Resistance to Neurotoxicity in Cortical Cultures from Neuronal Nitric Oxide Synthase-Deficient Mice

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In addition to its functions as a neuronal messenger molecule, nitric oxide (NO) has also been implicated in playing a major role in ischemic damage and glutamate neurotoxicity. Using primary cortical cultures from transgenic neuronal NO synthase (NOS) null (nNOS⁻) mice, we definitively establish NO as a mediator of NMDA and hypoxic neurotoxicity. Neurotoxicity elicited by NMDA is markedly attenuated in nNOS⁻ cortical cultures compared with wild-type cultures. The NOS inhibitor nitro-L-arginine is neuroprotective in wild-type but not nNOS⁻ cultures, confirming the role of nNOS-derived NO in glutamate neurotoxicity. Confirming that the nNOS⁻ cultures lack NMDA-stimulated nNOS activity, NMDA did not stimulate the formation of cGMP in nNOS⁻ cultures, but markedly elevates cGMP in wild-type cultures. Both wild-type and nNOS⁻ cultures are sensitive to toxicity induced by NO donors, indicating that pathways stimulated by NO that result in neuronal cell death are still intact in the transgenic mice. Superoxide dismutase is neuroprotective against NMDA and NO neurotoxicity in both wild-type and nNOS⁻ cultures, highlighting the importance of superoxide anion in subsequent neuronal damage. The unknown cellular factors that endow differential resistance to NMDA neurotoxicity and differential susceptibility to quisqualate neurotoxicity remain intact in the nNOS⁻ cultures, because the response of somatostatin-immunopositive neurons in nNOS⁻ cultures to high-dose NMDA and low-dose quisqualate is identical to the response of NOS-immunopositive neurons in the wild-type cultures. There is no difference in susceptibility to kainate neurotoxicity between nNOS⁻ and wild-type cultures and only a modest resistance to quisqualate neurotoxicity, confirming observations that NO-mediated neurotoxicity is associated primarily with activation of the NMDA receptor. The nNOS⁻ cultures are markedly protected from 60 min of combined oxygen–glucose deprivation neurotoxicity compared with wild-type cultures. Wild-type cultures are protected from neuronal cell death by the NMDA receptor antagonist MK-801 and the NOS inhibitor L-nitroarginine methyl ester, but not its inactive stereoisomer D-nitroarginine methyl ester. nNOS⁻ cultures were not additionally protected. These data confirm that activation of NMDA receptors and production of NO are primary mediators of neuronal damage after ischemic insult.

Key words: nitric oxide; nitric oxide synthase; glutamate; NMDA; toxicity; ischemia

Nitric oxide (NO) is a biologically important messenger molecule in many diverse tissues throughout the body (Nathan, 1992; Southam and Garthwaite, 1993; Dawson and Snyder, 1994). NO is synthesized from L-arginine by NO synthase (NOS), of which three isoforms have been identified representing the products of three distinct genes: neuronal NOS (nNOS; NOS-1), inducible NOS (iNOS; NOS-2), and endothelial NOS (eNOS; NOS-3) (Marletta, 1993; Dawson and Snyder, 1994; Marletta, 1994). Excess production of NO by nNOS has been implicated in neurotoxicity elicited by glutamate acting through NMDA receptors and in vascular stroke. In primary cerebral cortical cultures, NMDA neurotoxicity is prevented by a variety of NOS inhibitors (Dawson et al., 1991b, 1993a,b, 1995). These results have been

independently replicated in numerous culture systems (for review, see Dawson and Snyder, 1994). Some difficulties in replicating these findings may relate to inadequate expression of nNOS neurons in the cultures used (Hewett et al., 1993, 1994). Because NMDA neurotoxicity has been implicated in vascular stroke damage, NOS inhibitors have been evaluated in various stroke models and have provided protection from stroke damage (Carreau et al., 1994; Dalkara et al., 1994; Yoshida et al., 1994). Moreover, mice with deletion of nNOS (nNOS⁻ mice) manifest a substantial reduction in infarct size after permanent focal ischemia (Huang et al., 1994).

nNOS⁻ mice have clarified a number of aspects of NO physiology (Huang et al., 1993). The mutant mice are viable, procreate, and display grossly normal locomotor activity (Huang et al., 1993). They possess enlarged stomachs with hypertrophied pyloric sphincters, and thus provide a model for the human disease infantile hypertrophic pyloric stenosis (Huang et al., 1993). Long-term potentiation in hippocampal slices (O'Dell et al., 1994) and long-term depression in cerebellar cultures (Linden et al., 1995) are normal in nNOS⁻ animals. Carbon dioxide-induced relaxation of cerebral blood vessels occurs normally in nNOS⁻ mice. This effect of hypercapnia is blocked by NOS inhibitors in wild-type but not in mutant mice, indicating that a non-NO system compensates for the loss of NO (Irikura et al., 1994, 1995). nNOS⁻ male mice

Table 1. Neurotoxicity in wild-type versus NOS⁻ primary cortical cultures after exposure to NMDA, NOS inhibitors, and PARS inhibitors

Treatment	Wild-type % Cell death Mean ± SEM	nNOS⁻ % Cell death Mean ± SEM
500 µM NMDA	57.1 ± 3.4	17.4 ± 2.3**
NOS inhibitor		
+ 100 µM NArg	17.4 ± 2.3*	15.6 ± 3.3
+ 100 µM NArg + 1 mM LArg	55.8 ± 3.8	15.0 ± 4.4
500 µM NMDA	61.7 ± 4.2	20.7 ± 1.8**
Flavoprotein inhibitor		
+ 100 nM DPI	23.4 ± 3.2*	25.7 ± 3.2
Calcineurin inhibitor		
+ 100 nM FK506	9.3 ± 3.6*	12.4 ± 0.9*
PARS inhibitors		
+ 100 µM BZD	25.5 ± 3.3*	27.8 ± 3.2
+ 100 µM DHIQ	24.2 ± 3.9*	27.0 ± 3.7

Data are the means ± SEM (n = 8–16). Each data point represents a minimum of 4000–12,000 neurons counted. Toxicity was assessed by trypan blue exclusion 20–24 hr after exposure to experimental conditions, as described in Materials and Methods. Significant overall values were obtained using a chi-square test on all possible combinations. *p ≤ 0.001 comparing WT/NMDA with WT/NMDA/NArg; WT/NMDA/NArg/LArg with WT/NMDA/NArg; WT/NMDA with WT/NMDA/DPI; WT/NMDA with WT/NMDA/FK506; WT/NMDA with WT/NMDA/BZD; WT/NMDA with WT/NMDA/DHIQ; and nNOS /NMDA with nNOS⁻/NMDA/FK506. **p ≤ 0.001 comparing WT/NMDA with nNOS⁻/NMDA. There was no significant difference in percent cell death in control conditions between wild-type cultures compared with the nNOS⁻ cultures. PARS, Poly(ADP-ribose)synthetase; DPI, diphenyleneiodonium; DHIQ, dihydroisoquinoline; NArg, nitro-L-arginine; LArg, L-arginine; BZD, benzamide.

display inappropriate, excessive sexual and aggressive behavior (Nelson et al., 1995).

In the present study, we have used primary cerebral cultures from nNOS⁻ mice to clarify the role of NO in neurotoxicity. We demonstrate a loss of neurotoxicity in response to NMDA stimulation and ischemic insult of combined oxygen–glucose deprivation.

MATERIALS AND METHODS

Cell culture. Primary cortical cell cultures were prepared from gestational day 16 fetal mice in a procedure modified from that described previously (Dawson et al., 1991b, 1993b). Briefly, the cortex is dissected and the cells dissociated by trituration in modified Eagle's medium (MEM), 20% horse serum, 25 mM glucose, and 2 mM L-glutamine after a 30 min digestion in 0.027% trypsin/saline solution. The cells are plated on 15 mm multiwell plates coated with polyornithine. Four days after plating, the cells are treated with 5-fluoro-2-deoxyuridine for 3 d to inhibit proliferation of non-neuronal cells. Cells are then maintained in MEM, 10% horse serum, 25 mM glucose, and 2 mM L-glutamine in an 8% CO_2 humidified 37°C incubator. The growth medium is refreshed twice per week, and the neurons are allowed to mature for 14 d in culture before being used for experiments. Preliminary ontogeny studies in wild-type cultures determined that nNOS is expressed at mature levels by day 14 in culture. Mature levels of nNOS neurons correspond to 1–2% of total neuronal population (Bredt et al., 1991; Dawson et al., 1993b).

Cytotoxicity. The cells are exposed to neurotoxic conditions as described previously (Dawson et al., 1991b, 1993b). Before exposure, the cells are washed with Tris-buffered control salt solution (CSS) containing (in mM): 120 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 25 Tris-HCl, pH 7.4, and 15 glucose. Except for kainate, the exposure solutions containing experimental reagents are administered briefly for 5 min and then washed off. The cells are then placed in MEM with 21 mM glucose and returned to the incubator overnight. Exposure to kainate is performed in MEM with 21 mM glucose overnight in the incubator.

The effects of various inhibitors (see Table 1) of NO-dependent cell death pathways were examined in both nNOS⁻ and wild-type cultures. The concentrations of all the inhibitors used were in the appropriate

range of specificity for their respective targets (Dawson et al., 1991b; 1993a,b; Zhang et al., 1994).

Combined oxygen–glucose deprivation is performed as described previously (Kaku et al., 1991; Monyer et al., 1992). The culture media are completely exchanged with deoxygenated, glucose-free Earle's balanced salt solution (EBSS) containing (in mM): 116 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 1 NaH_2PO_4, and 0.9 $CaCl_2$, bubbled with 5% H_2/85% N_2/5% CO_2. The cultures are kept in an anaerobic chamber containing the gas mixture 5% H_2/85% N_2/5% CO_2 maintained at 37°C. Combined oxygen–glucose deprivation is terminated by removal of the cultures from the chamber and replacement of the EBSS solution with oxygenated MEM containing 21 mM glucose. The cultures are returned to a humidified incubator containing 5% CO_2 and atmospheric oxygen at 37°C overnight.

Toxicity is assayed 20–24 hr after exposure to cytotoxic conditions by trypan blue exclusion (0.4% trypan blue in CSS) as described previously (Dawson et al., 1991b, 1993b). Both live cells (cells that exclude trypan blue and are raised dots under Hoffman modulation optics) and dead cells (cells that take up trypan blue and are flat under Hoffman modulation optics) are counted. Percent cell death is determined as the ratio of live to dead cells compared with the percent cell death in control wells to account for cell death attributable to mechanical stimulation of the cultures. At least two separate experiments using four separate wells is performed with a minimum of 4000–12,000 neurons counted per data point. The data are collected and counted by an observer blinded to the treatment protocol.

Data are analyzed with a one-way ANOVA and the Student–Newman–Keuls multiple comparison test or the Student's t test for independent means.

cGMP assays. For determination of cGMP, the cultures are washed with CSS followed by a 5 min exposure to experimental solutions containing 100 µM 1-methyl-3-isobutylxanthine (IBMX), a phosphodiesterase inhibitor. The experiment is terminated by the addition of 15% ice-cold trichloroacetic acid. The samples are extracted with water-saturated ether three times and then cGMP concentrations are determined by radioimmunoassay (RIA) according to the manufacturer's instructions (Amersham, Arlington Heights, IL).

Immunohistochemistry. Cells were washed three times with CSS and then fixed for 30 min at room temperature in freshly depolymerized 4% paraformaldehyde/0.1 M phosphate buffer. The cells were washed once in Tris-buffered saline (TBS) containing 50 mM Tris-HCl, 1.5% NaCl, pH 7.4. The cultures were permeabilized with 0.2% Triton X-100/TBS for 5 min and then blocked with 4% normal goat serum (NGS)/0.1% Triton X-100/TBS for 1 hr. The cells were incubated overnight at 4°C in affinity-purified anti-nNOS antibodies or anti-somatostatin antibodies (Dawson et al., 1991a, 1993a; Huang et al., 1993). The cells were rinsed three times in TBS, 10 min each rinse, followed by incubation in biotin-conjugated secondary antibody (goat anti-rabbit)/1.5% NGS/0.1% Triton X-100/TBS for 1 hr at room temperature. The cells were again rinsed three times for 10 min each in TBS. The stain was developed with a substrate solution containing 0.01% H_2O_2/0.5 mg/ml diaminobenzidine/TBS. The stain was terminated by rinsing the cells in 0.02% sodium azide/TBS. Photomicrographs were made, and all NOS-positive and somatostatin-positive cells were counted in each well with an inverted Zeiss Axioplan microscope (Thornwood, NY).

Materials. FK506 was a generous gift from Fujisawa Pharmaceuticals (Tokyo, Japan). 3-Morpholino-sydnonimine hydrochloride (SIN-1) was a generous gift from Dr. Rainer Henning, Cassella AG (Frankfurt, Germany). Dihydroisoquinoline (DHIQ) was purchased from Aldrich (Milwaukee, WI). Diphenyleneiodonium (DPI) was purchased from Kodak. MK-801 was purchased from Research Biochemicals (Natick, MA). Cell culture media and supplies were purchased from Gibco (Gaithersburg, MD). cGMP RIA kits were purchased from Amersham. Anti-somatostatin antibody was obtained from Incstar (Stillwater, MN). Diaminobenzidine and the biotin-conjugated goat anti-rabbit secondary antibody were obtained from Vector (Burlingame, CA). All other reagents were obtained from Sigma (St. Louis, MO).

RESULTS

Cultures from nNOS⁻ animals are resistant to NMDA neurotoxicity

In primary cerebral cortical cultures from fetal rats, exposure to 500 µM NMDA kills 60–80% of the neurons. NMDA-induced cell death is markedly reduced by NOS inhibitors (Dawson et al., 1991b, 1993b). In the present study, we have used primary cortical
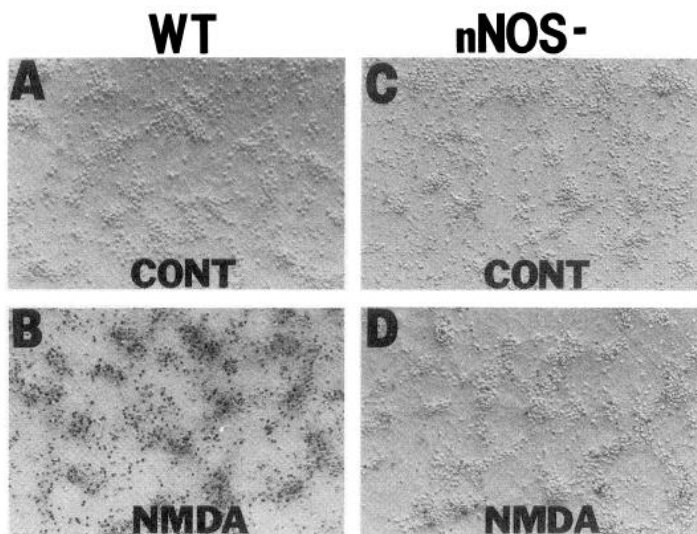
Figure 1. nNOS⁻ cultures are resistant to NMDA neurotoxicity. Hoffman modulation photomicrographs of cortical cell cultures 24 hr post-treatment after a 5 min application of 500 μM NMDA. The cultures were treated with 0.4% trypan blue to stain nonviable cells. Dead cells appear in the photomicrograph as *black dots*, live neurons are raised cells against the *gray background*. A and C are control cultures (*CONT*) from wild-type (*WT*) and *nNOS⁻* mice, respectively, that were not exposed to NMDA. B and D are cultures that were exposed for 5 min to 500 μM *NMDA*. Cultures from nNOS⁻ mice are markedly resistant to NMDA neurotoxicity compared with control cultures. Photomicrographs were taken randomly from culture wells.

Figure 2. Dose dependence of NMDA neurotoxicity. In primary cortical cultures from wild-type and nNOS⁻ mice, NMDA induces neurotoxicity in a dose-dependent manner. However, the neurotoxicity induced in the *nNOS⁻* cultures is markedly reduced compared with the wild-type (*WT*) cultures. Each data point represents the means $\pm$ SEM ($n = 8$–16) of at least two separate experiments assayed for toxicity 24 hr after a 5 min application of NMDA. Each data point represents a minimum of 4000–12,000 neurons counted. Significance was determined by the Student's *t* test for paired samples comparing wild-type with nNOS values for each concentration of NMDA. $^*p \leq 0.001$.

cultures from embryonic days 16–17 mice, which are maintained in culture for 2 weeks, exposed to NMDA for 5 min, and evaluated for toxicity 24 hr later. The 80% cell death elicited by 500 μM NMDA in wild-type cultures is almost completely abolished in nNOS⁻ cultures (Fig. 1). There remains a very small residual amount of cell death elicited by NMDA in the nNOS⁻ cultures, which displays a dose–response relationship with maximal effects at 500 μM NMDA (Fig. 2). At all NMDA concentrations, cell death is profoundly reduced.

Previously, we showed that NMDA neurotoxicity can be blocked by inhibiting NOS at both the catalytic site as well as regulatory sites distinct from the catalytic site. Nitro-L-arginine competes with L-arginine for the catalytic site and blocks synthesis of NO. As reported previously in rat cultures (Dawson et al., 1991b, 1993b), nitro-L-arginine blocks NMDA-induced neurotoxicity in cultures from wild-type mice, and its protective effect is reversed by L-arginine (Table 1). The much diminished neurotoxicity in the nNOS⁻ cultures is decreased slightly by nitro-L-arginine, but this effect is not influenced by L-arginine. DPI inhibits NOS and provides neuroprotection by preventing the shuttling of electrons through the flavin cofactors (Stuehr et al., 1991; Dawson et al., 1993b). The immunosuppressant FK506 also blocks neurotoxicity, possibly by increasing phosphorylated, inactive forms of nNOS (Dawson et al., 1993a). In cultures from wild-type mice, we confirm that DPI and FK506 are neuroprotective against NMDA neurotoxicity (Table 1). DPI has no effect on neurotoxicity in nNOS⁻ cultures, although FK506 does significantly reduce neurotoxicity in the nNOS⁻ cultures. The failure of NOS inhibitors to block NMDA neurotoxicity in nNOS⁻ cultures indicates that the residual toxicity is not derived from generation of NO from other NOS isoforms. Thus, nNOS-derived NO is the sole mediator of neurotoxicity.

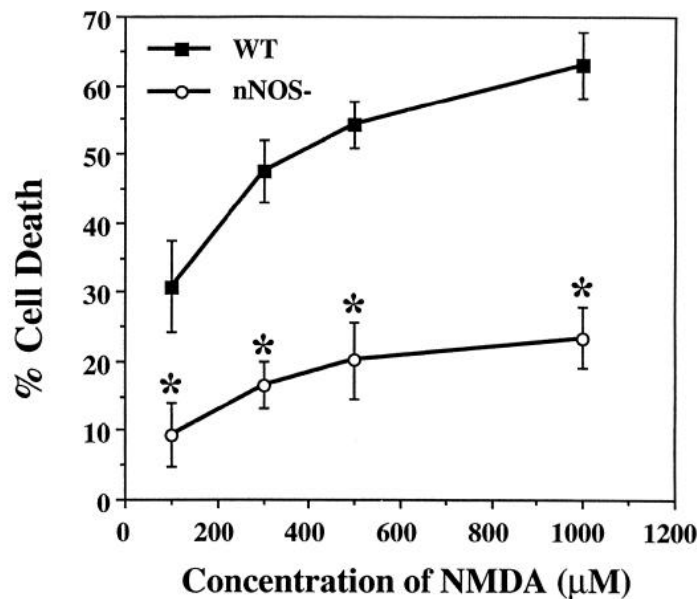We have reported previously that inhibitors of poly(ADP-ribose)synthetase (PARS) prevent NMDA neurotoxicity in rat cortical cultures (Zhang et al., 1994). PARS is activated by DNA fragments generated by NO damage to DNA (Berger, 1985; Lautier et al., 1993). The protective effect of PARS inhibitors implies that DNA damage by NO is a mediator of NMDA neurotoxicity (Zhang et al., 1994). In wild-type primary mouse cultures, the structurally distinct PARS inhibitors benzamide and DHIQ both prevent NMDA neurotoxicity. In nNOS⁻ cultures (Table 1), the PARS inhibitors cause a slight but statistically insignificant increase in cell death. The inability of PARS inhibitors to block NMDA neurotoxicity in nNOS cultures indicates that NO is the major activator of PARS in NMDA neurotoxicity in wild-type cultures. The lack of additional neuroprotection by the various inhibitors used in this study could conceivably be because of the small amount of residual toxicity in the nNOS⁻ cultures, which is not sufficient to activate these pathways.

In rat cortical cultures, we showed that NOS inhibitors are most effective in protecting against NMDA toxicity with only modest protective effects in cultures from different brain regions against toxicity induced by the non-NMDA receptor agonists quisqualate or kainate (Dawson et al., 1993b). Cortical cultures from nNOS⁻ animals are not protected against kainate toxicity and are only modestly protected against quisqualate neurotoxicity. The modest protection observed against quisqualate may be attributable to the secondary activation of NMDA receptors by quisqualate. However, in the same experiments, these cultures are profoundly resistant to NMDA neurotoxicity (Fig. 3).

NMDA stimulation of cGMP formation is abolished in nNOS⁻ cultures

The activation of guanylyl cyclase by NO to increase cGMP formation has been studied extensively (Moncada et al., 1989;
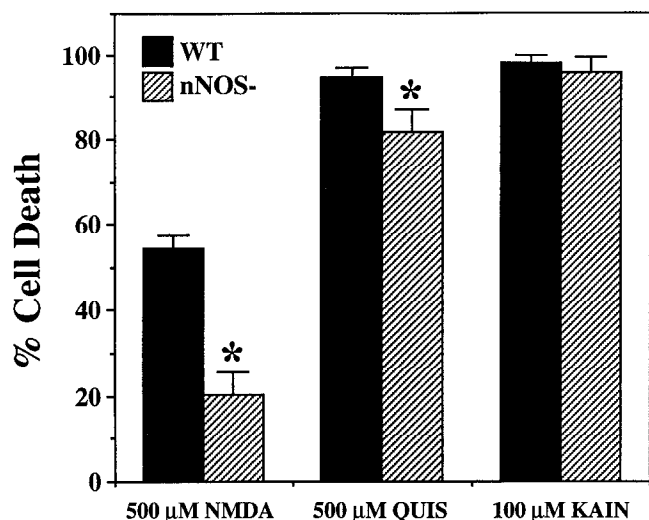
Figure 3. nNOS⁻ cultures are resistant to NMDA but not quisqualate or kainate neurotoxicity. Primary cortical cultures from wild-type (*WT*) and *nNOS⁻* mice are not resistant to a 24 hr exposure to 100 µM kainate (*KAIN*). nNOS⁻ cultures are modestly resistant to neurotoxicity induced by a 5 min application of 500 µM quisqualate (*QUIS*) and are markedly resistant to a 5 min exposure to 500 µM *NMDA* compared with the wild-type cultures. Each data point represents the means ± SEM (*n* = 8–20) of at least two separate experiments. Each data point represents a minimum of 4000–12,000 neurons counted. Significance was determined by the Student's *t* test for independent means. *$p \leq 0.001$ comparing WT/NMDA with nNOS⁻/NMDA; WT/QUIS with nNOS⁻/QUIS.
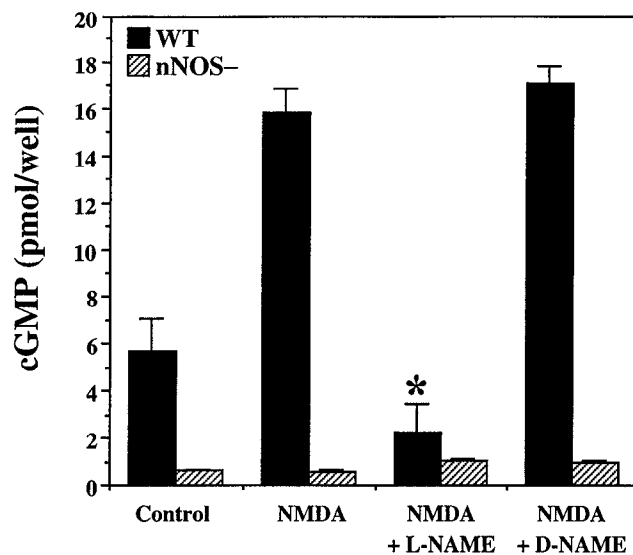

Figure 4. NMDA does not induce cGMP formation in nNOS⁻ cultures. cGMP formation after a 5 min exposure of cortical cultures to 500 µM *NMDA* in the absence or presence of 100 µM nitro-L-arginine methyl ester (*L-NAME*) or 100 µM nitro-D-arginine methyl ester (*D-NAME*). NMDA is effective in tripling the concentration of cGMP in wild-type (*WT*) but not in *nNOS⁻* cultures. This induction of cGMP is blocked by L-NAME but not by D-NAME, implicating activation of nNOS in the wild-type cultures. All experiments were performed in the presence of 100 µM IBMX. Data are means ± SEM (*n* = 8) of two separate experiments. Significant overall values comparing values within groups were obtained using a chi-square test. *$p \leq 0.001$ comparing WT/NMDA with WT/NMDA/L-NAME.

Ignarro, 1990; Moncada and Higgs, 1993; Southam and Garthwaite, 1993; Garthwaite, 1995; Garthwaite and Boulton, 1995). Recently, we showed that eNOS has discrete localizations in the CNS (Dinerman et al., 1994; O'Dell et al., 1994) and thus could contribute to NO-mediated neurotoxicity. In addition, in the CNS it is unclear whether NO is the sole or primary determinant of endogenous cGMP levels in the brain. The regional distribution of mRNA for guanylyl cyclase parallels that of hemeoxygenase-2 (HO-2) more closely than nNOS (Verma et al., 1993). HO-2 is an enzyme that generates CO, which also stimulates guanylyl cyclase (Verma et al., 1993). Moskowitz and associates (Huang et al., 1994) observed that basal levels of cGMP in adult nNOS⁻ mice are only 60% of those of wild-type mice. Moreover, the 30% increase in cGMP levels elicited by permanent focal ischemia in wild-type animals is absent in nNOS⁻ mice (Huang et al., 1994). Basal cGMP levels in nNOS⁻ cultures are only 10% of those of wild-type preparations (Fig. 4). Although NMDA triples cGMP levels in wild-type cultures, it elicits no effect in nNOS⁻ cultures. Thus, eNOS plays no role in NO-stimulated cGMP formation in cortical cultures, consistent with the lack of neuroprotection by NOS inhibitors in nNOS⁻ mice. L-Nitroarginine methyl ester (L-NAME) completely prevents the NMDA stimulation of cGMP levels in the wild-type preparations, but has no influence in the nNOS⁻ cultures. Interestingly, cGMP levels in the preparations treated with L-NAME plus NMDA are less than half those of basal control values, substantiating the importance of endogenous NO in regulating basal levels of cGMP. The specificity of NOS inhibition is indicated by the failure of D-nitroarginine methyl ester (D-NAME) to block the NMDA-induced increase in cGMP in wild-type cultures.

nNOS/somatostatin neurons are resistant to NMDA neurotoxicity but sensitive to quisqualate neurotoxicity in nNOS⁻ and wild-type cultures

In rat cortical cultures, we had demonstrated that neurons expressing nNOS are resistant to NMDA neurotoxicity to a much greater extent than other neurons (Dawson et al., 1993b). By contrast, the nNOS neurons are more susceptible to quisqualate toxicity than other neurons (Dawson et al., 1993b). In the cerebral cortex, all nNOS neurons also stain for somatostatin, and all somatostatin neurons are also nNOS-positive (Dawson et al., 1991a). In nNOS⁻ mice, the density of somatostatin-staining neurons is normal in the cerebral cortex, indicating that although nNOS has been deleted, the neurons remain intact (Huang et al., 1993). Accordingly, we used somatostatin to identify this neuronal population and examine the differential resistance and susceptibility to neurotoxicity of nNOS/somatostatin neurons in cultures from nNOS⁻ mice (Fig. 5). Although NMDA kills ~60% of the total population of neurons from wild-type mice, it kills only 20% of the nNOS/somatostatin-positive neurons in wild-type cultures. A similar percentage of cell death, 16–20%, occurs in somatostatin-staining neurons in nNOS⁻ cultures, while the total number of neurons killed is markedly reduced in nNOS⁻ cultures. The differential susceptibility to quisqualate of nNOS/somatostatin-positive neurons in both wild-type and nNOS⁻ cultures is maintained. Only 15–18% of the total neuronal population is sensitive to 20 µM quisqualate, whereas >85% of the nNOS/somatostatin-positive neurons are killed by this low concentration of quisqualate (Fig. 5). These results indicate that the factor(s) that render NOS neurons resistant to NMDA toxicity and sensitive to quisqualate toxicity remain intact in the nNOS⁻ cultures, and therefore are not likely to be nNOS itself (Fig. 5).
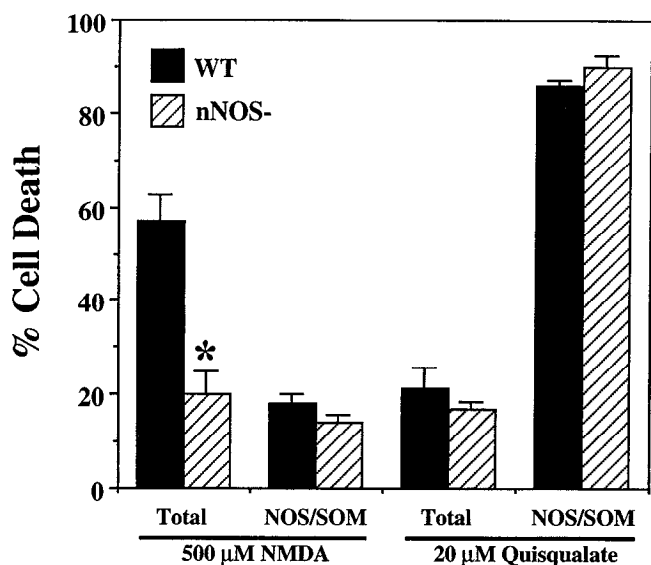
Figure 5. Differential resistance and susceptibility of nNOS/somatostatin-positive neurons. In the cerebral cortex, neurons that express nNOS also express somatostatin and vice versa. In the nNOS⁻ mice, immunoreactivity for somatostatin is used to identify the neurons that express nNOS in the wild-type (WT) mice. Primary neuronal cultures from wild-type (*WT*) mice and *nNOS⁻* mice were exposed for 5 min to either 500 μM *NMDA* or 20 μM *Quisqualate* and 24 hr later were stained by immunohistochemistry for nNOS or somatostatin and by trypan blue for overall (*Total*) cell death. The number of nNOS/somatostatin-positive (*NOS/SOM*) neurons were counted per well and compared with the total neuronal cell death determined by trypan blue exclusion. Differential resistance to NMDA and sensitivity to quisqualate of NOS/SOM neurons are maintained in the nNOS⁻ cultures when compared with wild-type cultures. Each data point represents the means ± SEM (*n* = 8) of at least two separate experiments. Significance was determined by the Student's *t* test for independent means comparing wild-type with control cultures within a treatment protocol. *p ≤ 0.001 comparing total WT/NMDA with total nNOS⁻/NMDA.

Superoxide dismutase prevents NMDA and NO neurotoxicity in nNOS⁻ and wild-type cultures

NO neurotoxicity has been suggested to involve a complex of NO and superoxide (O_2^- -) to form the highly toxic derivative peroxynitrite (ONOO−) (Beckman, 1991, 1994; Radi et al., 1991a,b). Evidence in support of this theory comes from our findings, as well as those of Lipton and collaborators (Dawson et al., 1991b, 1993b; Lipton et al., 1993), that superoxide dismutase (SOD) blocks both NO and NMDA neurotoxicity in rat cortical cultures (Dawson et al., 1991b, 1993b; Lipton et al., 1993). SOD also blocks NMDA neurotoxicity in wild-type mouse cultures (Fig. 6). The greatly attenuated NMDA-induced neurotoxicity in the nNOS⁻ cultures is further reduced by 60% after treatment with SOD. Because NMDA neurotoxicity in the nNOS⁻ cultures does not appear to have an NO component, the protection by SOD suggests that superoxide mediates cell death in the nNOS⁻ cultures by a mechanism other than ONOO−, perhaps involving the generation of other oxygen-free radicals and lipid peroxidation (Choi, 1988, 1994; Coyle and Puttfarcken, 1993; Lipton and Rosenberg, 1994; Schulz et al., 1995).

Despite the resistance to NMDA toxicity, nNOS⁻ cultures remain susceptible to direct damage by NO (Fig. 7). The percentage of cell death elicited by the NO donors sodium nitroprusside (SNP) and SIN-1 is the same in nNOS⁻ and wild-type cultures. SOD blocks SNP and SIN-1 toxicity to the same extent in wild-type and nNOS⁻ cultures, consistent with NO eliciting its toxicity by combining with superoxide to form peroxynitrite.
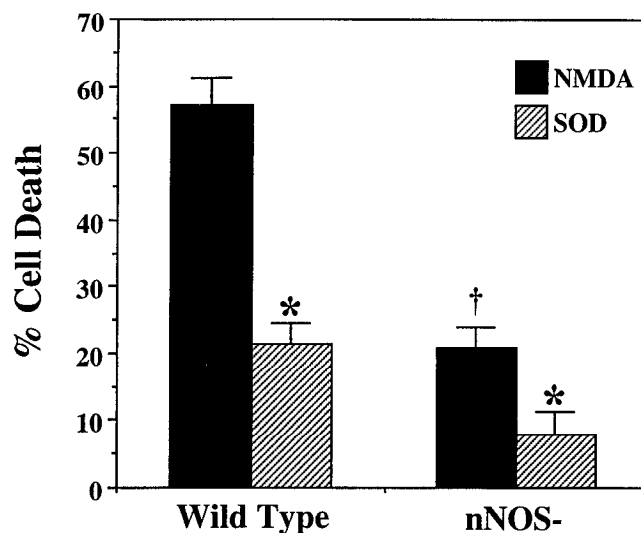

Figure 6. SOD is neuroprotective against NMDA in both wild-type and nNOS⁻ cultures. Primary cortical cultures exposed to 500 μM *NMDA* in the presence of 100 U of *SOD* are protected against neurotoxicity. Protection induced in wild-type cultures is equivalent to the protection elicited by the knockout of nNOS, suggesting that formation of peroxynitrite is a primary pathway toward cell death. Further protection by SOD is observed in the nNOS⁻ cultures, suggesting that there is a parallel but perhaps less active pathway involving oxygen-free radicals. Each data point represents the means ± SEM (*n* - 8) of at least two separate experiments assayed for toxicity 24 hr after a 5 min application of NMDA or NMDA and SOD. Each data point represents a minimum of 4000–8000 neurons counted. Significance was determined by the Student's *t* test for independent means. *p ≤ 0.001 comparing WT/NMDA with WT/NMDA/SOD; nNOS⁻/NMDA with nNOS⁻/NMDA/SOD; and †p ≤ 0.001 comparing WT/NMDA with nNOS⁻/NMDA.

Neurotoxicity elicited by ischemia–glucose deprivation is prevented in nNOS⁻ mice

Choi and collaborators (Kaku et al., 1991; Choi, 1993; Goldberg and Choi, 1993) have elegantly shown that neurotoxicity elicited in mouse cortical cultures by combined oxygen–glucose deprivation is blocked by NMDA antagonists. We have replicated these findings in mouse cortical cultures from wild-type animals (Figs. 8, 9). In the wild-type cultures, a major component of neurotoxicity induced by NMDA receptor activation elicited by oxygen–glucose deprivation appears to be through NO, because nitro-L-arginine methyl ester (L-NAME), but not the inactive stereoisomer nitro-D-arginine methyl ester (D-NAME), blocks this neurotoxicity (Fig. 9). Additionally, in nNOS⁻ cultures neurotoxicity is markedly attenuated after ischemia–glucose deprivation (Fig. 8). The residual toxicity is not affected by treatment with MK-801, L-NAME, or D-NAME, suggesting that non-NMDA and NO-independent mechanisms are activated (Fig. 9).

DISCUSSION

Our study using primary neuronal cultures from nNOS⁻ mice clarifies considerably the role of NO in neurotoxicity of cortical neurons. We only assessed cell death 20–24 hr after the initial insults, and the contribution of NO to toxicity that may occur after 24 hr is unknown. Numerous earlier studies had implicated NO as a mediator of NMDA neurotoxicity based on the neuroprotective properties of NOS inhibitors and hemoglobin, which binds NO (Dawson et al., 1991b, 1993b). Besides pharmacological agents targeted to the catalytic site of NOS, such as the arginine derivatives, agents targeted to regulatory elements of NOS were also
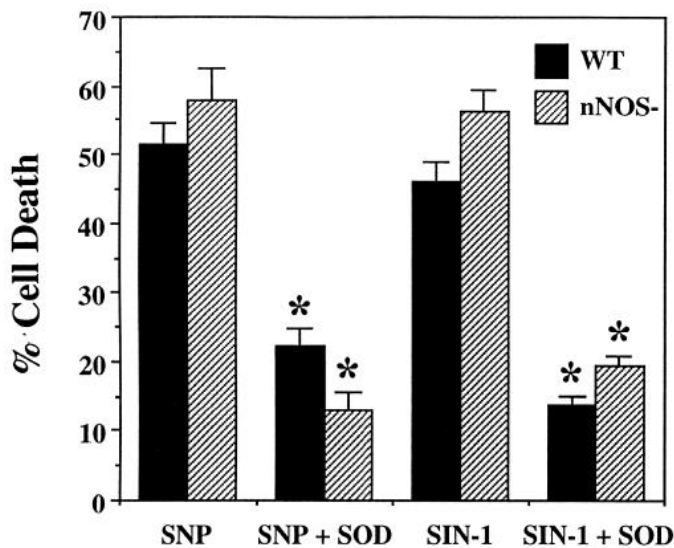
Figure 7. NO is neurotoxic to wild-type and nNOS⁻ cultures. Cultures were exposed to the NO donors [500 μM sodium nitroprusside (*SNP*) or 1 mM 3-morpholino-sydnonimine hydrochloride (*SIN-1*)] in the presence or absence of 100 U *SOD*. The mechanisms involved in NO-induced cell death remain intact in the nNOS⁻ cultures because the neurotoxicity induced by NO donors is identical to wild-type cultures. SOD is markedly neuroprotective against both NO donors and in both sets of cultures, further implicating the formation of ONOO⁻ as a major pathway toward neuronal cell death. Each data point represents the means ± SEM (*n* = 8) of at least two separate experiments assayed for toxicity 24 hr after a 5 min application of NO donors in the presence or absence of SOD. Each data point represents a minimum of 4000–8000 neurons counted. Significant overall values were obtained using a chi-square test. *$p \leq 0.001$ comparing WT/SNP with WT/SNP/SOD; nNOS⁻/SNP with nNOS/SNP/SOD; WT/SIN-1 with WT/SIN-1/SOD; nNOS⁻/SIN-1 with nNOS/SIN-1/SOD.

Figure 8. nNOS⁻ cultures are spared from ischemic insult. Hoffman modulation photomicrographs of cortical cell cultures 24 hr post-1 hr-combined oxygen–glucose deprivation and after treatment with 0.4% trypan blue to stain nonviable cells. Dead cells appear in the photomicrograph as *black dots*, live neurons are raised cells against the *gray background*. *A,C*, Control cultures (*CONT*) from *nNOS⁻* and wild-type (*WT*) mice, respectively, that were not exposed to combined oxygen–glucose deprivation. *B,D*, Cultures from nNOS and wild-type mice, respectively, that were exposed to 60 min of combined oxygen–glucose deprivation (*OGD*). *E*, Cultures from wild-type mice that were exposed to 60 min of combined oxygen–glucose deprivation in the presence of 10 μM MK-801 (*OGD+MK801*). *F*, Wild-type cultures that were exposed to 60 min of combined oxygen–glucose deprivation in the presence of 100 μM nitro-L-arginine methyl ester (*OGD+L-NAME*). *G*, Wild-type cultures that were exposed to 60 min of combined oxygen–glucose deprivation in the presence of 100 μM nitro-D-arginine methyl ester (*OGD+D-NAME*). Photomicrographs were taken randomly from culture wells under Hoffman modulation optics.

effective in eliciting neuroprotection. The flavoprotein inhibitor diphenyleneiodonium, which blocks the shuttling of electrons by flavin moieties, prevents neurotoxicity (Stuehr et al., 1991; Dawson et al., 1993b). Calmodulin inhibitors of various classes, including calmidazolium (Dawson et al., 1993b) and certain gangliosides (Dawson et al., 1995), prevent neurotoxicity, further confirming that calmodulin is an essential cofactor for NOS activity (Abu-Soud and Stuehr, 1993; Abu-Soud et al., 1994). The immunosuppressant drugs FK506 and cyclosporin-A inhibit calcineurin and increase phosphorylated levels of NOS, which results in diminished NOS activity (Dawson et al., 1993a). Both of these agents are also neuroprotective (Dawson et al., 1993a; Sharkey and Butcher, 1994). PARS inhibitors block NMDA neurotoxicity, presumably by preventing the activation of PARS elicited by DNA fragments attributable to damage from NO (Zhang et al., 1994). The overactivation of PARS consumes nicotinamide-adenine-dinucleotide and hence ATP, resulting in an irreversible energy depletion (Berger, 1985; Lautier et al., 1993).

NMDA neurotoxicity is thought to be involved in vascular stroke damage, because NMDA receptor antagonists decrease the infarct volume after middle cerebral artery occlusion, as well as other models of stroke (Choi, 1988; Meldrum and Garthwaite, 1990). A role for NO after NMDA receptor activation in stroke damage is supported by reduction of infarct volume with NOS inhibitors (Dawson and Snyder, 1994), as well as the substantial diminution in stroke damage in the brains of nNOS⁻ mice after permanent focal ischemic insult (Huang et al., 1994).

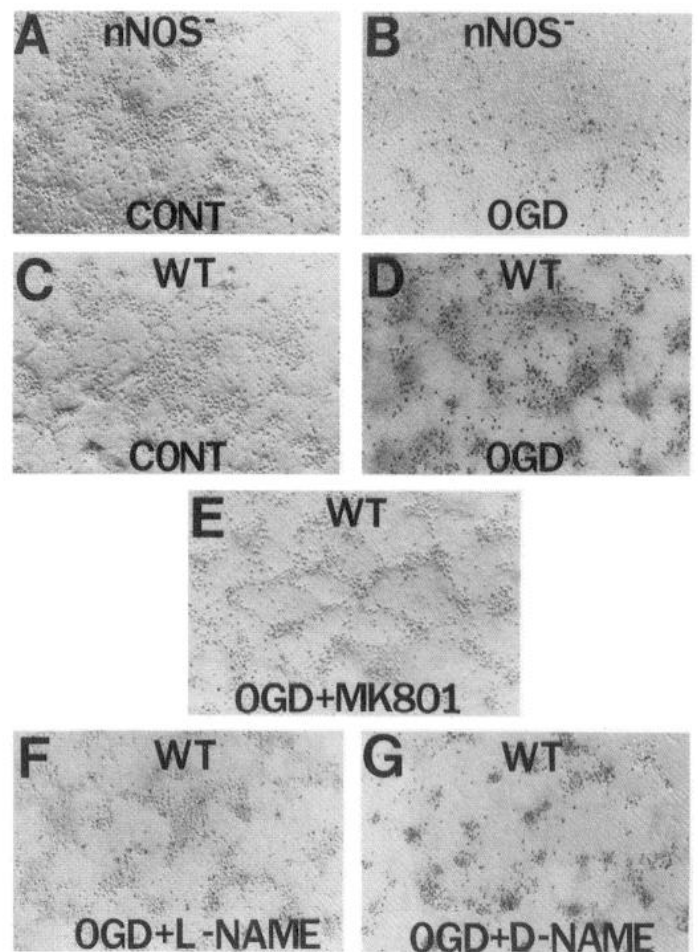Despite the abundance of data implicating NO in NMDA

neurotoxicity and stroke damage, there have been concerns that most of these studies are based on the use of drugs that may elicit nonspecific effects. Thus, most NOS inhibitors block all three isoforms. NOS inhibitors administered *in vivo* inhibit eNOS, raising blood pressure and reducing cerebral blood flow (Dalkara et al., 1994). Arginine analog NOS inhibitors, such as L-nitroarginine, might affect other arginine-dependent phenomena, such as the urea cycle and polyamine biosynthesis. nNOS⁻ animals overcome many of these pharmacological problems. The pronounced attenuation of NMDA neurotoxicity in nNOS⁻ cortical cultures establishes a major role for NO in neurotoxicity.

The profound decline in neurotoxicity in the nNOS⁻ cultures implies that NO may be responsible for as much as 80% of
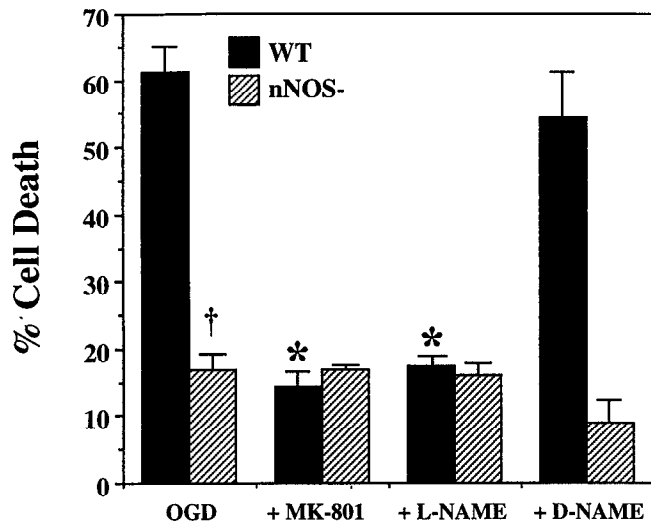
Figure 9. Blockade of NMDA receptors or NOS is neuroprotective against ischemic insult. Primary cortical cultures from wild-type (*WT*) mice exposed to 60 min of combined oxygen–glucose deprivation are protected against neurotoxicity by 10 μM MK-801 (*+MK-801*) or 100 μM nitro-L-arginine methyl ester (*+L-NAME*). Specificity of NOS inhibition is determined by the lack of protection from the inactive stereoisomer nitro-D-arginine methyl ester (*+D-NAME*). Cultures from *nNOS⁻* mice are resistant to toxicity from 60 min of combined oxygen–glucose deprivation. MK-801 or L-NAME do not further protect nNOS⁻ cultures. Each data point represents the means ± SEM (*n* = 8) of two separate experiments. Each data point represents a minimum of 4000–8000 neurons counted. Significant overall values were obtained using a chi-square test. $*p \leq 0.001$ comparing WT/OGD with WT/MK801; WT/OGD with WT/L-NAME; and $^{\dagger}p \leq 0.001$ comparing WT/OGD with nNOS⁻/OGD.

NMDA neurotoxicity. This does not rule out a role for other factors, such as oxygen-free radicals. If two factors act "in series," they may be equally responsible for an event, although blocking out only one of them will abolish the event. For instance, superoxide and NO react to form the toxic agent peroxynitrite so that elimination of superoxide with SOD or treating with NOS inhibitors would markedly reduce neurotoxicity. Consistent with this notion are the observations that both SOD and inhibitors of NOS protect against NMDA neurotoxicity (Dawson et al., 1991b, 1993b) and that transgenic mice that overexpress SOD or lack nNOS are protected against focal ischemia (Kinouchi et al., 1991; Chan et al., 1994; Huang et al., 1994). If two factors act "in parallel," then blocking only one of them should partially reduce toxicity. Superoxide may contribute to neurotoxicity independent of NO by interacting with other oxygen-free radicals leading to various types of damage such as lipid peroxidation. This would fit with our observations that SOD markedly reduces neurotoxicity in the nNOS⁻ cultures and that there is superoxide-dependent neuronal cell death in cerebellar granule cells (Lafon-Cazal et al., 1993a,b; Fagni et al., 1994). Choi and collaborators (Kaku et al., 1991; Monyer et al., 1992; Choi, 1993; Goldberg and Choi, 1993; Lynch et al., 1995) have provided evidence for parallel neurotoxic pathways of differing strengths after combined oxygen–glucose deprivation. The NMDA antagonist MK-801 is neuroprotective against 1 hr of combined oxygen–glucose deprivation (Choi, 1993; Goldberg and Choi, 1993). With prolonged combined oxygen–glucose deprivation, NMDA antagonists lose their efficacy, whereas non-NMDA antagonists provide protection (Kaku et al., 1991; Choi, 1993). Therefore, a single insult may initiate multiple

"parallel" pathways, but blocking the major "parallel" pathway may be protective. Our studies have focused on the initial neurotoxic insults associated with brief combined oxygen–glucose deprivation or brief exposures to excitotoxic agents and suggest that formation of NO from activation of nNOS is a major component toward neuronal cell death induced by excitotoxicity.

Basal cGMP levels in nNOS⁻ cultures are only 10% of levels in wild-type cultures. Clearly, NO is a major determinant of basal cGMP levels in these cultures, which derive from fetal-neonatal animals. Adult nNOS⁻ mice display only a 40% decline in cGMP levels (Huang et al., 1994). Moreover, in adult rats nitro-L-arginine treatment elicits only moderate reductions in cGMP levels. Thus, cGMP basal levels are determined by endogenous NO to a greater extent in fetal-neonatal animals than in adults. Absolute levels of cGMP are also substantially higher in fetal and neonatal rodent brain than in adult brain. The prominence of NO in determining the elevated levels of cGMP in fetal-neonatal brain may relate to the massive, transient nNOS neuronal system projecting from the cerebral cortex to the thalamus in fetal and neonatal rodents (Bredt and Snyder, 1994). Other factors that determine basal levels of cGMP in adult rodents have not been established. One strong candidate is CO, because its biosynthetic enzyme HO-2 displays similar localizations to guanylyl cyclase in the brain and hemeoxygenase inhibitors lower cGMP levels in some neuronal cultures (Verma et al., 1993).

Differential susceptibility of various neuronal populations to neurotoxicity has been well established. Neurons that stain for NADPH-diaphorase (NDP) are markedly resistant to destruction in Huntington's disease (Ferrante et al., 1985), Alzheimer's disease (Hyman et al., 1992), vascular stroke damage (Choi, 1988, 1990; Uemura et al., 1990), and excitotoxicity in cultures (Koh et al., 1986; Koh and Choi, 1988a,b). We showed that NDP-staining neurons are identical to nNOS neurons (Dawson et al., 1991a), and a major portion of NDP catalytic activity in brain homogenates is accounted for by NOS (Hope et al., 1991). In rat cortical cultures, we established that nNOS-staining neurons are resistant to NMDA neurotoxicity but manifest increased susceptibility to damage by low-dose quisqualate (Dawson et al., 1993b), confirming observations made with NDP (Koh et al., 1986; Koh and Choi, 1988a,b). Identifying nNOS catalytic activity as responsible for NDP in cells that were differentially resistant and susceptible raised the possibility that nNOS might have a role in either the differential resistance or susceptibility of these neurons. Previously, we showed that nNOS neurons, revealed by staining for somatostatin in the cerebral cortex, survive in nNOS⁻ animals (Huang et al., 1993). In the present study, we show that the nNOS/somatostatin neurons in the nNOS⁻ cultures are resistant to NMDA toxicity with the same percentage of cell death of nNOS/somatostatin-positive cells in both the wild-type and nNOS⁻ cultures. Because of the substantial resistance of nNOS⁻ cultures to NMDA neurotoxicity, we cannot make a definitive statement on the resistance of somatostatin neurons to NMDA neurotoxicity in nNOS⁻ mice. These nNOS/somatostatin-positive cells also retain their hypersensitivity to low concentrations of quisqualate. Thus, the unique responses of this subset of neurons to excitatory amino acids is not determined by the expression of nNOS or the ability to form NO. What factors then are responsible for this differential resistance and sensitivity? Recently, using the yeast-two-hybrid technique and other measures of protein-protein interactions, we identified two novel proteins that are closely associated with nNOS biochemically and display similar localizations in the brain (S. Jaffrey and S. Snyder, unpublished

observations). Conceivably, these nNOS-associated proteins could mediate this unique differential resistance and sensitivity of nNOS neurons to excitatory amino acid receptor stimulation. NDP (nNOS)-positive cells express cobalt permeant AMPA/kainate receptor channels (Weiss et al., 1994). These calcium-permeant channels probably account for the susceptibility of nNOS neurons to quisqualate and kainate. What accounts for the resistance to calcium influx via NMDA receptor channels and the exquisite sensitivity to calcium influx via AMPA/kainate receptor channels is not known.

Ischemia–glucose deprivation is a useful model of vascular stroke (Choi, 1993). Choi and associates (Kaku et al., 1991; Choi, 1993; Goldberg and Choi, 1993) showed that neurotoxicity after ischemia–glucose deprivation in cultures involves NMDA receptor activation. We confirm their observations that activation of the NMDA receptor plays a primary role in ischemic neurotoxicity, demonstrating in wild-type cultures that MK-801 provides pronounced neuroprotection. We extend these observations to identify the significant role of NO in ischemic insult. We show that wild-type cultures are protected from ischemic insult stereoselectively by the NOS inhibitor L-NAME and that nNOS⁻ cultures are markedly resistant to oxygen–glucose deprivation-induced neurotoxicity. In the nNOS⁻ cultures, the residual toxicity after 60 min of ischemia is not further attenuated by MK-801 or L-NAME. These data indicate that neurotoxicity induced by combined oxygen–glucose deprivation is mediated by stimulation of the NMDA receptor, activation of nNOS, and overproduction of NO.

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