

Effects of Dantrolene on Extracellular Glutamate Concentration and Neuronal Death in the Rat Hippocampal CA1 Region Subjected to Transient Ischemia

Ryosuke Nakayama, M.D.,* Toshiyuki Yano, M.D.,* Kazuo Ushijima, M.D.,† Eiji Abe, M.D.,‡ Hidenori Terasaki, M.D.§

Background: Excessive extracellular glutamate produced by cerebral ischemia has been proposed to initiate the cascade toward neuronal cell death. Changes in extracellular glutamate concentration are closely linked to changes in intracellular calcium ion concentration. Dantrolene inhibits calcium release from intracellular calcium stores. In this study, the authors investigated the effects of dantrolene on extracellular glutamate accumulation and neuronal degeneration in a rat model of transient global forebrain ischemia.

Methods: Male Wistar rats weighing 230–290 g were anesthetized with halothane in nitrous oxide–oxygen and were subjected to 10 min of transient forebrain ischemia using a four-vessel occlusion technique. Fifteen minutes before ischemic injury, dantrolene sodium (5 mM), dimethyl sulfoxide as a vehicle for dantrolene, or artificial cerebrospinal fluid as a control was intracerebroventricularly administered ($n = 8$ in each group). In the hippocampal CA1 subfield, the extracellular glutamate concentration *in vivo* was measured during the peri-ischemic period with a microdialysis biosensor, and the number of intact neurons was evaluated on day 7 after reperfusion.

Results: Both dantrolene and dimethyl sulfoxide significantly reduced the ischemia-induced increase in glutamate concentration to a similar extent, *i.e.*, by 53 and 51%, respectively, compared with artificial cerebrospinal fluid ($P < 0.01$). The number of intact hippocampal CA1 neurons (mean \pm SD; cells/mm) in dantrolene-treated rats (78 ± 21) was significantly higher than that in artificial cerebrospinal fluid– (35 ± 14 ; $P < 0.001$) and dimethyl sulfoxide–treated (56 ± 11 ; $P < 0.05$) animals. Dimethyl sulfoxide also significantly increased the number of preserved neurons in comparison with artificial cerebrospinal fluid ($P < 0.05$).

Conclusions: Intracerebroventricular dantrolene prevents delayed neuronal loss in the rat hippocampal CA1 region subjected to transient ischemia; however, this neuroprotection cannot be accounted for only by the reduced concentrations of extracellular glutamate during ischemia.

ISCHEMIA has been proposed to cause an excess increase in the extracellular concentration of glutamate, an excitotoxic amino acid, in the central nervous system.^{1–3} The increased glutamate in turn triggers a surplus influx of calcium ion (Ca^{2+}) from the extracellular space into the cytosol, resulting in the initiation of a neuronal cell death cascade.^{4,5} The extracellular glutamate concentra-

tion is tightly regulated by release from presynaptic membranes and uptake by postsynaptic membranes and glia.^{6–9} This regulation is closely linked to alterations in intracellular free calcium concentration; namely, an increase in intracellular Ca^{2+} may enhance glutamate release from glutamatergic neurons and astrocytes.^{10–12} Therefore, controlling the extracellular glutamate and intracellular Ca^{2+} concentrations could be a promising strategy for alleviating ischemic neuronal damage.

The endoplasmic reticulum (ER) is a putative primary intracellular storage site of dischargeable Ca^{2+} in neurons.¹³ Dantrolene, which has been clinically used to treat malignant hyperthermia, inhibits Ca^{2+} release from the ER through ryanodine receptors (RyR) and inositol 1,4,5-triphosphate receptor channels into cytosol.^{14,15} Moreover, dantrolene has been reported to stabilize the plasma membrane.¹⁶ An electron microscopic study showed that RyRs are also present in neuronal axons, dendritic spines, and dendritic shafts in the rat brain.¹⁷ The effects of dantrolene on extracellular glutamate concentrations and neuronal survival in cerebral ischemia remain to be elucidated. This study was conducted to test the hypothesis that dantrolene administered intracerebroventricularly before ischemia attenuates the extracellular glutamate accumulation and consequently ameliorates the neuronal injury in the rat hippocampal CA1 subfield, one of the most vulnerable regions to ischemia, after transient forebrain ischemia and reperfusion.

Materials and Methods

Subjects, Preparation, and Ischemia Model

All animal care procedures in this study were performed according to the Guidelines for Animal Experiments of the Kumamoto University School of Medicine (Kumamoto, Japan). The Animal Care and Use Committee of our institute approved the protocol. Adult male Wistar rats weighing 230–290 g, individually housed with free access to food and water, were used. They were anesthetized with 4% halothane in a 1:2 gas mixture of oxygen and nitrous oxide, intubated endotracheally with an 18-gauge intravenous Teflon (Dupont, Wilmington, DE) catheter connected to a rodent ventilator (7025; Ugo Basile, Comerio, Italy), and then mechanically ventilated (tidal volume, 10 ml/kg; respiratory rate, 60 breaths/min). The halothane concentration was then lowered to 1%, which was maintained throughout the surgery and cerebral ischemia. The pericranial temperature was monitored with an implantable thermocouple

* Staff Anesthesiologist, † Associate Professor and Vice Director, Surgical Center, ‡ Graduate Student, § Professor and Chair, Department of Anesthesiology.

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Address reprint requests to Dr. Ushijima: Surgical Center, Kumamoto University School of Medicine, 1-1-1 Honjo, Kumamoto 860-8556, Japan. Address electronic mail to: kazuo@kaiju.medic.kumamoto-u.ac.jp. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

microprobe (IT-14; Physitemp Instruments Inc., Clifton, NJ) and maintained at $37.0 \pm 0.2^\circ\text{C}$ during surgery, brain ischemia, and recovery from anesthesia using a temperature controller (TCAT-1A; Physitemp Instruments Inc.) and a radiant heating lamp. The femoral artery was cannulated for monitoring of arterial pressure and analysis of arterial blood gases, hematocrit, and glucose. A bilateral electroencephalogram of the temporal areas was continuously recorded with needle electrodes.

Global forebrain ischemia was induced by the four-vessel occlusion method previously described in detail.¹⁸ Briefly, bilateral vertebral arteries were electrocauterized at the alar foramina of the first cervical vertebra through a blunt needle with a diameter of 0.47 mm during halothane anesthesia. Twenty-four hours after the electrocauterization of vertebral arteries, each common carotid artery was tied off with a 3-0 silk suture by applying a weight of 15 g for 10 min. The electroencephalogram became isoelectric within 15 s after carotid occlusion in all animals. Thereafter, cutting the carotid sutures started reperfusion.

Microdialysis Biosensor

We used a microdialysis biosensor (General 20-10-2-2; Sycopel International Ltd., Boldon, Tyne and Wear, United Kingdom), 230 μm in OD, to measure extracellular glutamate concentration *in vivo*.¹⁹⁻²² The biosensor consists of a hollow tube with a semipermeable membrane, in which a platinum working electrode, a silver-silver chloride counter electrode, and a silver reference electrode are assembled. L-Glutamate is oxidized by L-glutamate oxidase (EC 1.4.3.11) and produces hydrogen peroxide. Extracellular glutamate passes through the semipermeable membrane into the biosensor and reacts with the glutamate oxidase contained in the biosensor. Therefore, extracellular glutamate concentration can be theoretically measured by detecting the current from hydrogen peroxide. The current evoked from hydrogen peroxide was amperometrically detected on the platinum electrode at +650 mV by an electrochemical detector (EPS-800; Eicom, Kyoto, Japan) and recorded on a polygraph in real time.

At first, the microdialysis biosensor was filled and perfused with phosphate buffered saline (PBS) at pH 7.4 (146 mM NaCl, 2.7 mM KCl, 4.3 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.4 mM KH_2PO_4 , 2.4 mM CaCl_2) by a perfusion pump (EP60; Eicom). To avoid interference from electroactive molecules, such as ascorbate, *O*-phenylenediamine was electropolymerized onto the platinum electrode in PBS perfused with 100% nitrogen. After stabilization of the current at +650 mV, the biosensor was perfused with 0.05 U/ μl L-glutamate oxidase (Yamasa Co. Ltd., Chiba, Japan) dissolved in PBS at a rate of 0.2 $\mu\text{l}/\text{min}$. The *in vitro* calibration, performed by placing the biosensor tips in PBS containing increasing concentrations of L-glutamate, showed that the current increased linearly

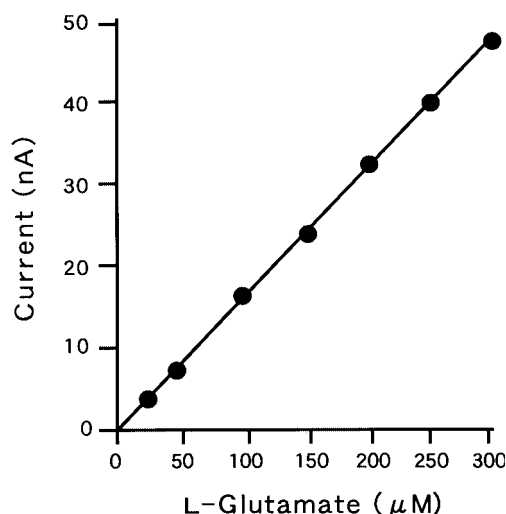


Fig. 1. An *in vitro* calibration curve of the microdialysis biosensor for increasing concentrations of L-glutamate. The linear regression line shows that the biosensor sensitivity is 0.16 nA/ μM .

with L-glutamate concentrations up to 300 μM . The sensitivity and response time of the biosensor were 0.16 nA/ μM and within 20 s for a 90% steady state response, respectively (fig. 1). Seventy-five minutes before the initiation of ischemia, the biosensor was stereotaxically inserted into the left hippocampal CA1 area through the burr hole at 2.1 mm lateral and 4.3 mm anterior to the bregma and was then perfused with PBS containing glutamate oxidase (0.05 U/ μl) at a rate of 0.2 $\mu\text{l}/\text{min}$ to start the real-time measurement of extracellular glutamate concentration. The area under the curve, defined as integrated increments of glutamate concentration from the preischemic baseline value between the beginning and 20 min after the end of ischemia, was calculated from the actual trace with the use of National Institutes of Health Image version 1.61 software (National Institutes of Health, Bethesda, MD).

Experimental Design

Rats were randomly assigned to one of three groups ($n = 8$ in each group) to be treated with one of the following: (1) artificial cerebrospinal fluid (aCSF; 126.5 mM NaCl, 2.4 mM KCl, 0.5 mM KH_2PO_4 , 1.1 mM CaCl_2 , 1.1 mM MgCl_2 , 0.5 mM Na_2SO_4); (2) 50% dimethyl sulfoxide (DMSO) in distilled water; or (3) 5 mM dantrolene sodium dissolved in 50% DMSO. Fifteen minutes before ischemic injury, these agents, 20 μl in volume and warmed to 37°C , were stereotaxically administered for 1 min into the bilateral cerebral ventricles (10 μl into each ventricle) through burr holes at 1.5 mm lateral and 0.8 mm posterior to the bregma. The glutamate analysis and anesthesia were stopped, and the injection cannulae and dialysis probes were removed 20 min after the initiation of reperfusion. After full awakening from anesthesia, the tracheas of the animals were extubated.

Table 1. Physiologic Data

	aCSF (n = 8)	DMSO (n = 8)	Dantrolene-DMSO (n = 8)
Body weight (g)	254 ± 21	254 ± 21	254 ± 21
Hematocrit (%)	39 ± 4	40 ± 2	41 ± 2
Blood glucose (mg/dl)	122 ± 14	129 ± 31	129 ± 25
Mean arterial pressure (mmHg)			
Baseline	74 ± 9	82 ± 11	79 ± 4
5 min before reperfusion	108 ± 18*	119 ± 8*	113 ± 7*
30 min after reperfusion	87 ± 16	92 ± 7	88 ± 6
Arterial pH			
Baseline	7.43 ± 0.04	7.42 ± 0.04	7.43 ± 0.03
15 min after reperfusion	7.43 ± 0.04	7.41 ± 0.08	7.44 ± 0.03
Paco ₂ (mmHg)			
Baseline	37 ± 3	39 ± 4	39 ± 2
15 min after reperfusion	37 ± 9	41 ± 9	35 ± 4
Pao ₂ (mmHg)			
Baseline	133 ± 17	149 ± 13	133 ± 12
15 min after reperfusion	153 ± 15	148 ± 23	144 ± 17
Pericranial temperature (°C)			
Baseline	37.0 ± 0.1	37.0 ± 0.1	37.0 ± 0.1
5 min before reperfusion	36.9 ± 0.1	37.0 ± 0.1	36.9 ± 0.1
30 min after reperfusion	37.0 ± 0.1	37.0 ± 0.1	37.0 ± 0.1

Values are expressed as mean ± SD.

* $P < 0.05$ versus baseline and 30 min after reperfusion.

aCSF = artificial cerebrospinal fluid; DMSO = dimethyl sulfoxide; Paco₂ = arterial carbon dioxide tension; Pao₂ = arterial oxygen tension.

Histology

The animals that survived for 7 days after reperfusion were perfused transcardially with 150 ml heparinized normal saline followed by 300 ml neutral buffered formalin, 10%, during pentobarbital anesthesia. The brains were removed and immersed in 10% neutral buffered formalin for 3 days and were then processed and embedded in paraffin. Coronal sections, 5 μ m in thickness, were taken from the dorsal hippocampus and stained with cresyl violet acetate. Pyramidal cells with a distinct nucleus and nucleolus were regarded as intact neurons. The number of intact neurons in a 1-mm length of the middle portion of the right hippocampal CA1 subfield was counted using a microscope at $\times 400$ magnification by an inspector blinded to intervention. The location of the microdialysis probe and stainless steel tube through which the agents were injected was ascertained in each 5- μ m-thick coronal section stained with hematoxylin and eosin.

Statistical Analysis

Values are presented as mean ± SD. Physiologic data and glutamate concentrations were assessed by one-way analysis of variance, followed by Tukey-Kramer honestly significant difference, to determine which pairs of means differed among the groups. Intact cell counts were analyzed using a Kruskal-Wallis test. A chi-square test was applied to identify differences in the survival rates. Significance was defined as $P < 0.05$.

Results

Table 1 presents the physiologic data showing no significant differences among the aCSF-, DMSO-, and

dantrolene-DMSO-treated groups. The mean arterial pressure 5 min before reperfusion was significantly higher than that at baseline and 30 min after reperfusion in all groups ($P < 0.05$). The intracerebroventricular administration of the agents tested *per se* did not influence the electroencephalogram.

Figure 2A shows the changes in extracellular glutamate concentration detected by the biosensor during the peri-ischemic period in the three groups. No significant differences in the baseline values were found among the aCSF ($28 \pm 9 \mu\text{M}$), DMSO ($30 \pm 24 \mu\text{M}$), and dantrolene-DMSO ($30 \pm 26 \mu\text{M}$) groups. An enormous increase in extracellular glutamate concentration was observed immediately after ischemic injury in the aCSF-treated rats, and the glutamate concentrations in the aCSF group were maintained significantly higher than in the other groups throughout the 4- to 10-min ischemic period ($P < 0.01$). Consequently, the area under the curve in the aCSF group was significantly greater than in the DMSO and dantrolene-DMSO groups by 105 and 115%, respectively ($P < 0.01$); however, DMSO and dantrolene-DMSO showed no significant difference in area under the curve (fig. 2B).

All aCSF-treated animals survived for the 7-day observation period. Two and one of eight rats died in the DMSO and dantrolene-DMSO groups, respectively, during 7 days of reperfusion. There were no significant differences in mortality among the groups. Representative microscopic photographs of part of the hippocampal CA1 subfield are presented in figure 3A, indicating that most pyramidal neurons were protected in this rat treated with dantrolene-DMSO. The number of intact

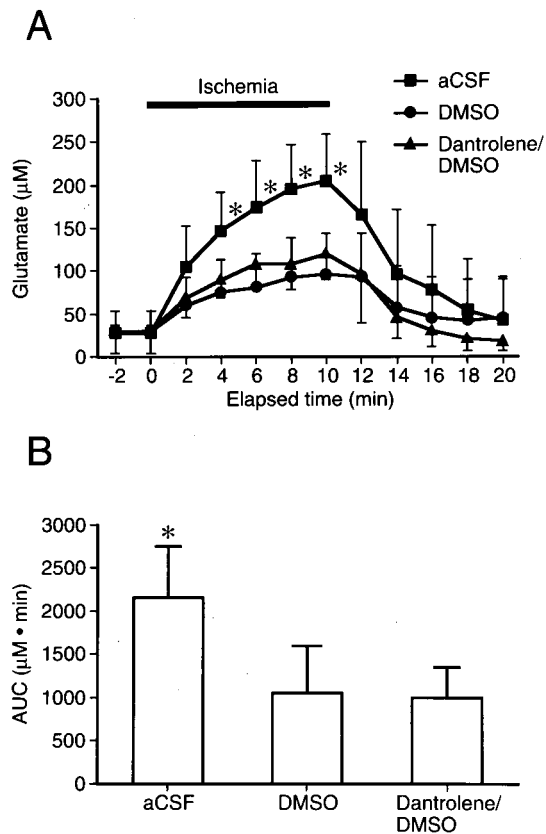


Fig. 2. Extracellular glutamate concentrations in artificial cerebrospinal fluid (aCSF), dimethyl sulfoxide (DMSO), and dantrolene-DMSO treatment groups. (A) Serial changes were obtained by plotting the symbols on the actual traces every 2 min. The treatments were conducted 15 min before the initiation of 10-min ischemia. Symbols indicate the mean, and bars are SD. * $P < 0.01$ versus other groups. (B) Area under the curve (AUC) calculated on the actual traces between the beginning and 20 min after the end of ischemia. Columns indicate the mean, and bars are SD. * $P < 0.01$ versus other groups.

CA1 neurons (cells/mm) in the dantrolene-DMSO group (78 ± 21) was significantly greater than that in the aCSF (35 ± 14 ; $P < 0.001$) and DMSO (56 ± 11 ; $P < 0.05$) groups (fig. 3B). Dimethyl sulfoxide also significantly increased the number of preserved cells in comparison with aCSF ($P < 0.05$, fig. 3B).

Discussion

The findings here indicate that dantrolene administered into the cerebral ventricles mitigated delayed neuronal death induced by transient forebrain ischemia in the rat hippocampal CA1 subfield. It is noteworthy that dantrolene was more neuroprotective than DMSO; however, the degree of reduction in glutamate concentrations during ischemia did not differ between them. This is, as far as we know, the first report on the effect of dantrolene on extracellular glutamate concentration *in vivo* measured with a microdialysis biosensor in cerebral ischemia.

Glutamate produced in excess by ischemia is believed to have a key role in initiating and exacerbating neurodegeneration in the central nervous system.²³ Glutamate causes Ca^{2+} influx through both *N*-methyl-D-aspartate and non-*N*-methyl-D-aspartate receptors, resulting in an increase in intracellular Ca^{2+} concentration. Glutamate is released as an excitatory neurotransmitter from nerve endings of glutamatergic neurons (vesicular release) and is uptaken by neurons, glia, or both *via* glutamate transporters.²⁴ Suggested mechanisms of excess glutamate accumulation in the extracellular space during brain ischemia are as follows: (1) vesicular release induced by an increase in transmembranous Ca^{2+} influx¹⁰; (2) vesicular release dependent on Ca^{2+} released from the intracellular stores¹¹; (3) Ca^{2+} -dependent release from astrocytes mediated by prostaglandins; and (4) reversed uptake by glutamate transporters in glia and neurons.^{6,8} Thus, glutamate dynamics are closely associated with changes in Ca^{2+} concentration. Dantrolene is well-known to inhibit Ca^{2+} release from the ER, leading us to hypothesize that dantrolene might attenuate the in-

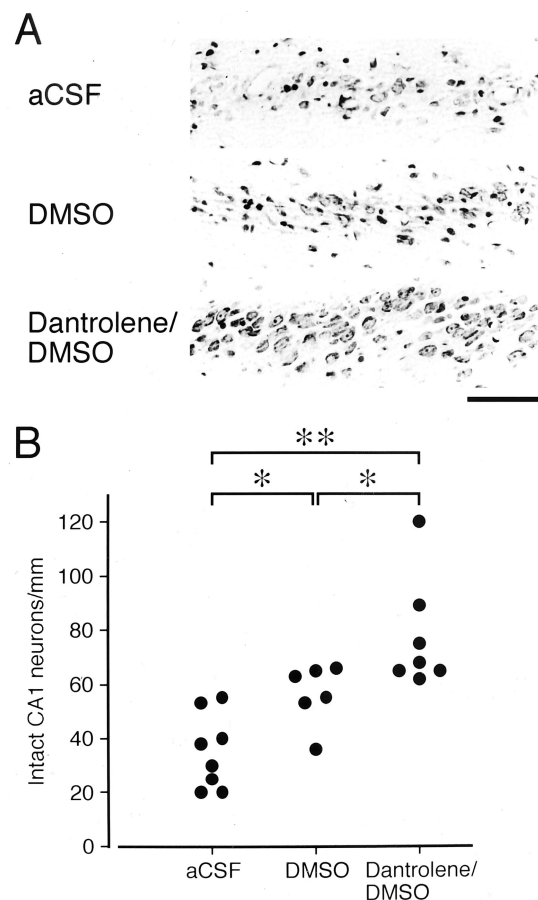


Fig. 3. Effects of artificial cerebrospinal fluid (aCSF), dimethyl sulfoxide (DMSO), and dantrolene-DMSO on neuronal damage 7 days after ischemia. (A) Representative microscopic photographs of the hippocampal CA1 subfield stained with cresyl violet. Bar = 50 μm . (B) Number of intact pyramidal cells in the hippocampal CA1 region counted on the sections. * $P < 0.05$; ** $P < 0.001$.

crease in extracellular glutamate during cerebral ischemia. Unexpectedly, dantrolene failed to reduce the extracellular glutamate concentration during ischemia beyond its vehicular DMSO in this experimental model. The reduction in glutamate concentration seems to be attributed to DMSO, not to dantrolene itself.

Calcium ions are of great importance in the development of ischemic neuronal injury. It has been postulated that Ca^{2+} overload in the cytosol may activate many Ca^{2+} -dependent enzymes, such as lipases, proteases, and endonucleases, which can initiate irreparable cell damage, resulting in neuronal death. The cessation or decrease of cerebral blood flow causes depolarization of the neuronal membrane because of energetic or metabolic failure. This neuronal depolarization promotes Ca^{2+} influx from the extracellular space into the cytosol by opening not only glutamate receptor-gated but also voltage-dependent calcium channels. The increased intracellular Ca^{2+} then triggers the Ca^{2+} efflux from intracellular stores, such as ER, leading to further increase of cytosolic Ca^{2+} . Given that the delayed neuronal death after ischemia is principally ascribable to this Ca^{2+} discharge due to the cytosolic Ca^{2+} overload, dantrolene could be neuroprotective by blocking Ca^{2+} efflux selectively *via* the RyR- Ca^{2+} release channels on the ER.^{25,26} Dantrolene has been shown to inhibit the Ca^{2+} -induced Ca^{2+} release caused by *N*-methyl-D-aspartate activation in cultured neurons and in *in vivo* rat hippocampus and dentate gyrus.^{14,27,28} Three genetically distinct isoforms of RyR protein have been identified to date: skeletal muscle, cardiac muscle, and brain types, each of which is heterogeneously distributed in the brain.^{29,30} All isoforms of RyR coexist in the hippocampal CA1 pyramidal layer, implicating the highly Ca^{2+} -dependent hippocampal functions and thus its selective vulnerability to ischemia.²⁸ Dantrolene might inhibit the brain type of RyR, although some reports have suggested that dantrolene does not bind to the RyR itself, but rather to some smaller associated proteins in the case of skeletal muscle.³¹⁻³³

It remains unclear whether dantrolene protects neurons against ischemia in the case of systemic administration.^{34,35} Furthermore, it has not been accurately documented how much dantrolene permeates the blood-brain barrier. Therefore, we circumvented this problem by administering dantrolene directly into the cerebral ventricles to clarify its neuroprotective properties. Several investigators, including us, have previously observed that intracerebroventricular dantrolene prevented delayed neuronal death in the rodent hippocampal CA1 region when administered before or immediately after, but not late after, ischemic injury.³⁶⁻³⁸ These findings are consistent with the fact that transient ischemia causes an immediate reduction in protein synthesis followed by a grad-

ual restoration by day 2 and a secondary decrease on day 3 of reperfusion, the time course of which coincides with that of intracellular Ca^{2+} accumulation, suggesting that a possible therapeutic window exists in the early reperfusion period.³⁷ We accordingly administered dantrolene just before ischemia in this study.

In the central nervous system, nitric oxide (NO) has a pivotal role as a second messenger being produced from L-arginine by the Ca^{2+} -calmodulin-dependent neuronal NO synthase (NOS), which is activated by an increase in intracellular Ca^{2+} . Neuronal NOS is primarily expressed constitutively in neurons throughout the nervous system, including the hippocampus, and is inducible under pathologic conditions.²³ However, abundant NO can be neurotoxic by acting as a free radical itself, and also by forming other free radicals, such as peroxynitrite, which is more reactive and toxic to neurons than NO. NO and peroxynitrite break deoxyribonucleic acids, leading to activation of the nucleic enzyme poly(adenosine diphosphate-ribose) polymerase (PARP). Excessive activation of PARP can cause energy depletion, leading to apoptotic cell death.^{39,40} Ischemia-induced delayed neuronal death may be in part due to apoptosis.³⁸ It is generally accepted that neuronal NOS mediates early neuronal injury and inducible NOS contributes to late neuronal injury, whereas endothelial NOS is neuroprotective.²³ Dantrolene has been reported to inhibit Ca^{2+} -dependent NO production by reducing the inositol 1,4,5 triphosphate-mediated Ca^{2+} signal in porcine skeletal muscle³³ and, moreover, to attenuate inducible NOS activity in rat alveolar macrophages.⁴¹ These findings *en masse* suggest that by inhibiting NO production, dantrolene could potentially protect neurons against free radical injury in cerebral ischemia.

Dimethyl sulfoxide is widely used as a solvent for a variety of agents. A substantial number of studies have shown the beneficial effects of DMSO on ischemic neuronal damage possibly by reducing intracranial pressure, scavenging free radicals, improving cerebral blood flow, and so on.⁴²⁻⁴⁴ In this study, the authors demonstrated that DMSO significantly ameliorated the ischemic neuronal loss in association with a reduction in extracellular glutamate concentrations compared with aCSF. Although the mechanism is unknown, the decrease in glutamate concentrations may contribute to the neuroprotective effects of DMSO.

In summary, intracerebroventricular dantrolene administered just before ischemia protects neurons against transient global ischemia in the rat hippocampal CA1 subfield. The mechanism by which dantrolene exerts this neuroprotective effect cannot be explained by the reduction in extracellular glutamate accumulation alone. It is possible that dantrolene could be of therapeutic interest for brain ischemia.

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