Introduction

Sodium nitroprusside (SNP) is widely used in clinical and pharmacological studies as a potent vasodilator (1, 2) and a nitric oxide (NO) donor (3), whereas cytotoxicity of SNP has been well documented. SNP releases several potentially toxic products such as cyanide anion, NO, and iron. We investigated the mechanisms of cell death and motor dysfunction induced by microinjection of SNP in mice to establish a brain oxidative stress model and then examined the anti-oxidant activity of glutathione. Intrastriatal microinjection of SNP (1 – 10 nmol) induced brain damage and motor dysfunction in a dose-dependent manner when the effects were evaluated with behavioral tests and TTC staining. NOC-18 (10 nmol), another NO donor, and KCN (10 nmol) did not cause motor dysfunction. However, FeCl₂ (10 nmol) caused motor dysfunction. In addition, simultaneous injection of SNP and deferoxamine (10 nmol), an iron-chelating agent, prevented SNP-induced brain damage and motor dysfunction, suggesting a role of iron-related radicals in SNP-toxicity. Moreover, reduced glutathione (1 – 10 nmol), a natural anti-oxidant substance, dose-dependently prevented motor dysfunction induced by SNP-toxicity. Finally, deferoxamine and glutathione (10 nmol) significantly protected against brain damage and motor dysfunction induced by FeCl₂ toxicity. These results suggest that cell death induced by injection of SNP is caused by iron-related radical reactions, but not by NO and cyanide anion.

Keywords: sodium nitroprusside, brain damage, behavior change, oxidative stress, anti-oxidant

In Vivo Brain Oxidative Stress Model Induced by Microinjection of Sodium Nitroprusside in Mice

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Abstract. Sodium nitroprusside (SNP) is widely used as a potent vasodilator and a nitric oxide (NO) donor, whereas the cytotoxicity of SNP has been well documented. SNP releases several potentially toxic products such as cyanide anion, NO, and iron. We investigated the mechanisms of cell death and motor dysfunction induced by microinjection of SNP in mice to establish a brain oxidative stress model and then examined the anti-oxidant activity of glutathione. Intrastriatal microinjection of SNP (1 – 10 nmol) induced brain damage and motor dysfunction in a dose-dependent manner when the effects were evaluated with behavioral tests and TTC staining. NOC-18 (10 nmol), another NO donor, and KCN (10 nmol) did not cause motor dysfunction. However, FeCl₂ (10 nmol) caused motor dysfunction. In addition, simultaneous injection of SNP and deferoxamine (10 nmol), an iron-chelating agent, prevented SNP-induced brain damage and motor dysfunction, suggesting a role of iron-related radicals in SNP-toxicity. Moreover, reduced glutathione (1 – 10 nmol), a natural anti-oxidant substance, dose-dependently prevented motor dysfunction induced by SNP-toxicity. Finally, deferoxamine and glutathione (10 nmol) significantly protected against brain damage and motor dysfunction induced by FeCl₂ toxicity. These results suggest that cell death induced by injection of SNP is caused by iron-related radical reactions, but not by NO and cyanide anion.

Keywords: sodium nitroprusside, brain damage, behavior change, oxidative stress, anti-oxidant
large amounts of ROS (16). Moreover, the brain contains high levels of fatty acids which are more susceptible to peroxidation. In addition, the brain is not particularly enriched in antioxidant defenses in comparison with other tissues (17). Oxidative stress in the brain is mediated mainly by ROS, including free radicals such as superoxide (O$_2^-$), OH·, and also NO, a RNS (18). Development of an in vivo brain oxidative-stress model is essential to evaluate the efficacy of anti-oxidant substances, in order to prevent oxidative damages accompanied by neurologic disorders.

Reduced glutathione (GSH) is the most abundant thiol-containing molecule in mammalian cells and plays a critical role in controlling intracellular burdens of oxidative stresses. GSH non-enzymatically reacts with O$_2^-$, NO, OH·, and peroxynitrite (ONOO$^-$) (19).

In this study, we first evaluated the cytotoxicity of SNP to establish a new brain oxidative-stress model in mice, and then we evaluated the anti-oxidant activity of glutathione.

Materials and Methods

Materials

ICR mice were obtained from (Nihon SLC, Shizuoka). SNP, potassium cyanide (KCN), 2,3,5-triphenyltetrazolium chloride (TTC), and ferrous chloride tetrahydrate (FeCl$_2$) were purchased from Nacalai Tesque (Kyoto). Deferoxamine was from Sigma (St. Louis, MO, USA). 2,2$'$-(Hydroxynitrosohydrazono) bisethanamine (NOC-18) and GSH were obtained from Wako Pure Chemicals (Osaka). Nembutal (sodium pentobarbital) was obtained from Dainippon Sumitomo Pharmaceutical Co., Ltd. (Osaka).

Animals

Six-week-old male ICR mice (25 – 30 g) were used in the present study. The animals were housed in a temperature-controlled room; lighting was maintained on a 12-h light–dark cycle, with ambient temperatures maintained at 20°C – 22°C. Food was available ad libitum. The experiments were conducted in accordance with the Ethical Guidance of the Kyoto University Animal Experimentation Committee and the Guidance of The Japanese Pharmacological Society.

Rotarod test

The rotarod test is used to assess motor coordination and balance in mice (20). In the present study, the rotational speed tested in the steady state mode was 20 rpm for 180 s. The rotational speed in the accelerated mode was 0 – 40 rpm for 300 s. The time the mouse was able to walk on the rod before falling off was recorded (maximum time: 180 s for the steady state mode and 300 s for the accelerated mode).

Locomotor activity test

The locomotor activity test is used to assess spontaneous activity in mice (21). The locomotor activity was tested individually in an open field using boxes, equipped with infrared beams. The mice were placed into the box and their movements were measured for 5 min. The interruptions of photo beams for 5 min per mice were registered as the number of transitions (horizontal activity). In the same time, the number of rearings (vertical activity) was counted.

Surgery and intrastriatal microinjection

Mice were anesthetized with Nembutal (60 mg/kg, i.p.) and placed in the streotaxic frame. An incision was made along the midline of the skull to expose the skull. Coordinates for injections were: AP +0.4 mm, ML +2 mm, DV $-3.5$ mm. Then a hole was drilled into the skull and a 30-gauge blunt tip needle was inserted into the striatum through the hole. Drugs dissolved in 1 $\mu$l of 0.9% sterile saline solution was injected into the right striatum, using a 10-$\mu$l Hamilton microsyringe mounted on a motorized pump, at a rate of 0.2 $\mu$l/min over 5 min. After the injection, the needle was held in place for an additional 5 min to prevent back flow and allow diffusion of the drugs.

Histopathological examination

Mice were decapitated 24 h after microinjection of drugs under deep anesthesia with sodium pentobarbital (60 mg/kg B.W.). Brains were rapidly removed after intracardial infusion of phosphate-buffered saline (PBS). The brains then were coronally cut into eight (1-mm thickness) slices, using a brain slicer. Brain slices were immediately incubated in TTC solution (2% solution in PBS) for 30 min at 37°C. Areas not stained red with TTC were considered to be damaged. Finally, the slices were photographed by scanning. The unstained areas in slices were quantified with the Image J 1.42 program, and the damage volume was calculated by summing up the damaged area in all slices.

Statistical analyses

The results were expressed as the mean ± S.E.M. One way analyses of variance (ANOVA) followed by Dunnett’s test was used to determine statistical significant among three or more groups of mice. Between two groups of mice, Student’s t-test was performed to determine statistical significance. Results were considered statistically significant at $P < 0.05$. All statistical analyses were performed with GraphPad InStat (GraphPad Soft-
Results

Effect of intrastriatal microinjection of SNP on motor function and brain damage

To assess the cytotoxicity of SNP, we injected SNP (1 – 10 nmol) into the right striatum of the mice. Then, one day after microinjection of SNP, the mice were examined for motor function. As shown in Fig. 1, A and B, SNP (10 nmol) caused significant decrease in rotarod performance compared with the vehicle control. In the locomotor test, microinjection of SNP did not alter horizontal activities (transitions), but SNP caused significant decrease in the vertical activities (rearings) of mice (Fig. 1: C and D). TTC staining also showed that SNP (10 nmol) caused marked brain damage compared with the vehicle control (Fig. 2B).

Effects of intrastriatal microinjection of NOC-18, KCN, and FeCl₂ on motor function

Previous reports showed that SNP releases several toxic compounds such as NO, CN⁻, and Fe²⁺ during its decomposition (8, 9). To investigate the genuine mediators of cytotoxic effects triggered by SNP, we injected NOC-18, KCN, and FeCl₂ (10 nmol), respectively, into the striatum to assess the role of different moieties in SNP-induced toxicity. In the rotarod test, FeCl₂ significantly decreased rotarod performance of mice compared with the vehicle control. In the locomotor test, FeCl₂ did not alter horizontal activities (transitions), but FeCl₂ caused significant decrease in vertical activities (rearings). However, NOC-18 and KCN did not cause motor dysfunction in both rotarod and locomotor tests (Fig. 3). Co-injection of NOC-18 plus KCN did not cause motor dysfunction (data not shown).

Effect of intrastriatal microinjection of deferoxamine on SNP-induced toxicity

For further evaluation of the role of the iron moiety of

![Fig. 1.](image1.png)

![Fig. 2.](image2.png)
SNP on motor dysfunction and brain damage, we injected deferoxamine, an iron chelating agent, with SNP (10 nmol) into the mice striatum. In the rotarod test, co-injection of deferoxamine (10 nmol) significantly prevented SNP-induced impairment in mice performance compared with SNP treatment (Fig. 4A). In the locomotor test, deferoxamine (10 nmol) did not alter horizontal activities (transitions) (B), but increased vertical activities (rearings) (C). **P < 0.01, compared with SNP treatment.

Effect of intrastriatal microinjection of glutathione on SNP-induced toxicity

GSH is the major cellular anti-oxidant found in higher levels in the brain (19). To evaluate the anti-oxidant activity of GSH, we injected GSH with SNP into the mice striatum and then performed behavioral tests and TTC staining. In the rotarod test, co-injection of GSH (1 – 10 nmol) dose-dependently prevented SNP-induced impairment in mice performance (Fig. 6A). In the locomotor test, GSH did not alter horizontal activities (transitions) (B), but increased vertical activities (rearings) (C). **P < 0.01, compared with vehicle, ##P < 0.01, compared with SNP treatment. Values represent the mean ± S.E.M. (n = 7).

Effects of intrastriatal microinjection of deferoxamine and glutathione on FeCl₂-induced toxicity

We examined the effects of deferoxamine and GSH in FeCl₂-induced motor dysfunction and brain damage, followed by behavioral tests and TTC staining. In the rotarod test, co-injection of deferoxamine or GSH (10 nmol), significantly prevented FeCl₂-induced impairment...
in mice performance (Fig. 7A). In the locomotor test, deferoxamine and GSH did not alter horizontal activities (transitions), but GSH significantly increased vertical activities (rearings) (Fig. 7: B and C). Quantitative analysis of TTC staining showed that deferoxamine and GSH (10 nmol) significantly decreased brain damage volume induced by FeCl₂-toxicity (Fig. 7D).

Discussion

In the present study, microinjection of SNP into the mice striatum caused brain damage and motor dysfunction. However, in the locomotor activity test, the striatal damage induced by SNP injection did not alter horizontal activities. The important factors in the behavioral impairment are the degree of depletion or damage in the nigrostriatal system and the behavioral test used for motor function (22, 23). We did not see alterations in the horizontal activities of the mice possibly because of the lower sensitivity of the test and the limited amount of damage induced by injection of SNP in the striatum.

There have been several reports regarding SNP-induced toxicity (4 – 7), but the mechanisms of cell death underlying of SNP toxicity remain to be characterized. Although SNP is widely used as an NO donor, it has been reported that SNP can release several other toxic compounds such as CN⁻, iron, and ROS (8, 9). It has been suggested that the pentacyano nitroxide radical [Fe(CN)₅NO]− of SNP rapidly releases a single cyanide ion (11). CN⁻ has been reported to react readily with cytochrome enzymes in mitochondria, which consequently causes inhibition of cellular respiration, reduction of ATP production, and cytotoxicity (24, 25). On the other hand, several other reports have recently suggested that cyanide ion has no important role in SNP-induced cytotoxicity, using in vivo and in vitro experiments (4, 5). In line with these reports, we also demonstrated here that upon SNP treatment, cyanide has no role in the establishment of the stress condition leading to brain dam-

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Fig. 5. Representative photographs showing protective effects of DFO on brain damage induced by SNP. SNP (10 nmol) (A) or DFO + SNP (10 nmol) (B) was injected into the right striatum. After one day, the brains were sliced into coronal sections for TTC staining. Co-injection of DFO protected against brain damage induced by SNP (B). Quantitative analysis showed a significant protective effect of DFO compared with SNP treatment (C). ***p < 0.001, compared with SNP. Values represent the mean ± S.E.M. (n = 5). Scale bar = 1 cm.

Fig. 6. Effect of intrastriatal microinjection of GSH on SNP-induced motor dysfunction. Behavioral changes were examined one day after microinjection of drugs. In the rotarod test, intrastriatal co-injection of GSH (1 – 10 nmol) prevented SNP-induced impairment in mouse performance (A). In the locomotor test, GSH did not alter horizontal activities (transition) (B), but increased vertical activities (rearings) (C). **p < 0.01, compared with vehicle, ###p < 0.01, compared with SNP treatment. Values represent the mean ± S.E.M. (n = 7).
Microinjection of KCN did not cause motor dysfunction and brain damage, suggesting that CN\textsuperscript{−} does not mediate the toxic effects of SNP.

After release of CN\textsuperscript{−}, the tetracyano nitroxide radical [Fe(CN)\textsubscript{4},NO]\textsuperscript{−} of SNP releases its NO (11). It has been reported that NO can increase intracellular ROS by inhibiting mitochondrial respiratory chain enzymes and cause cell death (26). Furthermore, NO readily reacts with O\textsubscript{2}·\textsuperscript{−} to form ONOO\textsuperscript{−} and OH·, potent oxidant substances capable of attacking proteins, lipids, and DNA as well as depleting antioxidant defenses of the cells, such as decreasing glutathione level (27). However, in this study we used another NO donor, NOC-18, which has a long half-life (t\textsubscript{1/2} = 21 h), and it did not induce toxic effects. On the other hand, it has been suggested that brain neurons express high levels of superoxide dismutase enzyme (SOD) (28, 29). SOD converts superoxide anion to H\textsubscript{2}O\textsubscript{2} and consequently inhibits the conversion of NO to harmful peroxynitrite. Furthermore, it has been proposed that nNOS-positive neurons are resistant to NO-induced cytotoxicity and that this resistance mechanism may be attributable to nNOS-positive neurons with abundant Mn-SOD (30). It shows that NO which is released by SNP, has no important role in SNP toxicity.

In our results, SNP-induced toxicity was prevented by deferoxamine, an iron chelator. Moreover, intrastriatal microinjection of FeCl\textsubscript{2} induced motor dysfunction and brain damage. Deferoxamine also significantly protected against FeCl\textsubscript{2}-induced motor dysfunction. Further, the toxic effect of SNP was more potent and constant than the FeCl\textsubscript{2}, which might be caused by FeCl\textsubscript{2} having different diffusion properties than SNP. Thus, it can be concluded that Fe\textsuperscript{2+} mediates the toxic effects of SNP in our model. After release of NO, the iron moiety of SNP may lead the formation of ROS, such as OH· radicals, via the Fenton reaction (11). Moreover, it has been reported that the OH· radical is involved in cytotoxicity induced by SNP (4).

The reduced form of glutathione (γ-glutamylcysteinylglycine, GSH) is the major cellular antioxidant and is found at high levels in the brain. GSH has several functions and its most important function is protection against oxidative damage caused by ROS, many of which are generated during normal metabolism in cells. GSH non-enzymatically reacts with superoxide, NO, OH· radical, and ONOO\textsuperscript{−}. Furthermore, there is no known enzymatic defense against OH· radicals, making GSH the only compound capable of scavenging these radicals in the cells (19). Therefore, we examined the effect of GSH on SNP-induced toxicity using our model and found that GSH protected against motor dysfunction and brain damage induced by SNP-toxicity. GSH also protected against motor dysfunction and brain damage induced by FeCl\textsubscript{2} injection. The anti-oxidant effect of GSH may be exerted by its scavenging of OH· radicals produced by the Fenton reaction catalyzed by the iron moiety of SNP.

In conclusion, the present study demonstrates that SNP-induced brain damage is caused by iron-related radicals but not NO and CN\textsuperscript{−} anion. This in vivo SNP-toxicity method is a useful model in mice to evaluate the
References


