

Neuroprotective effect of nitrite-derived NO in brain injury mediated through the NOS-independent but not the GC/COX/xanthine oxidase/PGIS-dependent pathways

Hisae Ando, Yoshihiro Nara, Yasuharu Kosaka, Masayasu Arai, Hirotsugu Okamoto

Department of Anesthesia, Kitasato University School of Medicine

Purpose: Nitrite-derived nitric oxide (NO) has been shown to provide neuroprotection against brain ischemia-reperfusion injury. The present study was designed to examine the effect and mechanism of nitrite-derived NO on cerebral infarct volume in a chronic rat model.

Methods: Male Sprague-Dawley rats were divided into eight treatment groups: saline only for the control, one group further divided into three subgroups receiving different doses of sodium nitrite (NaNO_2), and six groups with saline and NaNO_2 with: nitro-L-arginine (L-NNA) (a NO synthase [NOS] inhibitor), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (a soluble guanylate cyclase [GC] inhibitor), 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (C-PTIO) (a NO scavenger), allopurinol (a xanthine oxidase inhibitor), indomethacin (a cyclooxygenase [COX] inhibitor), and U-51605 (a prostacyclin synthase [PGIS] inhibitor). The rats were injected intraperitoneally with one of the above combinations, followed by 1-hour occlusion of the middle cerebral artery by suture ligation and subsequent reperfusion. Five days later, the brains were excised, sectioned, and stained to quantify the cerebral infarct area as a percentage of the whole brain area.

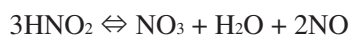
Results: Nitrite significantly reduced the cerebral infarct area in a dose-dependent manner. The nitrite-induced reduction in the cerebral infarct area was unaffected in rats injected with C-PTIO, ODQ, allopurinol, indomethacin, and U-51605. However, injection of L-NNA augmented the reduction in the nitrite-induced cerebral infarct area.

Conclusion: Nitrite-derived NO protects the brain against ischemia-reperfusion injury through NOS-independent but GC/COX/xanthine oxidase/PGIS-dependent pathways.

Key words: nitrite, nitric oxide synthase, ischemia-reperfusion injury, guanylate cyclase, cyclooxygenase, prostacyclin synthase, rat brain

Introduction

Nitrite-derived nitric oxide (NO) has several physiological effects. Nitrite generates NO by the oxidation-reduction reaction as follows.



One peculiar property of nitrite-derived NO is its concentration-dependent multimodal effects, acting as a neuroprotective agent, e.g., in ischemia-reperfusion injury, or as a neurotoxic compound.^{1,2} However, the mechanisms and/or the pathways of these effects remain elusive. The present study was designed to examine the mechanisms and the optimum dose of nitrite-derived NO in reducing the cerebral infarct volume following focal

cerebral ischemia-reperfusion in rats.

Materials and Methods

Surgical and experimental design

The experiments described in this study were conducted using 132 male Sprague-Dawley rats (weight, 300–400 g) after approval of the institutional animal care ethics review committee. To induce focal cerebral ischemia-reperfusion injury, the rats were anesthetized intraperitoneally (i.p.) with pentobarbital sodium (50 mg/kg).³ An endotracheal tube was inserted for mechanical ventilation and end-tidal CO_2 was monitored continuously and maintained within the normal range (35–40 mmHg) throughout the experiment. A catheter was inserted into

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Correspondence to: Hisae Ando, Department of Anesthesiology, Kitasato University School of Medicine
1-15-1 Kitasato, Minami-ku, Sagami-hara, Kanagawa 252-0374, Japan
E-mail: dm12012p@st.kitasato-u.ac.jp

the femoral artery to monitor blood pressure and heart rate and another one into the femoral vein for administration of the muscle relaxant. Vecuronium bromide was infused continuously at a rate of 0.015–0.02 mg/kg/min for muscle paralysis. Pentobarbital sodium was administered as needed to maintain an adequate level of anesthesia. Brain temperature was also measured with a 22-gauge stainless steel needle thermometer placed in the left temporal muscle, and normothermia was maintained using an overhead heating lamp and a heating pad throughout the experiment. The middle cerebral artery was completely occluded using a nylon suture then released after 1 hour. Regional cerebral blood flow (rCBF) was monitored ipsilaterally to the occlusion using a laser-Doppler probe (Omegawave; Tokyo) placed 6 mm laterally and 2 mm posteriorly to the bregma, and during middle cerebral artery occlusion the regional cerebral blood flow was maintained at <50% of the baseline.

Experiment protocol

Rats were divided into eight treatment groups: (1) The saline only control group (n = 8), (2) three doses of sodium nitrite (NaNO₂) (0.1 [n = 8], 1.0 [n = 7], and 10 mg/head [n = 10]), (3) nitro-L-arginine (L-NNA) (a NO synthase [NOS] inhibitor) 10 mg/kg with saline (n = 9) and NaNO₂ 1.0 mg/head (n = 8), (4) 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (a soluble guanylate cyclase [sGC] inhibitor) 20 mg/kg with saline (n = 9) and with NaNO₂ 1.0 mg/head (n = 9), (5) 10 mg/head of 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-

3-oxide (C-PTIO) (a NO scavenger) with saline (n = 9) and with NaNO₂ 1.0 mg/head (n = 7), (6) allopurinol (a xanthine oxidase inhibitor) 10 mg/head with saline (n = 8) and with NaNO₂ 1.0 mg/head (n = 7), (7) indomethacin (a cyclooxygenase [COX] inhibitor) 5 mg/head with saline (n = 12) and with NaNO₂ 1.0 mg/head (n = 5), and (8) U-51605 (a prostacyclin synthase [PGIS] inhibitor) 400 μg/head with saline (n = 11) and with NaNO₂ 1.0 mg/head (n = 5). After surgical preparation, the above compounds were injected i.p. 1 hour before, and each NaNO₂ was injected i.p. 30 minutes before ischemia. The doses used in the present study for each drug were selected based on previous studies¹⁻³ and our preliminary observations (data not shown).

Five days after ischemia-reperfusion injury, the brains were carefully excised, each one immediately sectioned into seven 2-mm-thick coronal sections, which were then stained with TTC (2,3,5-triphenyltetrazolium chloride). This method is commonly used for determining brain infarct volume and is highly reproducible.⁴⁻⁷ Hematoxylin-and-eosin staining was also performed to confirm the infarction site (Figure 1). The infarct area was measured in all brain slices and the infarct volume was expressed as a percentage of the total brain area (NIH ImageJ; Wayne Rasband, Bethesda, MD, USA).

Statistical analyses

All data are expressed as mean ± SEM. Differences in the infarct area between the control group and the NaNO₂-treated groups, and baseline rCBF and mean arterial blood pressure (MABP) were compared by non-repeated

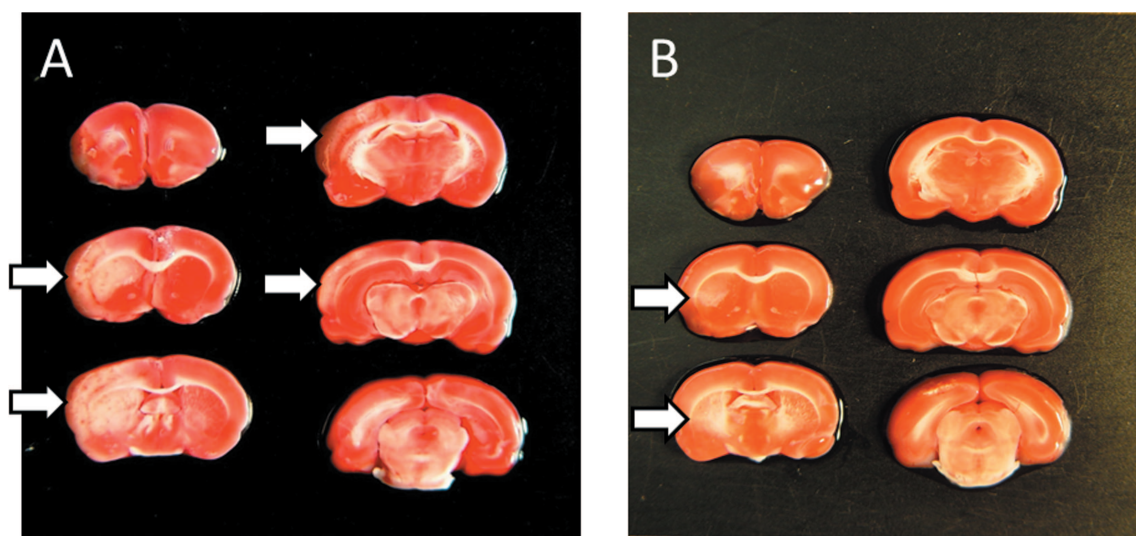


Figure 1. Images of 2,3,5-triphenyltetrazolium chloride-stained sections of a representative rat from (A) the control group and (B) the 10-mg NaNO₂-treated group. Note the lack of staining of the infarct area (white arrows). The infarct area was 15.5% in the control group and 2.8% in the NaNO₂-treated group.

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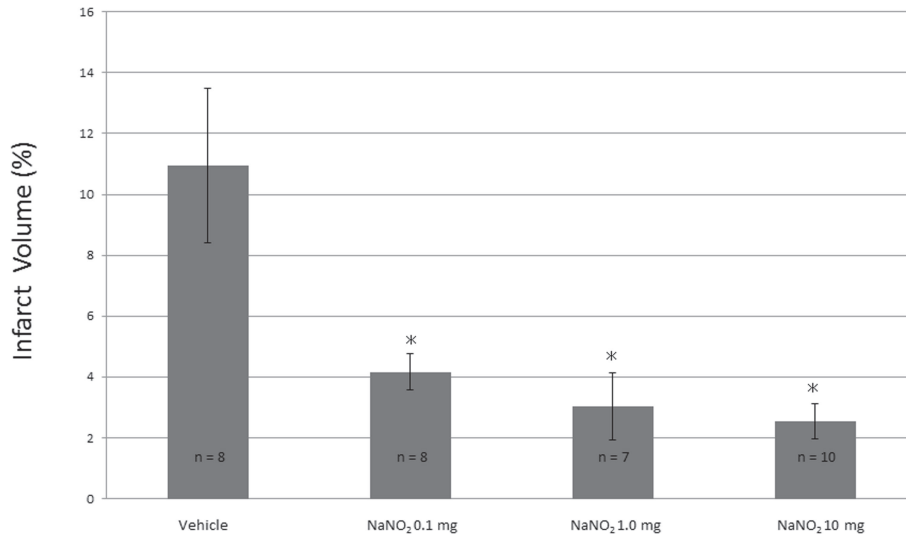


Figure 2. Comparison of the infarct area in the control group and the NaNO₂-treated groups

The infarct volume in the control group was 10.9% of the total brain volume. Intraperitoneal injection of NaNO₂ significantly decreased the infarct area to 4.1% in the 0.1-mg group, 3.0% in the 1.0-mg group, and 2.5% in the 10-mg group. Data are mean \pm SEM (*P < 0.05 vs. the control group).

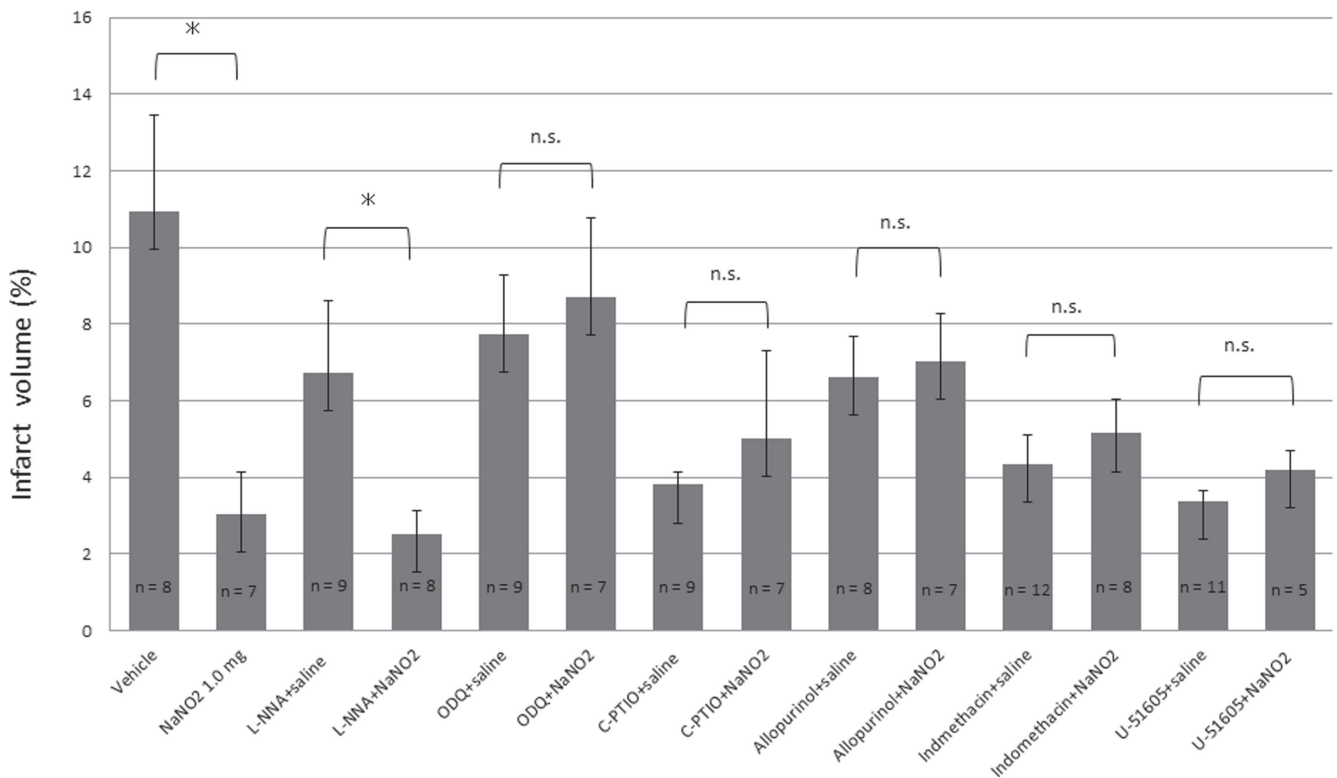


Figure 3. Effects of L-NNA, ODQ, C-PTIO, allopurinol, indomethacin, and U-51605 on the NaNO₂-induced reduction of the infarct volume

The L-NNA + NaNO₂ group decreased the cerebral infarction area significantly compared with the L-NNA+saline group. In other groups, there were no significant differences in the infarct volume after the injection of each compound followed by injection of NaNO₂ compared with the compound alone. Data are mean \pm SEM (*P < 0.05).

ANOVA (analysis of variance), post-hoc with the Student-Newman-Keuls test for multiple comparisons while the effects of L-NNA, ODQ, C-PTIO, allopurinol, indomethacin, and U-51605 on the NaNO₂ induced reduction of the infarct area were conducted by unpaired *t*-test. Differences were considered statistically significant when *P* < 0.05.

Results

NaNO₂ significantly reduced the cerebral infarct area compared with that in the saline group, and a weak dose-response effect was evident (Figure 2). Further experiments designed to determine the mechanism of the neuroprotective effect of NaNO₂ showed that only L-NNA (a NOS inhibitor) significantly augmented the reduction in the cerebral infarct area when injected before NaNO₂, relative to the saline control. On the other hand, ODQ (a guanylate cyclase inhibitor), C-PTIO (a NO scavenger), allopurinol (a xanthine oxidase inhibitor), indomethacin (a nonselective inhibitor of COX), and U-51605 (a prostacyclin synthase inhibitor) had no effect on NaNO₂-induced reduction of the infarct area (Figure 3). There were no significant differences in the changes of the heart rate among all the experimental groups.

Baseline rCBF and MABP are shown in Tables 1 and 2.

Discussion

First, the results of the present study confirmed those of previous reports that NO derived from NaNO₂ exerts neuroprotective effects against cerebral ischemia-reperfusion injury, and this effect tended to be dose-dependent. We used the second highest dose of nitrite, because the highest dose lowered blood pressure, which could worsen the cerebral ischemia. Second, we examined the further mechanisms of this neuroprotective effect of nitrite-derived NO. Nitrite-derived NO-induced effect was observed in the presence of NOS-inhibitor, L-NNA; however, the effects disappeared in the presence of other inhibitor compounds, including ODQ (a soluble guanylate cyclase inhibitor), C-PTIO (a NO scavenger), allopurinol (a xanthine oxidase inhibitor), indomethacin (nonselective inhibitor of COX), and U-51605 (prostacyclin synthase inhibitor). The neuroprotective effect of nitrite was inhibited in the presence C-PTIO, suggesting the NO-dependent action of nitrite. This was the same result as the previous study of Jung et al.¹ These findings suggest that the neuroprotective effects against brain ischemic-reperfusion injury is NOS-independent,

Table 1. Baseline rCBF (perfusion unit)

(a)	Saline	L-NNA + Saline	ODQ + Saline	C-PTIO + Saline	Allopurinol + Saline	Indomethacin + Saline	PGISI + Saline
	21.39 ± 7.56	26.58 ± 8.86	26.02 ± 8.67	24.31 ± 8.10	21.51 ± 7.60	23.02 ± 7.67	20.99 ± 6.32
(b)	NaNO ₂ 1.0 mg	L-NNA + NaNO ₂	ODQ + NaNO ₂	C-PTIO + NaNO ₂	Allopurinol + NaNO ₂	Indomethacin + NaNO ₂	PGISI + NaNO ₂
	23.02 ± 8.70	21.27 ± 7.52	22.73 ± 8.59	20.81 ± 7.86	19.44 ± 7.35	19.78 ± 7.47	22.98 ± 10.27

We compared rCBF of rats in (a) the saline- and saline+inhibitor-treated groups, and (b) the nitrite- and nitrite+inhibitor-treated groups. Baseline rCBF was not significantly different among the groups. Data are mean ± SEM of the indicated number of animals (**P* < 0.05).

Table 2. Baseline MABP (mmHg)

(a)	Saline	L-NNA + Saline	ODQ + Saline	C-PTIO + Saline	Allopurinol + Saline	Indomethacin + Saline	PGISI + Saline
	135.20 ± 47.80	118.92 ± 39.64	110.66 ± 36.88*	124.45 ± 41.48	116.78 ± 41.29	120.59 ± 40.19	122.74 ± 37.00
(b)	NaNO ₂ 1.0 mg	L-NNA + NaNO ₂	ODQ + NaNO ₂	C-PTIO + NaNO ₂	Allopurinol + NaNO ₂	Indomethacin + NaNO ₂	PGISI + NaNO ₂
	113.38 ± 42.85	126.21 ± 44.62	124.54 ± 47.07	126.44 ± 47.79	118.16 ± 44.66	114.47 ± 43.26	114.45 ± 51.18

We compared MABP of rats in (a) the saline- and saline+inhibitor-treated groups, and (b) the nitrite- and nitrite+inhibitor-treated groups. The MABP of rats in the saline group treated also with ODQ was significantly lower than that in the saline-alone group. Data are mean ± SEM of the indicated number of animals (**P* < 0.05).

and rather GC-, COX-, xanthine oxidase-, and PGIS-dependent. L-NNA, ODQ, C-PTIO, allopurinol, indomethacin, and U-51605, all seem to have infarct-reducing effect themselves, like NaNO₂ alone, compared with the saline group. However, we still found significant difference in experiments using L-NNA. Therefore, infarct-reducing effects of these inhibitors may not have critical impact on the present report, while having some confounding effects. The reduction of MABP was seen by the vasodilator effect of NaNO₂; however, this reduction did not exacerbate the cerebral infarction. Furthermore, although MABP was increased along with L-NNA administration, the cerebral infarction area was not reduced. Therefore, this suggests that blood pressure fluctuation did not affect our results. This conclusion is, in part, in agreement with the results of a recent study that demonstrated the generation of NO in a manner dependent on nitrite but independent on NOS inhibitors in the re-perfused myocardium.⁸ Brain hypoxemia during occlusion-reperfusion injury could result in impairment of brain NOS since oxygen is essential for the reaction involved in the production of NO from L-arginine and catalyzed by NOS. Similar to myocardial ischemia, nitrite produced NO in our study, which could potentially serve to alleviate ischemic brain injury.

Our results showed that ODQ inhibit the effect of NO. This finding suggests that the neuroprotective effect of nitrite-derived NO in brain injury is mediated via GC, in agreement with the results of two previous studies.^{2,9} Another recent study showed that xanthine oxidase can generate NO by reducing nitrite in the presence of NADH.¹⁰ The reaction occurred even under low tissue oxygen levels. Our results also showed that xanthine oxidase inhibitor suppressed the neuroprotective effects of nitrite-derived NO, in agreement with the results of previous studies.¹⁰⁻¹²

Both indomethacin and U-51605 inhibited the neuroprotective effects of nitrite-derived NO. Considered together with the other findings in the present study, the results suggest that the neuroprotective effects of NO are mediated through COX and prostacyclin. In this regard, previous studies reported that the NO/COX/cAMP (cyclic adenosine 3',5'-monophosphate) pathway is independent of GC and cGMP (cyclic guanosine monophosphate), and is involved in the opening of potassium channels and various physiological processes.^{2,13,14} The results in the present study also demonstrated the involvement of this pathway in the brain vasculature, consistent with other studies that showed the involvement of the same pathway in ocular vascular beds.¹³⁻¹⁵ This study may provide the therapeutic insight of using nitrite for patients suffering

from stroke and/or brain injury. Herein, we have demonstrated that nitrite-derived NO protects the brain against ischemia-reperfusion injury likely through NOS-independent but GC-, COX-, xanthine oxidase-, and PGIS-dependent pathways.

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