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Nitric oxide-enhanced excitotoxity-independent apoptosis of glucose-deprived neurons

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Abstract

Glucose deprivation has been shown to elicit neuronal death via extracellular glutamate accumulation. Here we report that immunostimulated glial expression of inducible nitric oxide synthase enhances the apoptotic death of glucose-deprived cerebellar granule cells (CGC) via the excitotoxicity-independent pathway. CGC cultures were immunostimulated by interferon-γ (100 U/ml) and lipopolysaccharides (1 μg/ml) and 2 days later were challenged by glucose deprivation. Neither a 2-h Glucose deprivation nor a 2-day immunostimulation altered the viability of CGC. A 2-day immunostimulation, however, markedly enhanced the apoptotic death of CGC glucose-deprived for 1 h. The increased apoptotic death of glucose-deprived CGC after immunostimulation was mimicked by the nitric oxide (NO) releasing reagent 3-morpholinosydnonimine (200 μM, 30 min) and was partially prevented by the NO synthase (NOS) inhibitor N^G-nitroarginine. The enhanced apoptotic death was not blocked by the *N*-methyl-D-aspartate (NMDA) receptor antagonists D-2-amino-5-phosphovalerate (APV) and dizocilpine (MK-801) or the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). Moreover, the NO-induced enhanced apoptotic death occurred without a significant increase of the concentration of glutamate in the bathing medium. Our data indicate that immunostimulated glial cells potentiate the apoptotic death of glucose-deprived CGC in part through the expression of inducible NOS but not through NMDA receptor activation. Potentiation of glucose-deprived CGC death by immunostimulated glial cells may be clinically implicated in the tendency of recurrent ischemic insults to be more severe and fatal than an initial ischemic insult.

Keywords: Glucose-deprivation; Cerebellar granule cells (CGC); Nitric oxide (NO); Apoptosis; DNA fragmentation; Cycloheximide; Glutamate

1. Introduction

The concentrations of cytokines in the cerebral spinal fluid are increased in cerebral ischemia resulting in an activation of glia including astrocytes and microglia (Minami et al., 1992; Lees, 1993; Rothwell and Relton, 1993). Immunostimulated glia react to disease through the secretion of bioactive agents including cytotoxins that ultimately determine the pattern and degree of functional recovery of the central nervous system (Giulian, 1990, 1992). One of these glia-derived cytotoxins is nitric oxide, which has been implicated in a large number of pathologies (Bruhwyler et al., 1993). In brain exposed to hypoxic-ischemia a continuous and marked increase in inducible nitric oxide synthase (iNOS) expression occurs in non-neuronal cells such as

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astrocytes (Giulian and Lachman, 1985; Endoh et al., 1994; Nakashima et al., 1995) and microglia (Giulian and Lachman, 1985; Nakashima et al., 1995). Nitric oxide (NO) may adversely affect the neuronal cells by several mechanisms. NO, either directly or through its derived species, causes oxidation of protein sulfhydryls (Radi et al., 1991), DNA deamination (Wink et al., 1991), and formation of iron-NO complexes of respiratory enzymes (Drapier et al., 1988; Reif and Simmons, 1990). NO promotes oxidative damage by reacting with superoxide anion to form peroxynitrite, a strong oxidant (Beckman et al., 1990).

The understanding of NO pathophysiology is further complicated by its interaction with other neurodegenerative mediators. The interaction between NO and the excitatory neurotransmitter glutamate is particularly important in neurons. Glutamate stimulated the rise of intracellular Ca^{2+} concentration and elicited NO production by a Ca^{2+}- and calmodulin-sensitive NO synthase (Garthwaite et al., 1998; Bredt and Snyder, 1989). NMDA-induced NO production may at least in part involve in glutamate toxicity in cortical neurons (Dawson et al., 1991). Conversely, NO itself triggered neuronal death in part by eliciting autocrine excitotoxicity (Bonfoco et al., 1996; Palluy and Rigaud, 1996; Leist et al., 1997). NO also enhanced *N*-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity (Hewett et al., 1994, 1996; Kim et al., in press). In the present study, however, we provide evidence that NO synergistically enhanced the apoptotic death of glucose-deprived CGC via the excitotoxicity-independent pathway.

2. Materials and methods

2.1. Cell culture

Primary culture of CGC was prepared as previously described (Kim and Pae, 1996). In brief, cerebella from 2- to 4-day old rat (Sprague-Dawley) were minced into small pieces in Dulbecco's modified Eagle medium (DMEM). Cells were dissociated by mild trypsinization, passed through 135 μm nylon mesh and preplated for 15 min at 37°C on a culture flask coated with poly-D-lysine (10 μg/ml) to remove astrocytes. The medium containing unattached neurons was then passed through two sizes of sterile nylon sieves (80 mm and then 25 μm). Cells (about 50 000 cells/well) were then plated on poly-D-lysine (100 μg/ml)-coated 24-well culture plates. For pure CGC cultures, cells were treated for 24 h with 10 μM cytosine arabinoside after 1 day and again after 5 days in culture in order to remove any residual astroglia and other proliferating cells. CGC were used for the neurotoxicity study 4–7 days after the second treatment with cytosine arabinoside.

Glial cells were cultured from the prefrontal cortices of 2–4-day-old Sprague-Dawley rat pups, as previously described (Kim and Pae, 1996). Cells were dissociated by mild trypsinization and passed through sterile nylon sieves (80 μm pore size). Cells were then plated onto poly-D-lysine (2 μg/ml)-coated 75-cm^2 culture bottles (four cortices/bottle) and maintained for 1 week in DMEM supplemented with 10% FBS. Glia were then trypsinized, washed and re-plated in the growth medium onto poly-D-lysine (2 μg/ml)-coated 24-well plates. Glial cells were further cultured for 7–10 days before use in the present study.

2.2. Immunostimulation and glucose deprivation

Cells were pre-treated for 48 h with IFN-γ (100 U/ml) and lipopolysaccharides (LPS, 1 μg/ml). After immunostimulation, glucose deprivation was achieved by repeated rinsing and incubation in glucose-free DMEM (Gibco BRL) that was not supplemented with serum, which interfered with the lactate dehydrogenase (LDH) assay.

2.3. Measurement of NO

NO production was determined by measuring nitrite, a stable oxidation product of NO, as described previously (Green et al., 1990). In brief, nitrite levels were determined by adding the Greiss reagent (mixing equal volumes of 0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid). After 10 min, the absorbance at 540 nm was determined using a microtiter plate reader (Molecular Devices, USA).

2.4. Measurement of cell death

Neuronal cell death was assessed by morphological observation and quantified by measuring lactate dehydrogenase (LDH) release into the medium 14–22 h after starting glucose deprivation. Previously, we have showed that in immunostimulated CGC/glia cultures 24-h exposure to 2 mM NMDA resulted in a complete death of neuronal cells without any damage of glial cells (Kim and Ko, 1998). Our preliminary experiments, however, showed that glucose deprivation for over 8 h induced significant LDH releases from immunostimulated pure glial cultures. Therefore, cell viability was expressed as a percent of total LDH, which corresponds to nearly complete neuronal death without glial damage and was measured in immunostimulated sister cultures exposed 24 h to 2 mM NMDA. Apoptotic cell death was evidenced by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) and DNA fragmentation using agarose gel electrophoresis 14–25 h after NMDA treatments.

Apoptotic neuronal cell death was quantified by counting the dark-brown (TUNEL-positive) spots in three random field at 400 × magnification.

2.5. In situ labelling of free 3′-hydroxyl ends of DNA

Internucleosomal fragmentation of DNA, which is characteristic for apoptotic dead cells, was visualized as previously described (Gavrieli et al., 1992). In brief, cells were fixed 5 min with 2% buffered paraformaldehyde and, to inactivate the endogenous peroxidase, covered with 2% H_2O_2 for 5 min at room temperature. Cells were incubated 60 min at 37°C with terminal deoxynucleotidyl transferase (TdT, 0.05 U/μl, Boehringer Mannheim) and biotinylated dUTP (0.01 nmol/μl, Boehringer Mannheim). The reaction product was visualized with Extra-avidin Peroxidase (30 min, 37°C) and diaminobenzidine. Negative controls included omission of TdT or dUTP. Positive controls included treatment of the samples with DNase I (200 ng/ml in 10 mM Tris–HCl (pH7.4) for 15 min at 37°C, Boehringer Mannheim).

2.6. DNA fragmentation-agarose gel electrophoresis

In vitro DNA fragmentation was determined using agarose electrophoresis. In brief, cells were harvested in 10 mM Tris–HCl buffer (pH 7.4) containing 30 mM EDTA (TE buffer). Immediately after this, sodium dodecyl sulfate (0.5%) and proteinase K (200 μg/ml) were added and then incubated at 37°C overnight. DNA was extracted using equal measures of phenol/chloroform/isoamyl alcohol (25:14:1; v/v/v), followed by centrifugation at 2000 × *g* for 5 min. The DNA in the phenol supernatant was precipitated with 2-fold isopropanol containing 1 M ammonium acetate, followed by centrifugation at 2000 × *g* for 5 min. The pellet was washed in 70% ethanol and treated with RNase A (500 μg/ml) at 37°C for 1 h. The nucleic acid and protein contents were measured by spectrophotometry at wavelengths of 260 and 275 nm, respectively. Samples yielding a nucleic acid to protein ratio of >1.8:1 were used for DNA fragmentation analysis. Then, 15 μg of DNA or 1 μg of a 123-bp DNA ladder (Gibco BRL, Gaithersburg, MD, USA) was used for electrophoresis on 1.5% agarose containing ethidium bromide (0.5 mg/ml). DNA was visualized by a UV (302 nm) transilluminator and the gels were photographed with a polaroid camera.

2.7. Measurement of glutamate concentration

The concentration of glutamate in the bathing medium was measured by high-performance liquid chromatography. Briefly, samples (standards or releasates) were derivatized by combining with 200 μl of sample with 40 μl of internal standard (3.3 μM homoglutamine) and 100 μl of *o*-phthalaldehyde solution (22 mg *o*-phthalaldehyde, 0.5 ml 0.0313 M Na_2SO_3, 0.5 ml ethanol, and 9 ml 0.1 M Borax). The mixture was allowed to react for 15 min at 37°C. Glutamate content of the samples was measured by reverse-phase high-pressure liquid chromatography using a μBONDAPAK C18 3.9 × 300 mm column (Millipore, Milford, MA.) and an LC-4 amphoteric electrochemical detector (Bioanalytical Systems, West Lafayette, IN). Samples were eluted with a mobile phase containing 0.1 M Na_2HPO_4, 25 μM EDTA, 5% MeOH, pH 5.2, and the separate biogenic amines were measured by electrochemical detection after oxidation at 0.6 V. The flow rate was 1.2 ml/min and the injection volume was 100 μl. The amount of glutamate per sample was quantified by comparison of peak heights with known glutamate standards.

2.8. Measurement of $^{45}Ca^{2+}$ uptake

For the measurement of $^{45}Ca^{2+}$ uptake, cells were deprived of glucose with the inclusion of SIN-1 and $^{45}Ca^{2+}$ (0.5 μCi/well) in the exposure medium. Immediately after the deprivation period, the exposure solution was washed out rapidly with the DMEM supplemented with 5 mM $LaCl_3$ to block nonspecific binding of $^{45}Ca^{2+}$ and remove any residual extracellular $^{45}Ca^{2+}$. Radioactivity in 0.1% Triton X-100 cell digests was counted by liquid scintillation spectroscopy.

2.9. Statistical analysis

Data are expressed as the mean ± standard error of mean (S.E.M.) and analyzed for statistical significance by one-way ANOVA followed by Newman-Keul's test for multiple comparison. A P value <0.05 was considered significant.

2.10. Materials

Cytosine arabinoside, glycine, interferon-γ (IFN-γ), lipoplysaccharide (LPS), naphthylethylenediamine, N^G-nitroarginine (N^G-NA), superoxide dismutase, sulfanilamide, D-2-amino-5-phosphovalerate (APV), 7-chlorokynurenate (7-Cl-kyn), dizocilpine (MK-801) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's media (DMEM) and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY, USA). 3-morpholinosydnonimine (SIN-1) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) were obtained from Research Biochemicals International (Natick, MA, USA).

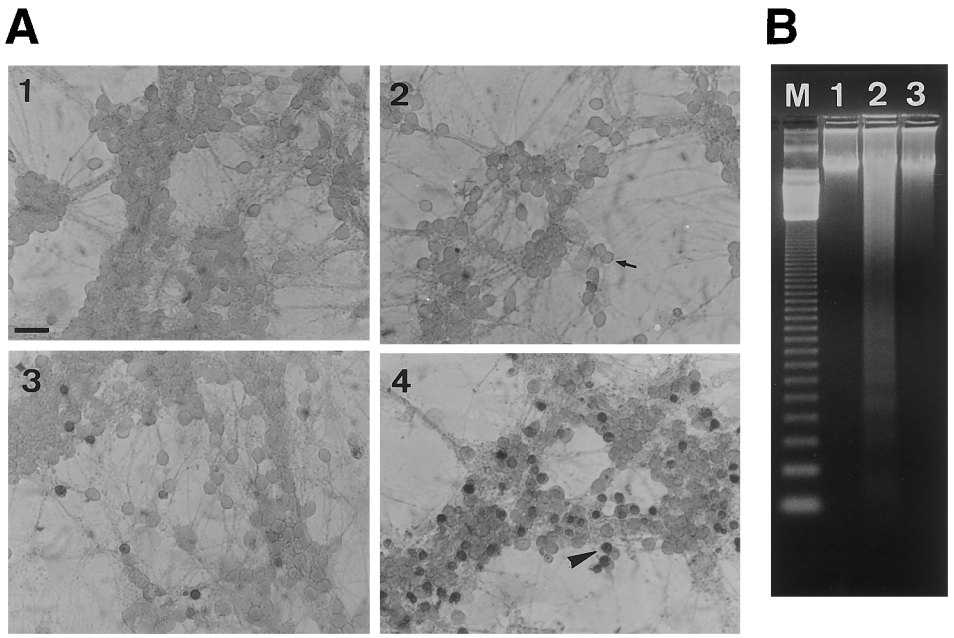

Fig. 1. Augmented apoptotic death of glucose-deprived CGC after immunostimulation. (A) In situ labelling of free 3′-hydroxyl ends of DNA. (A-1) Control. (A-2) CGC cultures were incubated for 5 h in the glucose-free DMEM. (A-3) Cells were immunostimulated with INF-γ and LPS for 48 h. (A-4) Immunostimulated glia were incubated for 1 h in the glucose-free DMEM. Glucose deprivation was terminated by repeated rinsing and incubation in glucose (5 mM)-containing DMEM; 18–22 h later apoptotic cell death in situ was measured by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL). Neurons and their processes were clearly visualised using anti-MAP-2 antibody following TUNEL. Arrow in A-2 and arrow head in A-4 representatively indicate a healthy cell and an apoptotic dead cell, respectively. Bar = 15 μm. N = 4. (B) In vitro DNA fragmentation. DNA extracts were prepared from CGC 18–24 h after glucose deprivation with or without immunostimulation. Lane 1; glucose-deprivation. Lane 2; glucose-deprivation/immunostimulation. Lane 3; immunostimulation. M: 123 bp marker. Photographs are representative of three separate experiments.

3. Results

Glucose deprivation for 2 h did not induce apoptotic death in CGC cultures (Fig. 1A-1, control; Fig. 1A-2, 5-h glucose deprivation). Immunostimulation with IFN-γ (100 U/ml) and LPS (1 μg/ml) for 48 h did not induce the apoptotic death of CGC (Fig. 1A-3). After immunostimulation, however, CGC became highly vulnerable to glucose deprivation. Thus, glucose deprivation for as short as 1 h significantly induced apoptotic death in immunostimulated CGC cultures (Fig. 1A-4). In contrast, 1-h glucose deprivation following immunostimulation produced no apoptotic death in pure glial cultures (data not shown).

Blockade of cell death by protein synthesis inhibitors have generally been interpreted as supporting involvement of apoptosis, since new macromolecular synthesis is required for several forms of apoptosis. In the present study, the protein synthesis inhibitor cycloheximide (100 nM) prevented $63 \pm 17.2\%$ the glucose deprivation-induced apoptotic death of immunostimulated CGC (Fig. 2; $P < 0.01$, $N = 4$). Cycloheximide also blocked $43 \pm 16.2\%$ the LDH release from glucose-deprived/immunostimulated CGC (Fig. 2; $P < 0.05$, $N = 4$).

Immunostimulated CGC cultures produced NO (measured as nitrite) in a time-dependent manner (Choi and Kim, 1998; Kim et al., in press). Immunostimulated CGC cultures continued to produce NO after removal of immunostimulants (Choi and Kim, 1998;

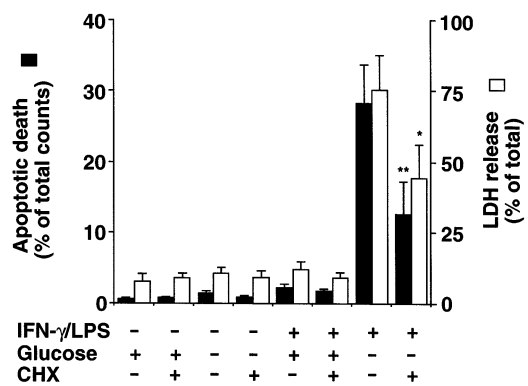

Fig. 2. Cycloheximide blocks NO-induced apoptotic death of glucose-deprived CGC. Immunostimulated CGC cultures were incubated for 1 h (for apoptosis) or 4 h (for LDH release) in the glucose-deprived DMEM. CGC cultures were treated with 100 nM cycloheximide (CHX) 16 h before and during the glucose deprivation. Apoptotic death and LDH levels were determined 14–18 h after termination of glucose deprivation. $N = 4$. *$P < 0.05$, **$P < 0.01$: compared with the apoptotic count or LDH release in glucose-deprived/immunostimulated groups.

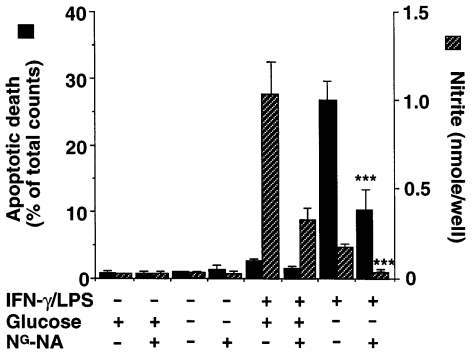

Fig. 3. NOS inhibitor blocks NO production and apoptotic death of glucose-deprived CGC enhanced after immunostimulation. CGC cultures were stimulated with IFN-γ and LPS. 1 mM N^G-nitroarginine (N^G-NA) was added 12 h before removal of immunostimulants from the cultures. NO production was measured after 1-h glucose deprivation and apoptotic cell death were measured 16–20 h after starting the glucose deprivation. $N = 4$. ***$P < 0.001$: compared with the apoptotic count or nitrite formation in glucose-deprived/immunostimulated groups.

Kim et al., in press). Immunostimulation with IFN-γ and LPS did not evoke NO production in pure CGC cultures (Choi and Kim, 1998; Kim et al., in press). Therefore, the NO production from immunostimulated CGC cultures in the present study might origin from the immunostimulated glial cells coexisting in CGC cultures. Glucose deprivation for 1 h decreased 84 (5.1% the nitrite formation of immunostimulated CGC cultures (Fig. 3). In order to block the production of NO, immunostimulated CGC cultures were incubated for 12 h with the nitric oxide synthase inhibitor N^G-nitroarginine (N^G-NA, 1 mM): N^G-NA blocked 79 (9.1% NO production from immunostimulated CGC cultures during 1-h glucose deprivation (Fig. 3). Cycloheximide also blocked 68 ± 7.2% NO production from immunostimulated CGC cultures (data not shown). Enhanced apoptotic death of glucose-deprived/immunostimulated CGC cultures was partially blocked 63 (8.9% by N^G-NA (Fig. 3). More selective iNOS inhibitors N^G-nitro-L-arginine-methyl ester (2 mM) and aminoguanideine (1 mM) also blocked the enhanced apoptotic death to the similar extents (data not shown). Despite large production of NO, however, immunostimulated CGC cultures did not undergo apoptotic death in the presence of glucose (Fig. 3)

We further studied whether the NO releasing reagent 3-morpholinosydnonimine (SIN-1) enhanced apoptotic death of glucose-deprived CGC. Neither 30-min glucose deprivation nor 30-min exposure to 200 μM SIN-1 alone induced the apoptotic death of CGC (Table 1). However, 30-min exposure to 200 μM SIN-1 synergistically enhanced the apoptotic death of glucose-deprived CGC, as evidenced by internucleosomal DNA fragmentation. Thus, the number of apoptotic dead (TUNEL-positive) cells increased after 30-min glucose deprivation in the presence of SIN-1(Table 1), whereas the amounts of lactate dehydrogenase (LDH) released into the medium from the CGC did not (Table 1). Other NO-releasing reagents, *S*-nitroso-*N*-acetylpenicillamine (SNAP) and *S*-nitrosoglutathione also showed qualitatively similar results: thus, 200 uM SNAP and 1 mM *S*-nitrosoglutathione increased 5–9-fold the apoptotic death of glucose-deprived CGC ($N = 3$ each, data not shown). The SIN-1-induced apoptotic death of glucose-deprived CGC was blocked by cycloheximide (data not shown). Higher concentrations of SIN-1 ((1 mM, 30 min) induced the necrotic death of CGC, as determined by cellular disintegration (data not shown). In contrast to the enhanced apoptosis in glucose-deprived neuronal cultures, 30-min exposure to 200 μM SIN-1 did not induce the apoptotic glial death in glucose-deprived pure glial cultures (data not shown). Longer (2 h) deprivation of glucose in the presence of 200 μM SIN-1 synergistically augmented the LDH re-

Table 1
NO synergistically enhances the apoptotic death of glucose-deprived CGC[a]

Treatments	Time (h)	Apoptotic counts (% of total)	LDH release (% of total)
Basal	0.5	2.3 ± 0.56	13.2
	2.0	2.0 ± 0.82	14.5 ± 3.27
SIN-1	0.5	3.4 ± 0.68	13.2 ± 2.07
	2.0	4.2 ± 2.15	16.2 ± 4.12
GD	0.5	3.5 ± 3.20	15.6 ± 5.13
	2.0	9.5 ± 3.04**	28.8 ± 4.45**
GD/SIN-1	0.5	26.8 ± 6.74	17.0 ± 3.28
	2.0	ND[b]	76.5 ± 6.99***

[a] Cells were deprived of glucose and/or treated with SIN-1 for 0.5 or 2 h. GD was tereminated by repeated rinsing and incubation in the clucose (5 mM)-containing DMEM. Apoptotic death and KDH levels were determined 14-18 h after termination of glucose deprivation. $N = 4$ for each.
[b] ND, not determind to be due to necrotic cell death.
** $P < 0.01$.
*** $P < 0.001$, compared with appropriate basal values as determined by ANOVA followed by the Student–Newman–Keuls test.

Table 2
NO-evoked accumulation of extracellular glutamate and rinse in intracellular calcium levels in glucose-deprived CGC[a]

Treatments	Time (h)	Apoptotic counts (% of total)	LDH release (% of total)
Basal	0.5	0.08 ± 0.01	680 ± 167
	2.0	0.13 ± 0.05	1212 ± 201
SIN-1	0.5	0.09 ± 0.03	728 ± 164
	2.0	0.14 ± 0.05	1496 ± 187
GD	0.5	0.08 ± 0.03	812 ± 112
	2.0	0.29 ± 0.08*	2567 ± 218***
GD/SIN-1	0.5	0.11 ± 0.04	831 ± 194
	2.0	[a]1.73 ± 0.18***	[a]709 ± 7930***

[a] Glutamate accumulation and $^{45}Ca^{2+}$ uptake were measured immediately before LDH assay (shown in Table 1). The relaease of glutamate from and the $^{45}Ca^{2+}$ uptake by the CGC deprived of glucose and treated with SIN-1 for 2 h (marked as 'a') was determined immediately before replacing the glucose deprived medium with glucose containing DMEM. $N = 4$ for glutamate measurements, and 5 for $45Ca^{2+}$ uptake.

* $P < 0.05$.

*** $P < 0.001$, compared with appropriate basal values as determined by ANOVA followed by the Student–Neuman-Keuls test.

lease from glucose-deprived CGC (Table 1) and induced their necrotic death, as assessed by morphological examination (characterised by cellular disintegration; data not shown).

In order to study whether NO enhanced apoptotic death of glucose-deprived CGC via releasing endogenous glutamate, we measured extracellular accumulation of glutamate and intracellular rises in $[Ca^{2+}]i$. The concentration of glutamate in the bathing medium did not significantly increase after 30-min exposure to SIN-1 and glucose deprivation (Table 2). Similarly, the simultaneous exposure to SIN-1 and glucose deprivation for 30 min did not induce neuronal $^{45}Ca^{2+}$ accumulation (Table 2). Further, the augmented apoptosis was not inhibited by the NMDA receptor antagonists D-2-amino-5-phosphovalerate (APV) and dizocilpine (MK-801) or the non-NMDA receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) at concentrations that they nearly completely block the activities of NMDA or non-NMDA receptors (Fig. 4). In contrast, significant glutamate accumulation in the bathing medium and neuronal $^{45}Ca^{2+}$ uptake were observed after 2-h exposure to SIN-1 and glucose deprivation, which evoked enhanced LDH release of CGC (Table 2).

4. Discussion

Necrosis and apoptosis are distinct mechanisms of cell death with different characteristics. In the same pathophysiological condition different neurons can die by different death mechanisms. Thus, cerebellar purkinje cells died by necrosis but not by apoptosis following a transient hypoxic-ischemic insult (Yue et al., 1997). In contrast, CGC underwent frequently apoptotic, but much less necrotic after the same hypoxic-ischemic insult (Yue et al., 1997). Cells, however, often coexist with either necrotic or apoptotic features at a given time. Further complication comes from the observation that secondary necrosis may take place in apoptotic model systems either in vitro and in vivo (Ankarcrona et al., 1995; Leist et al., 1995). In our model, apoptotic death of glucose-deprived CGC was observed with no necrotic features after short (0.5 h) treatment with SIN-1. Longer (2 h) treatment with SIN-1, however, released a large amount of LDH from glucose-deprived CGC cultures. The LDH release from glucose-deprived CGC treated longer with SIN-1 could

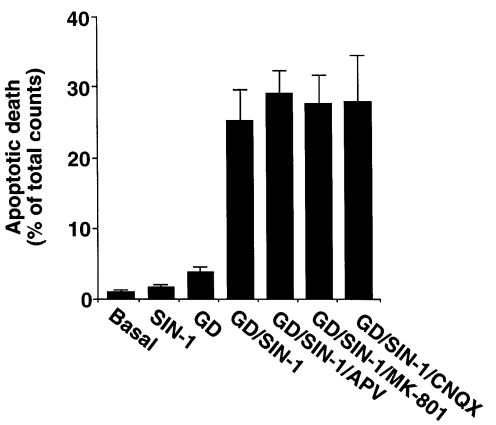

Fig. 4. Glutamate receptor antagonists do not block the augmented apoptotic neuronal death. Cells were incubated for 30 min (for TUNEL-staining) or for 2 h (for LDH assay) in glucose-free (GD)/SIN-1 (200 μM)-containing DMEM, thrice rinsed with and further incubated in glucose (5 mM)-containing DMEM. Glutamate receptor antagonists were treated during the glucose deprivation period. Apoptotic death was expressed as percentages of total cell counts. LDH levels in the bathing medium were determined 20–22 h after termination of glucose deprivation. NMDA receptor antagonists APV (200 μM) and MK-801 (10 μM) and non-NMDA receptor antagonist CNQX (100 μM) were added during and after the glucose deprivation until the apoptotic cell death was determined. $N = 4$.

be caused by necrotic death by increasing intensity of exposure and/or by secondary necrosis after early apoptosis.

Recent studies suggest that preconditioning of glial cells with immunostimulants aggravated neurotoxicity caused by over-activation of NMDA receptor (Hewett et al., 1994; Kim and Ko, 1998) or by hypoxia and/or glucose deprivation (Hewett et al., 1996; Kim et al., in press). However, a recent paper showed that intravenous injection of LPS induced tolerance against subsequent focal cerebral ischemic damage in spontaneously hypertensive rats (Tasaki et al., 1997). Although exact mechanisms responsible for the discrepancy remain to be determined, the effect of LPS on the cerebral vasculature and circulating cells may account for much of the induced tolerance. Different experimental conditions may also cause different results.

Three observations indicate that the synergistic enhancement of apoptotic death of glucose-deprived CGC was mediated by the NO produced from IFN-γ- and LPS-treated glial cultures coexisting in CGC cultures. First, a combined treatment of IFN-γ and LPS was needed for induction of glial NO production and the augmented death of glucose-deprived CGC. Second, the enhanced CGC death by immunostimulation was blocked by the NOS blocker N^G-NA. Third, the enhancing effect of immunostimulated glial cells was mimicked by exogenous application of the NO releasers SIN-1, SNAP and *S*-nitrosoglutathione. The protein synthesis inhibitor CHX also decreased the NO production in immunostimulated CGC cultures, which may result in a decrease of the apoptotic death.

NO has been closely associated with various neurodegenerative diseases including cerebral ischemia (Wood, 1995; Kosel et al., 1997). NO, mostly produced from activated glial cells in the central nervous system, may damage neuronal cells depending on its concentration. At fairly high concentrations, NO itself damages neuronal cells. At low concentrations produced by 200 μM SIN-1, however, NO itself did not exert cytotoxicity. Little cytotoxicity of NO was also reported by other investigators using cerebellar granule cells (Lafon-Cazal et al., 1993) and cortical neurons (Lipton et al., 1993). Many neurodegenerative processes are thought to be mediated by a combination of energy impairment, free radical damage and excitotoxic mechanisms. Thus, the cytotoxicity evoked by NO shown in the present study could be due to the synergistic interaction with other cytotoxic molecules. NO has been reported to have a synergistic interaction with oxygen free radicals (Lipton et al., 1993; Hewett et al., 1994). Glucose deprivation was shown to damage neurons by producing reactive oxygen species (Cheng and Mattson, 1995). Thus, NO might enter the neuronal cells and form a toxic peroxynitrite with the superoxide produced by glucose deprivation.

Glucose deprivation evoked arachidonic acid release from cultured striatal neurons (Williams et al., 1995) and damaged cultured hippocampal neurons via a release of glutamate (Geng et al., 1997). NO also induced neuronal degeneration by eliciting autocrine excitotoxicity (Bonfoco et al., 1996; Palluy and Rigaud, 1996; Leist et al., 1997). Also, the apoptotic neuronal death elicited by NO donors in CGC cultures required intracellular Ca^{2+} overload induced by activation of NMDA receptors (Bonfoco et al., 1996; Leist et al., 1997). Furthermore, NO increased glutamate receptor-mediated excitotoxicity on cortical neurons (Hewett et al., 1994 and 1996) and cerebellar granule neurons (Kim et al., in press). NO was further reported to increase the death of oxygen/glucose-deprived cortical neurons via activation of NMDA receptors by the glutamate released from the neurons (Hewett et al., 1996). Thus, it is plausible to think that simultaneous treatment of NO and glucose deprivation increased neuronal death simply via enhancing the glutamate-mediated excitotoxicity. However, the augmented apoptosis was not inhibited by various NMDA and non-NMDA receptor antagonists used in this study. Furthermore, significant glutamate accumulation in the bathing medium and neuronal $^{45}Ca^{2+}$ uptake were not observed after a short (30 min) exposure of glucose-deprived CGC to NO (released from 200 μM SIN-1). Thus, the present data indicate that NO can modulate the apoptotic cell death via a mechanism(s) independent of the excitotoxic pathway, at least in the present experimental model. In some non-neuronal cells apoptotic cell death was also reported to be induced in a calcium-independent manner (Shibasaki and McKeon, 1995; Waring et al., 1997).

In brain hypoxic-ischemia a continuous and marked increase in inducible nitric oxide synthase (iNOS) expression occurs in non-neuronal cells such as microglia (Giulian and Lachman, 1985; Nakashima et al., 1995), astrocytes (Giulian and Lachman, 1985; Endoh et al., 1994; Nakashima et al., 1995) and endothelial cells (Nakashima et al., 1995). While glutamate and aspartate have been considered as major excitotoxic mediators, NO most possibly produced by neighboring cytokine-activated glial cells can participate synergistically in the neuronal damage by excitotoxicity (Hewett et al., 1994 Hewett et al., 1996; Kim et al., in press) and energy depletion including glucose deprivation shown in the present study. Exact mechanisms responsible for the NO-evoked potentiation of glucose deprivation-induced neurotoxicity remain to be further determined. Regardless of the exact mechanisms, NO may act on neurons to enhance excitotoxicity-independent brain injuries in various pathophysiological conditions. Understanding the NO pathophysiology in the neurodegenerative diseases would advance the development of new neuroprotective agents.

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