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Nitric oxide-induced cytotoxicity attenuation by thiopentone sodium but not pentobarbitone sodium in primary brain cultures

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1 We describe the effects of barbiturates on the neurotoxicity induced by nitric oxide (NO) on foetal rat cultured cortical and hippocampal neurones. Cessation of cerebral blood flow leads to an initiation of a neurotoxic cascade including NO and peroxynitrite. Barbiturates are often used to protect neurones against cerebrovascular disorders clinically. However, its neuroprotective mechanism remains unclear.

2 In the present experiment, we established a new *in vitro* model of brain injury mediated by NO with an NO-donor, 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (NOC-5) on grid tissue culture wells. We also investigated the mechanisms of protection of CNS neurones from NO-induced neurotoxicity by thiopentone sodium, which contains a sulphhydryl group (SH-) in the medium, and pentobarbitone sodium, which does not contain SH-.

3 Primary cultures of cortical and hippocampal neurones (prepared from 16-day gestational rat foetuses) were used after 13–14 days in culture. The cells were exposed to NOC-5 at the various concentrations for 24 h in the culture to evaluate a dose-dependent effect of NOC-5.

4 To evaluate the role of the barbiturates, neurones were exposed to 4, 40 and 400 μM of thiopentone sodium or pentobarbitone sodium with or without 30 μM NOC-5. In addition, superoxide dismutase (SOD) at 1000 u ml^{-1} and 30 μM NOC-5 were co-administered for 24 h to evaluate the role of SOD.

5 Exposure to NOC-5 induced neural cell death in a dose-dependent manner in both cortical and hippocampal cultured neurones. Approximately 90% of the cultured neurones were killed by 100 μM NOC-5.

6 This NOC-5-induced neurotoxicity was significantly attenuated by high concentrations of thiopentone sodium (40 and 400 μM) as well as SOD, but not by pentobarbitone sodium. The survival rates of the cortical neurones and hippocampal neurones that were exposed to 30 μM NOC-5 were $11.2 \pm 4.2\%$ and $37.2 \pm 3.0\%$, respectively, and in the presence of 400 μM thiopentone sodium, the survival rate increased to $65.3 \pm 3.5\%$ in the cortical neurones and $74.6 \pm 2.2\%$ in the hippocampal neurones.

7 These findings demonstrate that thiopentone sodium, which acts as a free radical scavenger, protects the CNS neurones against NO-mediated cytotoxicity *in vitro*. In conclusion, thiopentone sodium is one of the best of the currently available pharmacological agents for protection of neurones against intraoperative cerebral ischaemia.

Keywords: Nitric oxide; nitric oxide-releasing compounds; NOC-5; thiopentone sodium; pentobarbitone sodium; primary cultured neurones

Introduction

Nitric oxide (NO), derived from a semiessential amino acid, L-arginine, is a small diffusible molecule. NO has been identified as a neurotransmitter involved in glutamate, acting on N-methyl-D-aspartate (NMDA) receptors (Snyder & Bredt, 1991; Moncada *et al*., 1991; Garthwaite, 1991; Dawson & Snyder, 1994). It has been also demonstrated that glutamate agonists stimulate NO synthase (NOS) activity (Kiedrowski *et al*., 1992) and increase guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels via NO-soluble guanylate cyclase-cyclic GMP pathway (Bredt & Snyder, 1989; Wood *et al*., 1990).

Acute traumatic or ischaemic injury to the brain triggers a cascade of biochemical events that cause a prolonged secondary injury to neurones surrounding the local injury site. It is considered that NO and free radicals are involved in one of the most important cascades under these conditions (Dawson *et al*., 1996). Following cerebrovascular infarction, NMDA receptors are stimulated by release of excitatory amino acids into the extracellular space. During focal ischaemia, increased NOS activity and marked increases in NO production occur in the brain (Malinski *et al*., 1993). NO rapidly reacts with superoxide anion to form a reactive oxidant species, peroxynitrite anion ($ONOO^-$) (Blough *et al*., 1985; Saran *et al*., 1990). This peroxynitrite anion is protonated to form peroxynitrous acid, an unstable species, and this acid spontaneously decomposes to form OH and NO (Beckman, 1990; Radi *et al*., 1991). NO, peroxynitrite and superoxide have all been considered to mediate cellular damage under conditions of shock, inflammation and oxidative stress (Halliwell & Gutteridge, 1990; Beckman & Crow, 1993). Therefore, preventing accumulation of superoxide anion, or decreasing production of NO, would attenuate brain injury and improve outcome after focal ischaemia. Consistent with this notion, a couple of experiments showed that superoxide dismutase (SOD) and NOS inhibitors were markedly effective at attenuating neural necrosis (Widdowson *et al*., 1996) and the infarct volume (Kinouchi *et al*., 1991; Dawson & Snyder, 1994).

Barbiturates have the ability to protect the central nervous system (CNS) (Hall, 1990). One of the mechanisms by which

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barbiturates exert a cerebral protective effect is decreasing cerebral metabolic rate (CMR), that is, reducing energy expenditure required for synaptic transmission, while maintaining the energy required for basic cellular functions (Steen & Michenfelder, 1980; Hall, 1990). Another possible mechanism that contributes to the protective effect of barbiturates during cerebral ischaemia is scavenging free radicals (Weiss *et al*., 1994). Although several studies have identified the efficacy of barbiturates as neuroprotective agents during cerebral ischaemia (Milde *et al*., 1988; Kass *et al*., 1992; Sano *et al*., 1993; Guo *et al*., 1995), the mechanism remains unclear.

In the present study, we attempted to establish a new model of neurotoxicity that is caused by NO using primary cultures of cerebral cortical and hippocampal neurones. We investigated the pharmacological property of NO using 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (NOC-5), as an ideal NO-donor. NOC-5 releases a large quantity of NO constantly through a simple mechanism without producing any metabolites (Hrabie *et al*., 1993). Its utility has been demonstrated in many fields (Shibuta *et al*., 1995; 1996; Zhang *et al*., 1996). We clarified the mechanisms of protection of CNS neurones from NO-induced neurotoxicity by two barbiturates, thiopentone sodium, which contains a sulphhydryl group (SH-) in the medium, and pentobarbitone sodium, which does not contain a sulphhydryl group, *in vitro* without the involvement of haemodynamics and other physiological elements.

Methods

Cell culture

The treatments of all animals were in strict accordance with institutional and NIH guidelines for care and treatment of laboratory animals. The study protocol was approved by the Animal Care Committee at Osaka University Medical School.

Rat foetuses at embryonic day 16 were removed surgically from anaesthetized pregnant Wistar rats, which were obtained from Nihon SLC (Hamamatsu, Japan). Their brains were isolated under a microscope. Cerebral cortical and hippocampal neurones were treated with 0.25% trypsin at 37°C for 20 min, and triturated with a pasteur pipette (Dawson *et al*., 1993). Dispersed cells were diluted to a concentration of 1×10^6 cells ml^{-1} in DMEM with 8% FCS and 4% HS, 50 μg ml^{-1} streptomycin and 50 iu ml^{-1} penicillin. This suspension was put onto poly-L-lysine-coated 35 mm diameter/2 mm grid tissue culture dishes (1.5 ml/well) (Nunc Inc., Naperville, IL). We used the grid tissue culture dishes to observe the same neurones of a certain area at any time.

After 4 days in culture the cells were treated with 5 μg ml^{-1} of 5-FU for 3 days to prevent proliferation of non-neuronal cells. The neurones were maintained in DMEM containing 8% FCS and 4% HS in 5% CO_2, 95% air and 100% humidity condition at 37°C. The medium was changed twice weekly. In this study, experiments were carried out after 13–14 days in culture.

Cytotoxicity

All experiments were performed at 37°C. To evaluate the cytotoxicity of the drugs, three to four microphotographs were made of each well shortly before the administration of drugs and at the end of the experiments. The cells, including control, were exposed to 0.4% trypan blue in phosphate buffer saline (PBS) to stain the dead cells. Microphotographs were taken at the same area as before the administration of drugs according to the grid arrangement of the dish. Stained and non-stained cells were counted by an additional observer blinded to the arrangement of photographs, study design, and treatment protocol, with approximately 500–1000 cells counted per well. Survival rates were calculated by following formula; (non-stained cells at the end of the experiment)/(whole cells shortly before the administration of the drugs). Cells were exposed to the drugs, which were added into the medium, as follows. (1) To evaluate time course of neurotoxicity induced by NOC-5, the NO-donor, we administered 100 μM NOC-5 into each well. According to manufacturer's instructions, the half-life of this NO donor in PBS at pH 7.4 and 37°C is 25 min. One mol of NOC-5 can release 2 mols of NO. The cytotoxicity of this drug was investigated at 1, 2, 3, 6, 12, 18 and 24 h after the administration of NOC-5. (2) To evaluate a dose-dependent effect of NOC-5, the cells were exposed to NOC-5 at concentrations of 1, 10, 20, 30, 100, 300 and 1000 μM for 24 h in the culture. As control, we also observed neurones that were not exposed to NOC-5 for 24 h. (3) SOD at 1000 u ml^{-1} and 30 μM NOC-5 were co-administered into each well to evaluate the role of SOD. Observations were performed 24 h after the administration of the drugs. (4) To evaluate the role of the barbiturates, neurones were exposed to 4, 40 and 400 μM, thiopentone sodium or pentobarbitone sodium with or without 30 μM NOC-5. The effects of these drugs were observed 24 h after exposure to the drugs.

Chemical reagents

Chemicals used in this study were obtained from the following sources: copper/zinc superoxide dismutase (SOD) from Cellular Products Inc. (Buffalo, NY); Dulbecco's modified Eagle's medium (DMEM) from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan); NOC-5 from Dojindo (Kumamoto, Japan); 5-fluoro-2′-deoxyuridine (5-FU), poly-L-lysine, thiopentone sodium and pentobarbitone sodium were obtained from Sigma Co. (St. Louis, MO); trypsin from DIFCO Lab., (Detroit, MI); foetal calf serum (FCS) from ICN Biochemicals, Inc., (Costa Mesa, CA); horse serum (HS) from Gibco BRL (Grand Island, NY).

Statistical analysis

Results are expressed as the mean$\pm$s.e.mean. Student's unpaired t test was used to determine the statistical significance of differences between the means, and a P value of <0.05 was considered significant.

Results

The neurotoxic effect of NOC-5 in primary brain cultures

To establish a new model of NO-mediated neurotoxicity using NOC-5, we examined the time course and concentration-response relationships of neural cell death for the NO-donor in both cortical and hippocampal primary cultured neurones. Figure 1 shows the time course of the survival rate of the cultured neurones exposed to NOC-5. In the presence of 100 μM NOC-5, approximately 30% of both cultured neurones at 1 h were already stained with trypan blue. The survival rates of both of the neurones at 24 h after drug administration did not significantly differ from those at 18 h after drug administration. Mean survival rates at 18 h after the drug exposure were 9.3$\pm$4.1% in the cortical neurones and

15.2±5.3% in the hippocampal neurones, whereas, mean survival rates at 24 h after the drug exposure were 9.4±2.6% in the cortical neurones and 13.5±3.3% in the hippocampal neurones. Figure 2 demonstrates decreased survival rates at 24 h after NOC-5 exposure in a dose-dependent manner in both cell groups. Only about 5% of the neurones that were not exposed to NOC-5 were dead within 24 h. In cortical neurones, 11.2±4.2% of cells were surviving with 30 μM NOC-5. On the other hand, 37.2±3.0% cells were viable with the same concentration of NOC-5 in hippocampal neurones. More than 95% cells were killed by exposure to 300 μM NOC-5 in both cortical and hippocampal cultured neurones. Figure 3b and d show the microphotographs of cultured neurones stained with trypan blue that had been exposed to 30 μM NOC-5 for 24 h. In these pictures, similar localization patterns of the neurones were observed compared to Figure 3a and c, before the administration of NOC-5. While the morphology of neurites and cell bodies of viable cells did not appear to change after the exposure to NOC-5, neurites of dead cells were not detected.

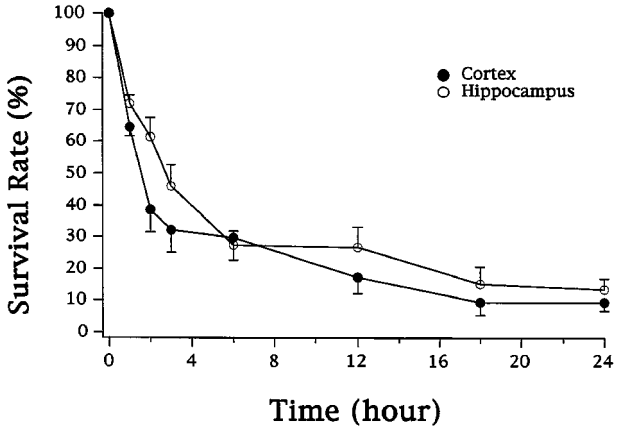

Figure 1 Time course of 100 μM NOC-5-induced neurotoxicity in primary cultured neurones. The survival rates of both cortical and hippocampal neurones at 1 h after the administration of NOC-5 decreased significantly. Each data point represents the mean of at least two separate experiments in which at least seven wells per experiment; 500–1000 neurones were counted per well. Vertical lines show s.e.mean.

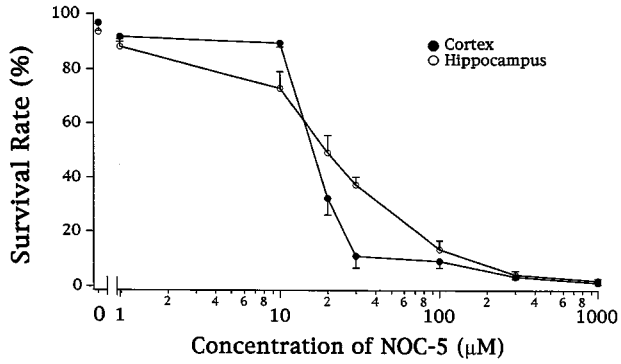

Figure 2 Dose-dependence of NOC-5-induced neurotoxicity in primary cultured neurones. Survival rates of the cells were calculated as (viable cells at 24 h after the administration of NOC-5)/(whole cells shortly before the administration of the drug). All data points represent the mean of at least two separate experiments in which at least seven wells per experiment; 500–1000 neurones were counted per well. Vertical lines show s.e.mean.

The role of superoxide dismutase

To evaluate the role of superoxide anion in NOC-5, we investigated the effects of SOD, an enzyme able to scavenge the superoxide anion. Although no significant neuroprotective effect was observed with 100 u ml^{-1} SOD (data not shown), 1000 u ml^{-1} SOD significantly attenuated 30 μM NOC-5-induced neurotoxicity in both cortical and hippocampal cultured neurones; the mean survival rate of the cortical neurones was increased to 65.1±9.5%, whereas that of the hippocampal neurones was increased to 67.2±7.4% (Table 1).

The effects of barbiturates on NO-mediated neurotoxicity

Figure 5 shows that thiopentone sodium attenuated the neurotoxicity elicited by 30 μM NOC-5. While the survival rates of cortical neurones and hippocampal neurones exposed to 30 μM NOC-5 were 11.2±4.2% and 37.2±3.0%, respectively, after treatment with 400 μM thiopentone sodium, the survival rate increased to 65.3±3.5% in the cortical neurones and 74.6±2.2% in the hippocampal neurones. Figure 4b and d shows the microphotographs of rescued cultured cortical neurones in the combined presence of thiopentone sodium

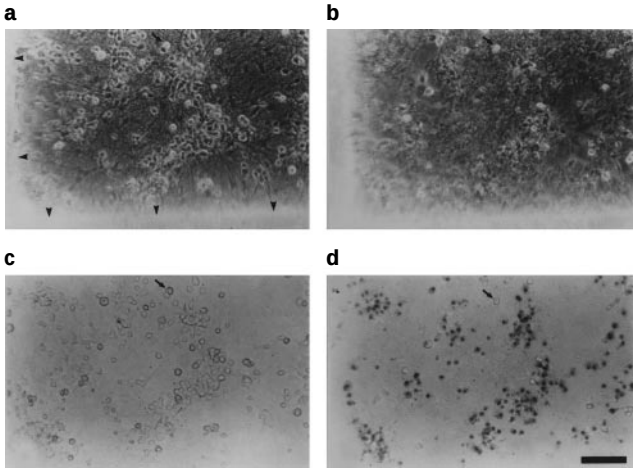

Figure 3 Neurones were killed by NOC-5. Microphotographs of cortical neurones in cultures shortly before the administration of NOC-5 with phase microscopy (a) and with transmitted light (c). Twenty-four hours after the administration of 30 μM NOC-5 to the same area, dead neurones had disappeared or stained with trypan blue in these photographs with phase microscopy (b) and with transmitted light (d). An arrow indicates the same neurone in all pictures. The neurone was viable 24 h after the administration of NOC-5. Arrowheads indicate the edges of the grid. Scale bar = 100 μm.

Table 1 Superoxide dismutase (SOD) attenuated 30 μM NOC-5-induced neurotoxicity

	Survival rate (%)
Cortex	
NOC-5 30 μM	11.2±4.2
+SOD 1000 u ml^{-1}	65.1±9.5***
Hippocampus	
NOC-5 30 μM	37.2±3.0
+SOD 1000 u ml^{-1}	67.2±7.4***

Data are means±s.e.mean. ***$P<0.005$ *vs* administration of 30 μM NOC-5 alone.

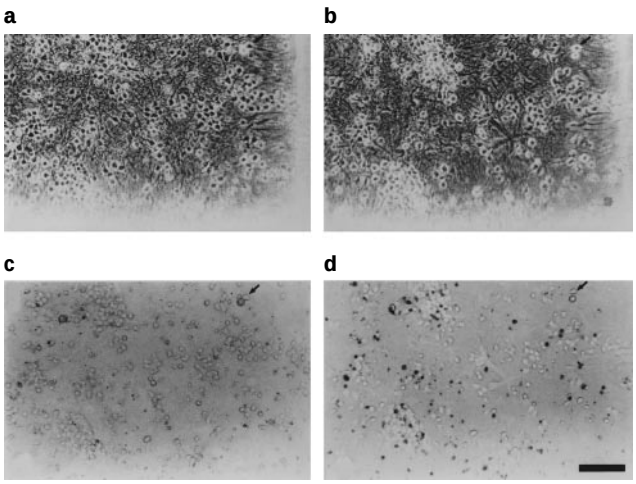

Figure 4 Thiopentone sodium (400 μM) protected neurones in cortical cultures from 30 μM NOC-5-induced neurotoxicity. Microphotographs of neurones in cortical cultures shortly before the administration of the drugs with phase microscopy (a) and with transmitted light (c). Twenty four hours after administration of the drugs to the same area, dead neurones had disappeared or stained with trypan blue in the micrographs with phase microscopy (b) and with transmitted light (d). An arrow indicates the same neurone which was still surviving 24 after the administration of the drugs. Scale bar = 100 μm.

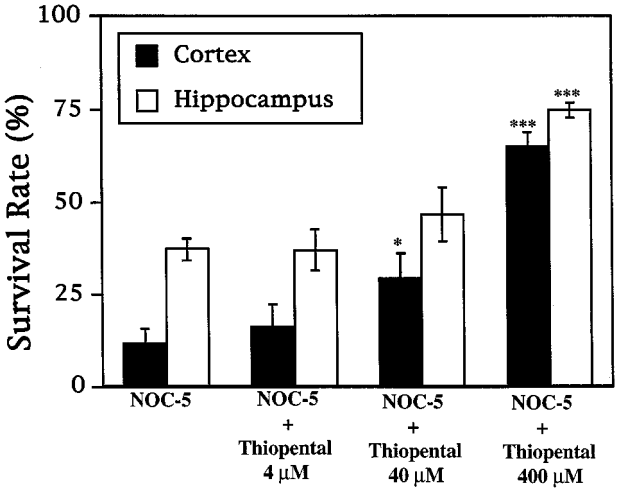

Figure 5 Attenuation of 30 μM NOC-5-induced neurotoxicity by different concentrations of thiopentone sodium (Thiopental). Columns represent the mean ± s.e.mean of at least two separate experiments with at least seven wells per experiment; 500–1000 neurones; were counted per well. $^{*}P<0.05$, $^{***}P<0.005$ *vs* administration of 30 μM NOC-5 alone.

and NOC-5. In cultured cortical neurones, 40 μM thiopentone sodium also exhibited a significant neuroprotective effect (mean survival rate of the neurones; 28.9 ± 7.1%), on the other hand, no statistically significant reduction in cell death was observed in hippocampal cultured neurones (mean survival rate of the neurones 46.6 ± 7.6%). The lowest dose (4 μM) of thiopentone sodium used did not show a significant neuroprotective effect in either cortical or hippocampal cultured neurones. In contrast to thiopentone sodium, no neuroprotective effect was observed with pentobarbitone sodium at any dose in either cortical or hippocampal neurones, as shown in Figure 6. Neither thiopentone sodium nor

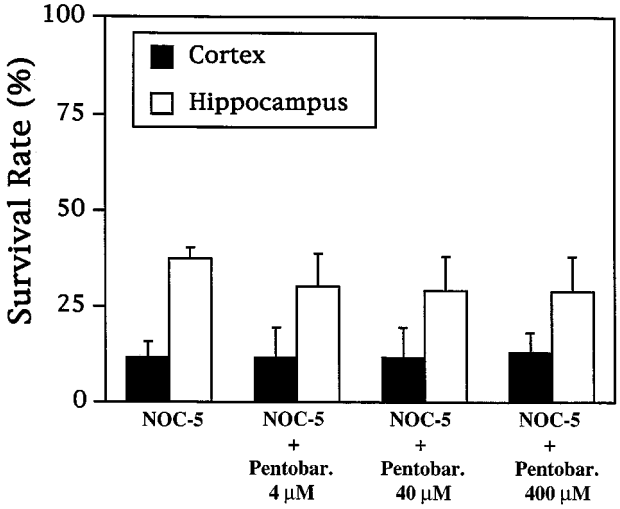

Figure 6 Pentobarbitone sodium did not influence 30 μM NOC-5-induced neurotoxicity at any dose. Columns represent the mean ± s.e.mean of at least two separate experiments with at least seven wells per experiment; 500–1000 neurones were counted per well.

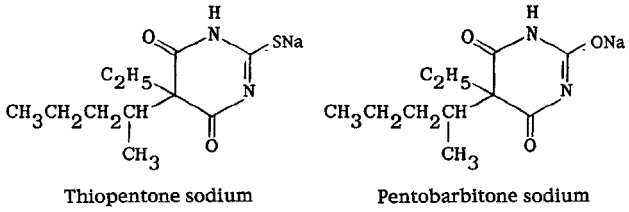

Figure 7 Chemical structures of thiopentone sodium and pentobarbitone sodium.

pentobarbitone sodium (400 μM, respectively) exhibited neurotoxicity alone in either cortical or hippocampal neurones (data not shown).

Discussion

In the present study, we established a new experimental model of NO-induced neurotoxicity in primary cultured neurones using grid tissue culture dishes and NOC-5. Using this model, we obtained the following findings. (1) NOC-5 elicited neural cell death in both cortical and hippocampal primary cultured neurones in a dose-dependent manner. (2) This neurotoxicity was attenuated significantly by SOD. (3) Thiopentone sodium, but not pentobarbitone sodium, protected both cortical and hippocampal primary cultured neurones from NOC-5-induced neurotoxicity in a dose-dependent fashion.

Establishment of a new model of NO-mediated neurotoxicity in vitro

NMDA and sodium nitroprusside (SNP) were used as NO-donors to examine the neurotoxic effects of NO (Dawson *et al.*, 1993; Globus *et al.*, 1995). NMDA needs NOS to form NO. SNP spontaneously releases NO, an effect that appears to be independent of the pH (Feelisch & Noack, 1987), but the mechanism of NO release from SNP remains obscure. Aqueous solution of NO is temperature- and light-sensitive (Bates *et al.*, 1991), and during decomposition, cyanide is released (Leeuwenkamp *et al.*, 1984). Moreover, SNP itself is likely to show neurotoxicity (Izumi *et al.*, 1993) and additional

effects on other regulatory systems unrelated to the generation of NO are suspected. Therefore, it has not been regarded as an ideal NO-donor (Feelisch, 1991). S-nitrosothiols, such as S-nitro-N-acetylpenicillamine (SNAP), also release NO spontaneously (Kowaluk *et al*., 1987). However, it is possible that S-nitrosothiols are catatlytically degraded at the plasma membrane (Kowaluk & Fung, 1990). Compared with these NO-donors, NOC-5 releases a large quantity of NO spontaneously, and its NO-releasing mechanisms is very simple (Hrabie *et al*., 1993). When the conditioned medium, withdrawn at 24 h after 1000 μM of NOC-5 had been administered into the culture well, was re-added into another culture, survival rate of the neurones did not decrease significantly compared to the negative control (data not shown). Hence, the effects of other metabolites on neurotoxicity need not be considered (Mitchell *et al*., 1993; Wink *et al*., 1993; Gelperin, 1994; Shibuta *et al*., 1995). Consequently, NOC-5 would be a powerful tool to examine directly the neurotoxic effect of NO *in vivo* and *in vitro*.

Using the grid tissue culture dishes, we were able to count the number of the cells that disappeared after drug exposure, as well as the cells that were stained with trypan blue, as shown in Figure 3. In our experimental model, non-viable cells are defined as not only the cells stained with trypan blue but the cells disappeared from the culture dish. Therefore, we estimated that had the survival rate by counting viable cells, that is unstained with trypan blue, on the same areas before and after exposure to the drugs. These measurements enable us to calculate survival rates of the cells more accurately and easily. Therefore, our present model is considered to be very useful for studying NO-elicited neurotoxicity in primary cultured neurones.

The effect of SOD on NO-induced neural death

SOD was an effective enzyme molecule for protecting neurones against NO-elicited neurotoxicity in the present study, as is shown previously (Dawson *et al*., 1993; Heales *et al*., 1994; Burkart *et al*., 1995; Salgo *et al*., 1995; Szabo *et al*., 1996). Under pathophysiological conditions, such as ischaemic damage, excessive glutamate is released, NOS is overactivated and a large amount of NO is produced. NO rapidly reacts with superoxide anion to form peroxynitrite anion ($ONOO^-$). $ONOO^-$ shows a strong oxidizing property and triggers several biochemical events, such as membrane lipid peroxidation and reaction with methionine (Radi *et al*., 1991). Moreover, both NO and $ONOO^-$ damage DNA and mitochondrial functions (Szabo *et al*., 1996; Dawson & Dawson, 1996). Thus, preventing accumulation of superoxide anion, or decreasing production of NO is very important to protect CNS during cerebral ischaemia.

The observation that SOD protected cells against NOC-5-induced neurotoxicity accords with recent findings demonstrating that oxygen radical neutralization by vitamin E protects islet cells against SNAP (Burkart *et al*., 1995), that a vitamin E analogue, trolox, protects cells against NO- or peroxynitrite-mediated injury (Heales *et al*., 1994; Salgo *et al*., 1995) and that Mn(III)tetrakis (4-benzoic acid) porphyrin inhibits the oxidation of dihydrorhodamine-123 by peroxynitrite (Szabo *et al*., 1996). We needed 1000 u ml^{-1} SOD to attenuate NOC-5-induced neurotoxicity in the present study, whereas Dawson *et al*. (1993) showed that 100 u ml^{-1} SOD was enough to exhibit a neuroprotective effect against 300 μM SNP. These results suggest that NOC-5 released a larger amount of NO compared to SNP.

Do barbiturates protect neurones against NO-induced neurotoxicity?

A possible mechanism that may contribute to the cerebral protective effect of barbiturates during CNS ischaemia is free radicals scavenging capacity. Weiss *et al*. (1994) showed that free radical scavenging property of the barbiturates caused a depression of neutrophil chemiluminescence. They also found that thiopentone had the strongest free radical scavenging capacity of all the barbiturates (Smith *et al*., 1980; Weiss *et al*., 1994). From chemical structures, as shown in Figure 7, only thiopentone sodium but not pentobarbitone sodium contains a sulphhydryl group and therefore can act as a free radical scavenger in the medium (Flaherty & Weisfeldt, 1988). These data are consistent with our findings that thiopentone sodium but not pentobarbitone sodium protected primary cultured neurones from NO-induced neurotoxicity.

When NOC-5 was used as an NO-donor, the protection of the neurones was not complete even with the combined application of SOD or thiopentone sodium, suggesting a contribution of other pathways to the neurotoxic effect of NO. The primary neuroprotective mechanism of the barbiturates *in vivo* is thought to be a reduction in the cerebral metabolic rate (CMR) and hence, an improved ratio of oxygen supply to oxygen demand. Associated with the administration of this class of agents, the reduction in CMR is directly attributable to effects on synaptic neurotransmission, while maintaining the energy required for basic cellular functions. A redistribution of cerebral blood flow to ischaemic tissues and a reduction in intracranial pressure may contribute secondary to the metabolic effect (Hall, 1990; Warner *et al*., 1996).

Recently, several anaesthetics have become available that share properties similar to barbiturates with respect to both EEG and CMR. Despite this, these compounds, for instance, halothane and isoflurane, failed to provide cerebral protection (Nehls *et al*., 1987; Warner *et al*., 1991). In conclusion, thiopentone sodium which contains a sulphhydryl group, works as a free radical scavenger effectively attenuating NOC-5 induced cytotoxicity in cultured cortical and hippocampal neurones. We consider that thiopentone sodium remains perhaps one of the best of the currently available pharmacological agents for the protection of neurones against intraoperative cerebral ischaemia.

Abbreviations

CMR, cerebral metabolic rate; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's minimum essential medium; FCS, foetal calf serum; 5-FU, 5-fluoro-2′-deoxyuridine; HS, horse serum; NMDA, N-methyl-D-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; $ONOO^-$, peroxynitrite anion; PBS, phosphate buffered saline; SNAP, S-nitro-N-acetylpenicillamine; SNP, sodium nitroprusside; SOD, superoxide dismutase

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