the entire lane between 112 kDa and 210 kDa.

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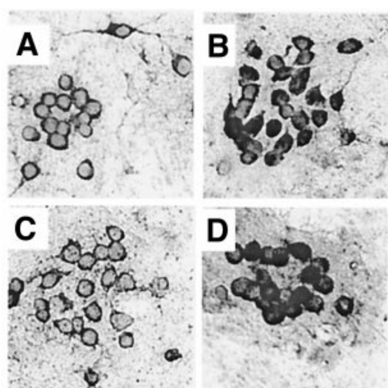

Fig. 8. Photomicrographs showing the effects of gallotannin and benzamide on MNNG-induced poly(ADP-ribose) formation in neurons. Astrocyte-neuron cocultures were treated with sham washes (*A*), 300 μM MNNG for 15 min (*B*), MNNG plus 3 mM benzamide (*C*), and MNNG plus 25 μM gallotannin (*D*).

Discussion

PARP1 activation has been established as a major component of both oxidative and excitotoxic neuronal death (12, 13). The present findings suggest that PARP1-mediated cell death requires the concomitant action of PARG. PARG inhibitors were found to be effective neuroprotective agents during exposure to several agents that activate PARP1, namely NMDA, H_2O_2, and the DNA alkylating agent MNNG. The PARG inhibitor gallotannin attenuated NAD^+ depletion but increased PAR accumulation. Together these results suggest that PARG inhibitors block PARP1-mediated cell death by slowing the rate of PAR turnover.

PARG is a 110-kDa protein that is ubiquitously expressed in mammalian cells (1, 37, 38). A 59-kDa cleavage product also has catalytic activity (1, 39). PARG functions as an exo- and endoglycosidase to rapidly degrade poly(ADP-ribose) and has no other known cellular functions (1, 40). The most potent known inhibitors of PARG are hydrolyzable tannins, including gallotannins and oligomeric ellagitannins (30, 31). These compounds inhibit PARG by competing with PAR at binding sites on PARG (30, 31). Gallotannin is composed of three closely related hydrolyzable tannins, trigalloyglucose, tetragalloyglucose, and pentagalloyglucose, which are obtained from green tea, pine cones, and other sources (30, 31). To our knowledge, gallotannin is the only commercially available hydrolyzable tannin. It has a K_i of $\approx$25 μM in studies using purified PARG (30, 31). Nobotanin B, which is extracted from the plant *Tibouchina semidecandra* Cogn., is a more potent PARG inhibitor, with a K_i of 4.8 μM (30, 31). Studies with purified enzymes also have shown that gallotannin and nobotanin B do not affect the activities of PARP1 at concentrations up to 1 mM (31); however, other pharmacological actions of these agents remain possible (41).

H_2O_2 and NMDA both activate PARP1 (12, 13, 42). It is likely that $^{\bullet}OH$, generated by H_2O_2 through the Fenton reaction (43), mediates the H_2O_2-induced DNA damage and PARP activation. The excitotoxin NMDA is thought to activate PARP by stimulating the production of nitric oxide and superoxide, which in turn form reactive species capable of damaging DNA (12, 13, 18). The results presented here show that both nobotanin B and gallotannin can substantially reduce cell death caused by H_2O_2 exposure. Nobotanin B and gallotannin provided significant, but less complete protection against NMDA-induced neuronal death. This difference may be due to the fact that NMDA can also induce cell death by mechanisms independent of DNA damage and PARP1 activation (44, 45).

Nobotanin B was a substantially more potent neuroprotective agent than gallotannin in all studies, consistent with the relative potencies of these compounds as PARG inhibitors. Of note, the maximal efficacy of nobotanin B, gallotannin, and benzamide were all similar, suggesting that these agents act along a common pathway. However, studies aiming to directly test for nonadditive effects of the PARP and PARG inhibitors were limited by intrinsic cytotoxicity of the inhibitors when used at high concentrations. The effects of PARG inhibitors on H_2O_2- and SIN-1-induced astrocyte death, along with the results of a prior study (21), indicate that the effects of PARG inhibitors are not limited to neurons. This is not surprising, because PAR metabolism is highly conserved in mammalian cells, and PARP inhibition can prevent oxidative death in many cell types (1, 13).

PARP1 is activated by oxidative stress, excitotoxicity, and by agents such as MNNG that directly react with DNA (8, 13, 42). PARG inhibitors were found in the present study to reduce cell death under each of these conditions, suggesting that PARG activity is necessary for PARP-mediated cell death to occur. The mechanisms leading from PARP1 activation to cell death have not been clearly defined, but several studies show a tight coupling to NAD^+ depletion (1, 13). Accordingly, a role for PARG in PARP-mediated cell death is supported by the finding that the PARG inhibitor gallotannin, like the PARP inhibitor benzamide, prevents the depletion of NAD^+ following H_2O_2 or MNNG exposure. NAD^+ is consumed by the rapid production of PAR by PARP1 (and possibly other PARP isozymes; refs. 13, 17, and 46). Because the only known function of PARG is the hydrolysis of poly(ADP-ribose), the effects of PARG inhibitors on NAD^+ levels and cell survival suggest that PAR turnover is required for PARP1-mediated cell death to occur. Western blots of poly(ADP-ribosyl)ated proteins prepared at several time points after MNNG-induced PARP1 activation confirmed that gallotannin slowed degradation of the PAR chains, and immunostaining of the neurons in cortical cultures confirmed that nuclear PAR formation was blocked by benzamide but enhanced by gallotannin. In principle, an increase in PAR formation could also result from accelerated PARP activity, but the fact that gallotannin also attenuated the decrease in cellular NAD^+ levels argues against this possibility. The preponderance of staining in the high molecular weight region of the Western blots may be due to the fact that long PAR chains can increase the molecular weight of acceptor proteins by 100 kDa or more. Of note, immunostaining provides an underestimate of PAR formation because PAR residues in proximal regions of long, branched PAR chains are shielded from antibody binding.

Importantly, the opposing effects of benzamide and gallotannin on PAR formation in these studies confirm that gallotannin does not act by inhibiting PARP directly. These results suggest instead that the PARG inhibitors slow NAD^+ consumption and attenuate PARP1-mediated cell death by slowing the normally fast rate of PAR turnover on acceptor proteins. This interpretation further suggests that PAR formation and degradation must occur repeatedly at the same acceptor sites. It follows that the number of available acceptor sites may become the rate limiting step in PARP activity and NAD^+ consumption during extensive PARP activation.

Other mechanisms could also contribute to the protective effects of PARG inhibition on cell death. Because PARP1 is itself strongly inhibited by poly(ADP-ribosyl)ation (1, 7), PARG inhibitors could in principle maintain PARP1 in an inactive state by preventing removal of these PAR groups. Ca^{2+}/Mg^{2+}-dependent endonuclease, which produces DNA fragmentation, is similarly inhibited by poly(ADP-ribosyl)ation (47, 48), and thus could be indirectly inhibited by PARG inhibition. In addition, the presence of substantial PARG outside of the cell

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nucleus under normal conditions (38, 39) implies that PARG inhibitors might also affect cell viability by influencing cytoplasmic targets. This possibility is supported by the recent discovery of a PARP isoform, VPARP, in the cytoplasm of mammalian cells (49). Further studies using targeted gene disruption of PARG and the PARP isoforms will be needed to confirm the mechanism by which the PARG inhibitors influence cell survival.

Blockade of PARP1 activation can substantially reduce cell death when DNA damage is sufficient to trigger extensive PARP1 activation and NAD^+ depletion (2). Because PARP1 normally functions in DNA repair, genetic or pharmacological inhibition of PARP1 can also produce detrimental effects. These include accumulated DNA strand breaks, genomic instability, altered gene expression, and oncogenesis (4, 50–52). Accordingly, PARP1 inhibition during less severe DNA damage may increase cell death, possibly because of impaired DNA repair in otherwise viable cells (50, 53). Effects of PARG inhibitors on DNA repair are not known. Because PARG inhibitors, unlike PARP1 inhibitors, do not prevent initial PAR formation after DNA damage, it is possible PARG inhibitors may have less deleterious effects on DNA repair. The present findings suggest that PARP1-mediated cell death requires the concomitant action of PARG. PARG inhibitors can effectively reduce cell death during excitotoxic and oxidative stress in cell cultures, and these compounds may have promise as cytoprotective agents *in vivo*.

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