Graphics 4D/240 GTX as described [W. Carrington, K. E. Fogarty, F. S. Fay, in *Non-Invasive Techniques in Cell Biology*, K. Foster, Ed. (Wiley-Liss, New York, 1990), pp. 53–72]. This software is now available for a personal computer from Scanalytics (Billerica, MA).
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Nitric Oxide Activation of Poly(ADP-Ribose) Synthetase in Neurotoxicity

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Poly(adenosine 5′-diphosphoribose) synthetase (PARS) is a nuclear enzyme which, when activated by DNA strand breaks, adds up to 100 adenosine 5′-diphosphoribose (ADP-ribose) units to nuclear proteins such as histones and PARS itself. This activation can lead to cell death through depletion of β-nicotinamide adenine dinucleotide (the source of ADP-ribose) and adenosine triphosphate. Nitric oxide (NO) stimulated ADP-ribosylation of PARS in rat brain. Benzamide and other derivatives, which inhibit PARS, blocked *N*-methyl-D-aspartate– and NO-mediated neurotoxicity with relative potencies paralleling their ability to inhibit PARS. Thus, NO appeared to elicit neurotoxicity by activating PARS.

Nitric oxide is a messenger molecule that regulates macrophage killing of tumor cells and bacteria (*1*) and blood vessel relaxation (*2*) and also is a neurotransmitter (*3*). When produced in large quantities in response to actions of the excitatory neurotransmitter glutamate acting at *N*-methyl-D-aspartate (NMDA) receptors, NO mediates neuronal killing (*4, 5*). Toxicity due to NMDA may account for neural damage in vascular stroke, as NO synthase (NOS) inhibitors prevent stroke damage (*6*). Mechanisms proposed for NO neurotoxicity as well as tumoricidal and bactericidal actions include monoADP-ribosylation and S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*7*), inhibition of mitochondrial enzymes such as *cis*-aconitase (*8*), inhibition of the mitochondrial electron transport chain (*1*), inhibition of ribonucleotide reductase (*9*), and DNA damage (*10, 11*). DNA damage activates PARS (E.C. 2.4.2.30) (*12, 13*). Here we show that NO activates PARS in association with damage to DNA, and that PARS inhibitors prevent NMDA neurotoxicity with relative potencies paralleling their inhibition of the enzyme.

In rat brain nuclear extracts, PARS activity was almost tripled in a dosage-dependent manner when DNA that had been preincubated with NO was added (Fig. 1A) (*14*). Addition of covalently closed circular DNA by itself had no effect on PARS activity. Both 4-amino-1,8-naphthalimide and 1,5-dihydroxyisoquinoline, two potent PARS inhibitors, reduced the activity to <5% of basal levels (Fig. 1A). The major protein ADP-ribosylated in the nuclear extracts was PARS itself (Fig. 1B). Similarly, DNA that had been treated with 3-morpholinosyndnonimine (SIN-1) and sodium nitroprusside (SNP), two NO donors, could stimulate poly(ADP-ribose) synthesis, which was inhibited by benzamide, another PARS inhibitor (*14*). Neither SNP nor SIN-1 alone had an effect on PARS (*14*).

McDonald and Moss have demonstrated that NO-enhanced modification of GAPDH by β-nicotinamide adenine dinucleotide (NAD) involves the transfer of the entire NAD to a thiol group rather than ADP-ribosylation (*7*). To ensure that the polymer formed from NAD in our study was poly(ADP-ribose), we used both [*adenine*-^{14}C]NAD and [*nicotinamide*-^{14}C]NAD and found radioactivity could only be incorporated into the polymers from the former compound (*14*).

To directly determine if PARS activation participates in NMDA neurotoxicity, we monitored neurotoxicity elicited by NMDA in rat cerebral cortical cultures in which NOS inhibitors provided protection (Fig. 2) (*4*). Increasing concentrations of NMDA progressively augmented neuronal killing. Benzamide (100 μM) provided 40 to 50% protection at all NMDA concentrations examined. At a benzamide concentration of 50 μM, there was no significant protection detected; but 500 μM benzamide provided ~30% more protection than 100 μM benzamide (Table 1). NO did not interact with benzamide (*15*). A variety of benzamide derivatives exist with differing potencies as PARS inhibitors. In the family of benzamide and its derivatives, benzamide is the most active, 3-aminobenzamide is about 50% as potent and 4-aminobenzamide is 1 to 2% as potent as benzamide, and benzoic acid is inactive (*16*). Benzamide provided the most protection against NMDA neurotoxicity and 3-aminobenzamide exerted somewhat less protection, but 4-aminobenzamide and benzoic acid did not protect at all (Table 1). A structurally unrelated PARS inhibitor, 1,5-dihydroxyisoquinoline (10 μM), was also neuropro-

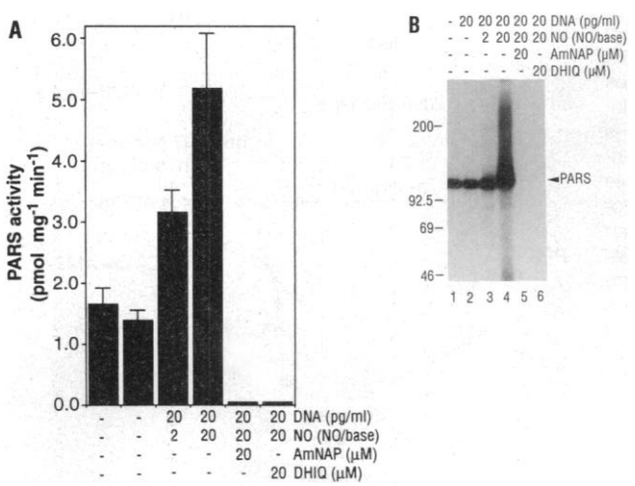

Fig. 1. Activation of PARS by NO-damaged DNA. (**A**) PARS activity after different treatments (mean ± SEM, n = 3) (*14*). (**B**) Autoradiography of poly-ADP-ribosylation of PARS on 7.5% SDS–polyacrylamide gel electrophoresis. Sizes are indicated at left in kilodaltons. Abbreviations: AmNAP, 4-amino-1,8-naphthalimide; DHIQ, 1,5-dihydroxyisoquinoline.

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tective against NMDA neurotoxicity (Table 1). It is unlikely that the PARS inhibitors could prevent NMDA neurotoxicity through inhibition of mono(ADP-ribose) synthetase as their IC_{50}'s (amount required to inhibit the activity of another substance by 50%) for mono(ADP-ribose) synthetase inhibition are at least 100 times as large as their IC_{50}'s as PARS inhibitors. Furthermore, novobiocin (1 mM), a relatively selective mono(ADP-ribose) synthetase inhibitor, was ineffective against NMDA neurotoxicity (Table 1).

Brief (5 min) NMDA exposure initiates "delayed neurotoxicity" in which poorly characterized irreversible processes ultimately lead to calcium overload and cell death (*17*). In most experiments benzamide was added before administration of NMDA (Fig. 2 and Table 1). However, in some experiments we added benzamide up to 1 hour after NMDA exposure and observed similar protection (Table 1).

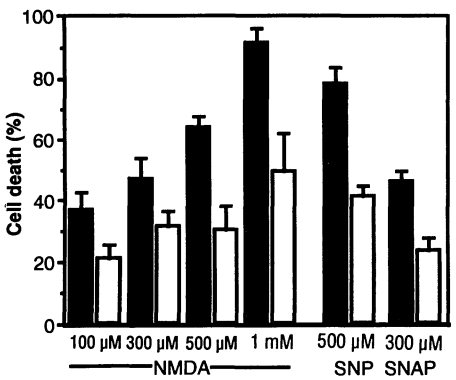


Fig. 2. Inhibition of NMDA- and NO-mediated neurotoxicity by PARS inhibitors. Benzamide (100 μM) (treatment indicated by open bars) reduces neurotoxicity mediated by NMDA (100 μM, 300 μM, 500 μM, and 1 mM), SNP (500 μM), and SNAP (300 μM). Data are the means ± SEM ($n \geq 8$). Each data point represents 4,000 to 12,000 neurons counted (*22*). All differences are significant ($P \leq 0.001$, Student's t test).

To ascertain whether NO itself elicits neurotoxicity by activation of PARS, we administered the NO releasers SNP and *S*-nitroso-*N*-acetylpenicillamine (SNAP). Benzamide (100 μM) reduced SNP and SNAP neurotoxicity by 45 to 50% (Fig. 2). Benzamide does not inhibit NOS. In human kidney 293 (HK 293) cells stably transfected with the complementary DNA (cDNA) of brain NOS, production of nitrite, a measure of NO synthesis, was not affected by 100 μM benzamide, but was blocked by nitroarginine (*18*). This experiment also confirms that benzamide did not interact with NO.

Our observation that NO activates PARS is consistent with other studies indicating that NO damages DNA (*10, 11*). The protection against NMDA neurotoxicity provided by PARS inhibitors appears to reflect PARS inhibition, as the relative potencies of these drugs in blocking neurotoxicity parallel their activities as PARS inhibitors.

The ability of PARS inhibition to provide substantial protection against NMDA neurotoxicity implicates DNA damage in neuronal killing. PARS activation rapidly leads to energy depletion. For each ADP-ribose unit transferred by PARS, one molecule of NAD is consumed and an equivalent of four molecules of adenosine triphosphate (ATP) are required to regenerate NAD from nicotinamide. PARS, whose density is up to one enzyme per 10 to 20 nucleosomes, can be activated 10- to 20-fold by DNA damage. PARS transfers 50 to 100 ADP-ribose moieties to each acceptor site of target proteins, and its overactivation by substantial DNA damage can markedly deplete cells of NAD and ATP (*13*). Neurotoxicity is similarly associated with a change in the NADH/NAD redox state and energy depletion (*19*). The protection against NMDA neurotoxicity afforded by PARS inhibition supports a mechanism of cell death in which DNA damage overwhelms repair mechanisms leading to energy depletion by activation of PARS (Fig. 3).

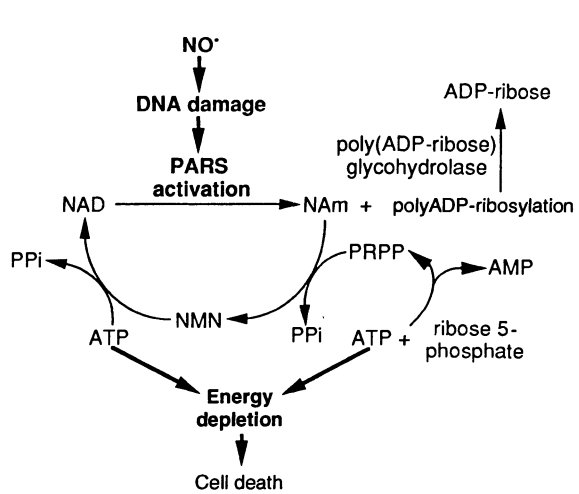
Table 1. Effects of PARS inhibitors on neurotoxicity elicited by NMDA. Application of PARS inhibitors started 30 min before NMDA was applied, unless otherwise indicated. Times in parentheses indicate that the inhibitor was applied after NMDA administration at the specified time, and the preparation was then incubated for 20 to 24 hours. Data points are the means ± SEM ($n \geq 8$). Each data point represents 4000 to 12,000 neurons counted (*22*).

Treatment	Cell death (%)
Protection by PARS inhibitors	
500 μM NMDA	57.1 ± 4.7
+ 50 μM benzamide	60.1 ± 12.1
+ 100 μM benzamide	39.5 ± 5.0*
+ 500 μM benzamide	21.9 ± 4.1*
+ 100 μM 3-aminobenzamide	45.9 ± 5.1*
+ 100 μM 4-aminobenzamide	55.8 ± 7.6
+ 1 mM benzoic acid	58.9 ± 5.7
+ 10 μM 1,5-dihydroxyisoquinoline	39.0 ± 4.5*
+ 1 mM novobiocin	62.4 ± 8.9
Rescue by PARS inhibitors	
500 μM NMDA	74.7 ± 7.8
+ 100 μM benzamide (5 min)	35.6 ± 8.7*
+ 100 μM benzamide (30 min)	40.4 ± 5.6*
+ 100 μM benzamide (60 min)	43.4 ± 3.9*

*$P \leq 0.001$, Student's t test.

Two major effects of glutamate neurotoxicity suggested to participate in neuronal killing are the formation of free radicals and energy depletion (*20*). Our findings indicate that both participate: NO, a free radical, triggers DNA damage that in turn activates PARS, which ultimately depletes energy sources from the cell. There is now evidence that neuronal injury can result from nitrosative chemistry as well as from free radicals via superoxide, NO, and peroxynitrite formation, as shown by the work of others. This mechanism of cell death might also account for the tumoricidal effects of NO generated by macrophages, which is consistent with observations that 3-aminobenzamide protects pancreatic islet cells from macrophage cytotoxicity (*21*).

Fig. 3. A model of NO-mediated cytotoxicity. NO-damaged DNA activates PARS which depletes NAD by polyADP-ribosylating nuclear proteins. Poly(ADP-ribose) is rapidly degraded by poly(ADP-ribose) glycohydrolase. The futile cycle continues during the prolonged PARS activation. It takes an equivalent of four ATPs to resynthesize NAD from nicotinamide (NAm) by means of nicotinamide mononucleotide (NMN), a reaction that requires phosphoribosyl pyrophosphate (PRPP) and ATP. AMP, adenosine monophosphate; PPi, inorganic pyrophosphate.



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14. Whole brains from 1-day-old rats were homogenized in 20% (w/v) buffer A [50 mM tris-HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 50 mM NaCl, 0.25 M sucrose, 0.2 mM phenylmethylsulfonyl fluoride, and the inhibitors chymostatin, leupeptin, pepstatin, and trypsin (1 μg/ml of each)]. The homogenate was centrifuged at 1000*g* for 15 min. The pellet was washed with buffer A and centrifuged again. The washed pellet, termed nuclear fraction, was resuspended in buffer A. PARS activity was assayed according to Schranfstatter *et al.* [*J. Clin. Invest.* **77**, 1312 (1986)]. Each 50-μl assay mixture contained 10 μg of the nuclear protein fraction and [*adenylate*-^{32}P]NAD (0.1 mM, 10 Ci/mmol), in the presence or absence of 4-amino-1,8-naphthalimide (20 μM), 1,5-dihydroxyisoquinoline (20 μM), benzamide (100 μM), SNP (1 mM), SIN-1 (1 mM), DNA (0.1 pg, pTrcA, InVitrogen), and DNA that had been treated with NO gas, SNP, or SIN-1. Incubation of DNA with NO gas was according to (*11*). DNA treated with SNP and SIN-1 doubled PARS activity, but neither compound had an effect on PARS by itself. When [^{14}C]NADs were used in place of [^{32}P]NAD in PARS assays, radioactive polymers were only formed from [*adenine*-^{14}C]NAD, not from [*carbonyl*-^{14}C]NAD.
15. To test whether NO interacts directly with benzamide, we incubated benzamide (1 mM) with SNP (1 mM) for 30 min, 1 hour, and 5 days at 25°C and analyzed it by high-pressure liquid chromatography (HPLC) on a C_{18} column. Benzamide treated with SNP was 100% recovered at the same elution time point with the same ultraviolet spectrum as that of untreated benzamide. When SNP-treated benzamide and benzamide were mixed and analyzed, only one peak was observed. Benzamide, at 0.03, 0.1, 0.3, and 1 mM, did not compete with cytochrome c for being reduced by superoxide generated from xanthine and xanthine oxidase.
16. The IC_{50} for benzamide in inhibiting PARS in vitro is 22 μM [M. Banasik, H. Komura, M. Shimoyama, K. Ueda, *J. Biol. Chem.* **267**, 1569 (1992)]. In intact cells, a higher value would be anticipated because benzamide would be competing with millimolar endogenous concentrations of NAD [R. McNerney *et al.*, *Biochim. Biophys. Acta* **1009**, 185 (1989)].
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18. Nitrite formation in a human kidney 293 cell line stably transfected with the cDNA for brain NOS was measured in response to A23187 (10 μM) as described [D. S. Bredt, C. D. Ferris, S. H. Snyder, *J. Biol. Chem.* **267**, 10976 (1992)]. A23187 (10 μM) elicited the formation of 17.1 ± 2.1 μM nitrite in 2 hours. Benzamide (100 μM) did not prevent the formation of nitrite (15.9 ± 3.8 μM nitrite), and 100 μM nitroarginine diminished nitrite formation to 3.9 ± 1.6 μM nitrite (mean ± SEM, n = 3).
19. See, for example, O. H. Lowry *et al.*, *J. Biol. Chem.* **239**, 18 (1964); D. Uematsu *et al.*, *Brain Res.* **482**, 129 (1989); K. M. Raley and P. Lipton, *Neurosci. Lett.* **110**, 118 (1990).
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22. Primary neuronal cultures from cortex were prepared from fetal Sprague-Dawley rats, gestation day 13 to 14. Mature neurons (more than 21 days in culture) were used in all experiments. We determined neurotoxicity by exposing the neurons to the various test solutions as described (*4*). NMDA, SNP, or SNAP were applied to the cells for 5 min, then the cells were washed and replaced with minimum essential medium and 21 mM glucose overnight in the incubator. After 20 to 24 hours of exposure to test solutions, the neurons were exposed to 0.4% Trypan blue in control salt solution to stain the residue of nonviable cells and to assess toxicity. Viable and nonviable cells were counted. At least two separate experiments using four separate wells were done for each data point shown. Significant overall values were obtained with a one-way, between-groups analysis of variance. Specific comparisons on all possible pair combinations were made with the Student's t test for independent means.
23. We thank S. Pou (Department of Pharmacology and Toxicology, University of Maryland School of Pharmacy) for NO, R. Henning (Leiter Der Pharma-Forschung, Cassella A.G., Frankfurt, Germany) for providing SIN-1, H. Zhou for providing purified pTrcA, and N. Bruce for manuscript preparation. Supported by USPHS grants DA-00266, contract DA-271-90-7408, Research Scientist Award DA-00074 (S.H.S.), a grant from Asahi Chemical Company, and a grant of the W. M. Keck Foundation. T.M.D. was supported by USPHS Clinical Investigator Development Award NS-01578 and a grant from the American Academy of Neurology. V.L.D. was supported by an Intramural Research Training Award from NIH.

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Adenosine Inhibition of Mesopontine Cholinergic Neurons: Implications for EEG Arousal

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Increased discharge activity of mesopontine cholinergic neurons participates in the production of electroencephalographic (EEG) arousal; such arousal diminishes as a function of the duration of prior wakefulness or of brain hyperthermia. Whole-cell and extracellular recordings in a brainstem slice show that mesopontine cholinergic neurons are under the tonic inhibitory control of endogenous adenosine, a neuromodulator released during brain metabolism. This inhibitory tone is mediated postsynaptically by an inwardly rectifying potassium conductance and by an inhibition of the hyperpolarization-activated current. These data provide a coupling mechanism linking neuronal control of EEG arousal with the effects of prior wakefulness, brain hyperthermia, and the use of the adenosine receptor blockers caffeine and theophylline.

Factors as diverse as prior wakefulness, brain hyperthermia, and adenosine blockers [such as caffeine and theophylline (*1*)] control the degree of arousal, usually measured as EEG activation (EEG arousal). Both the propensity to sleep and the intensity of delta waves upon falling asleep are proportional to the duration of prior wakefulness (*2*, *3*). Behavioral experiments have shown that a rise in brain temperature induces somnolence and a high level of EEG delta activity during sleep (*4*). The stimulating effects of coffee (caffeine) and tea (theophylline) are a nearly universal subjective experience, one whose EEG arousal effects have been documented (*5*). However, the neural mediator or mediators of the effects of these diverse events on EEG arousal are unknown.

Considerable evidence suggests that mesopontine cholinergic neurons play a key role in EEG arousal (*6*, *7*). The cholinergic neurons of this region form a continuum, extending from the laterodorsal tegmental nucleus (LDT) laterally to the pedunculopontine tegmental nucleus (PPT); they project heavily to the forebrain and thalamus in rat, cat, and monkey (*8*). In vivo extracellular data indicate that a majority of these cholinergic neurons selectively discharge during states of EEG arousal (*7*). Furthermore, both in vivo and in vitro data indicate that the cholinergic neurons promote EEG arousal by a cholinergic depolarization of thalamic neurons that, when hyperpolarized, oscillate in the delta EEG frequency range in concert with their cortical neuronal targets (*6*).

It seemed likely, therefore, that modulation of mesopontine cholinergic activity might be a key neural mediator of behavioral state. Adenosine (AD) was of particular interest as a modulator of these neurons because (i) the production and release of AD into the extracellular media is linked to neuronal metabolic activity (*9*); (ii) neural metabolism is much greater during wakefulness than in delta sleep and is also increased by hyperthermia (*10*); and (iii) caffeine and

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