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ORIGINAL ARTICLE

Dietary supplementation with sodium nitrite can exert neuroprotective effects on global cerebral ischemia/reperfusion in mice

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Abstract
Background Nitrite-derived NO protects against middle cerebral artery occlusion in mice. We developed a new mouse model of global cerebral ischemia and reperfusion (GCI/R) involving reversible occlusion of the major vessels from the aortic arch supplying the brain, and investigated neuroprotection with dietary sodium nitrite supplementation against GCI/R injury.
Methods Mice received drinking water with (nitrite group) or without (control group) sodium nitrite (2 mM) for 5 days and underwent 3-min GCI/R by reversible occlusion of major vessels from the aortic arch (i.e., brachiocephalic, left common carotid, and left subclavian artery). Survival rates and neurological function scores were evaluated for up to 5 days after GCI/R. Histopathological studies were performed to detect neurological degeneration and caspase-3 activation in serial hippocampal sections.
Results In the control group, 17/30 mice (57 %) survived 5 days after 3-min GCI/R, whereas in the nitrite group 25/30 mice (83 %) survived ($p < 0.05$). The neurological score at 5 days after GCI in control group was significantly higher than in the nitrite group. Cerebral blood flow (CBF) during GCI was significantly higher in the nitrite group than in the control group, while MABP did not differ significantly between groups. Degenerative changes and caspase-3 activation in hippocampal sections after GCI were observed in the control group but not in the nitrite group. Pretreatment with the NO scavenger c-PTIO abolished the neuroprotective effects of sodium nitrite.
Conclusions Sodium nitrite supplementation attenuated mortality and neurological impairment after 3-min GCI in mice; an effect likely mediated via vascular mechanisms involving NO.

Keywords Nitrite · Neuroprotection · Global cerebral ischemia and reperfusion

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Introduction

Nitrite is an oxidative breakdown product of nitric oxide (NO) that has traditionally been viewed as an acute marker of NO flux/formation in biological systems, and has recently been shown to be a storage pool for NO that can be released via several tissue-specific pathways during hypoxia and acidosis [1]. In short, as nitrite is converted to NO mainly in hypoxic regions, NO generation rates increase as oxygen concentrations and pH drop. In this manner, nitrite could act as a prodrug for NO and would be expected to have fewer off-target side effects than direct treatment with NO.

NO is known for its involvement in numerous physiological processes under normal conditions. Besides its role as a potent vasodilator, NO acts as an endothelial survival

factor preserving endothelial integrity [2], improves cell survival [3], inhibits platelet and neutrophil aggregation [4], scavenges free radicals [5], and inhibits mitochondrial respiration [6]. Recently, it has been reported that during ischemia/reperfusion, modulation of these processes by NO can contribute to the outcome of tissue injury. Furthermore, a number of animal studies have demonstrated that physiological and therapeutic levels of nitrite exert potent cytoprotection after prolonged ischemia and blood-flow reperfusion in liver [7, 8], heart [9, 10], kidney [11], and brain [5]. Additionally, Dezfulian and colleagues reported that administration of nitrite at the initiation of cardiopulmonary resuscitation improved outcomes in a murine cardiac arrest model, presumably by releasing NO [12]. The cardiac arrest model would result not only in neuronal injury but also damage to other vital organs (including heart, liver, kidney, etc.); therefore, attenuation of mortality and neurological impairment in this model following nitrite administration might result from preservation of the function of organs except the brain. Thus, studies using cardiac arrest models may be unable to reveal the precise mechanism underlying the neuroprotective effect of nitrite against global cerebral ischemia and reperfusion (GCI/R). Consequently, we have developed a new mouse model of GCI/R involving reversible occlusion of the major vessels originating from the aortic arch and supplying the brain, and we then investigated the neuroprotective effects of dietary sodium nitrite supplementation against GCI/R injury.

Materials and methods

Animals

All animal protocols were approved by the Institutional Animal Care Committee, University of the Ryukyus. Adult male C57/BL6 mice (8–10 weeks old) were used.

Anesthesia and monitoring

Male mice (C57/BL6) were anesthetized with 5 % isoflurane in a 100 % O_2. The mice were then orotracheally intubated and mechanically ventilated (Rodent Ventilator, Model 683, Harvard Apparatus) with isoflurane (1–2 %). Pharynx temperature was maintained at 37.0 °C by a feedback-controlled heating pad (Small Animals Heat Controller, Model ATC-101B, Unique Medical). Cerebral blood flow (CBF) was determined by laser-Doppler flowmetry (Advance Laser Flowmeter, Model ALF-21, Advance Co.) using a flexible fiber-optic extension. The tip of the probe was affixed to the surface of the skull over the brain. Mean arterial blood pressure (MABP) was monitored via a cannula in the left femoral artery (PowerLab, AD Instruments).

GCI model

Prior to surgery, mice received 100 IE of heparin intraperitoneally. A ventral midline cervicothoracic incision was made and the chest wall was incised from the apex of the caudal manubrium along the median sternum to the second rib. The major vessels originating from the aortic arch (i.e., brachiocephalic artery, left subclavian artery, left common carotid artery) were gently separated. A clip was placed on the left subclavian artery followed by the left common carotid artery and then the brachiocephalic artery. At 3 min post-ischemia, all clips were removed and the chest was closed in layers. Protamine sulfate (1 mg) was then administered intraperitoneally. At 5 min post-reperfusion, the arterial catheter was removed, the incision was closed, and the animals were allowed to recover from anesthesia.

Neurological function

On postoperative days 1–5, a neurological score based on a maximum of 14 points for the most severe deficits was utilized to evaluate post-ischemic neurological function [13]. Briefly, eight parameters were assessed and scored: grasping movement reflex (present at all paws = 0, 1 point for each missing reflex = 1~4), stop at the edge of a table (stopping at the end of table = 0, no stopping behavior = 1), turning the head when touching the ear from behind with a small rod (turning towards both directions = 0, 1 point for each failure to turn towards the stimulated ear = 2), falling reflex (extension of both front paws = 0, not reaching down towards the ground with both front paws = 1), spontaneous motility (immediate, spontaneous activity = 0, activity after 30–60 s = 1, no activity within 60 s = 2), circling behavior (no circling, moving in all directions = 0, spontaneous circling, turning only to one direction = 2), pelt property (clean, well kept = 0, coarse, scrubby = 1), and flight reaction (normal behavior = 0, anxious, jumpy behavior = 1). The total score was reported as the neurological function score (total possible score = 14).

Histological analysis of neuronal injury in hippocampal CA1 region

To process brain tissue for histological and immunohistochemical examination, mice were anesthetized with 5 % isoflurane. The mice were then transcardially perfused with heparinized saline followed by 4 % paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4). After postfixation for several hours, the brains were removed and embedded in paraffin, and cut into 6-μm-thick coronal sections. Slides were stained with cresyl violet according to the Nissl method and evaluated for evidence of cellular degeneration. Cells that contained Nissl substance in the cytoplasm,

loose chromatin, and prominent nucleoli were considered to be normal neurons, and ischemic neurons were identified by loss of Nissl substance and by the presence of pyknotic homogenous nuclei.

Activation of caspase-3 was assessed by immunohistochemistry in paraffin-embedded coronal sections using a rabbit monoclonal antibody against cleaved caspase-3 (1:50, Cell Signaling) according to the protocol recommended by the manufacturer of activated cleaved caspase-3 positive neuron in hippocampal CA1 region under high-power magnification (200×) by an investigator (TK) blinded as to the identify of mice.

Study 1: relationship between ischemic time and 5-day mortality

For the quantal bioassay of the relationship between GCI duration and 5-day mortality, the duration of GCI was selected to span of mortality from survival or death until 5 days after GCI. Based on our pilot experiments, the duration of GCI for individual animals was varied from 2.5 min up to 4 min (2.5 min $n = 5$, 3 min $n = 13$, 3.5 min $n = 8$ and 4 min $n = 5$). The P50 value represents the duration of ischemia (in minutes) associated with a 50 % probability of death 5 days after GCI.

Study 2: investigation of the neuroprotective effects of dietary sodium nitrite supplementation against GCI.

Sodium nitrite therapy

Mice were randomly divided into two groups as follows: the nitrite group ($n = 30$) was comprised of mice given drinking water containing sodium nitrite (2 mM) for 5 days before and after GCI; the control group ($n = 30$) received drinking water without sodium nitrite. According to the results derived from study 1, 3 min of GCI was determined as an adequate duration of ischemia for subsequent studies. Since our preliminary study using this GCI model showed that 2 mM sodium nitrite containing drinking water exerted a better survival rate than 0.5 or 10 mM sodium nitrite, we made a choice for the sodium nitrite concentration of drinking water as 2 mM.

For investigating whether NO might be involved into the neuroprotective effect of sodium nitrite therapy, Carboxy-PTIO [2-(4-carboxyphenyl)-4,4,5,5–tetramethylimidazoline–1–oxyl–3–oxide potassium salt] (Sigma-Aldrich), a direct intravascular NO scavenger, was used. Mice given drinking water containing sodium nitrite (2 mM) for 5 days were divided into two groups as follows; Nitrite + PTIO group ($n = 9$) was comprised of mice given intraperitoneally carboxy-PTIO dissolved in saline intraperitoneally at a dose of 1 mg/kg 30 min before GCI; Intraperitoneal saline instead of carboxy-PTIO was given in nitrite group ($n = 9$).

Determination of NOx level in plasma

Concentrations of NOx were measured in plasma samples obtained from mice with ($n = 5$) or without ($n = 5$) nitrite (2 mM) supplementation for 5 days by using the Griess method with an automated NO detector/high-performance liquid chromatography system (ENO-20; Eicom, Kyoto, Japan).

Cyclic guanosine monophosphate measurement in brain tissue

Cyclic guanosine monophosphate (cGMP) levels were measured in brain tissue taken from the mice during GCI. Mice were decapitated after 2 min of GCI, and brain tissue was immediately harvested and snap-frozen in liquid nitrogen. Because significant differences in CBF during GCI had already been observed at 2 min of GCI, we decided to measure cGMP levels in brain tissue harvested at 2 min of GCI. The cGMP measurements were performed using a sandwich enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. Two groups were studied: a nitrite supplementation group ($n = 6$) and a control group ($n = 6$).

Statistical analysis

All data are expressed as mean ± SEM. The number of animals for each measurement was determined by a power analysis using a type I error estimate of 0.05, a power of 90 %. Neurological function scores were compared between groups using the Wilcoxon Mann–Whitney rank sum test, and p values were adjusted for multiple comparisons according to Bonferroni's method. Quantal bioassay results were analyzed based by logistic regression analysis [14]. Differences in survival rates were analyzed by log-rank test. A p value <0.05 was regarded as statistically significant.

Results

Survival rate after GCI

All mice were subjected to 2.5, 3, 3.5, or 4 min GCI. The survival rates at 5 days after 2.5, 3, 3.5, or 4 min of GCI were 100, 61, 25, and 0 %, respectively (Fig. 1). Quantal bioassay analysis revealed that the P50 was 3.19 ± 0.81 min (Fig. 2). According to these results, 3 min of GCI was determined as an adequate ischemic interval in this study to evaluate neurological outcomes after 5 days of reperfusion.

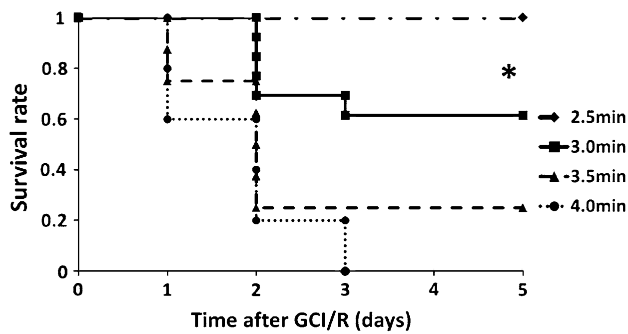

Fig. 1 Survival rate during the first 5 days after global cerebral ischemia–reperfusion. There was a significant difference in survival rates among groups after global cerebral ischemia–reperfusion for 2.5–4 min ($p < 0.05$). 2.5 min ($n = 5$), 3 min ($n = 13$), 3.5 min ($n = 8$), and 4 min ($n = 5$)

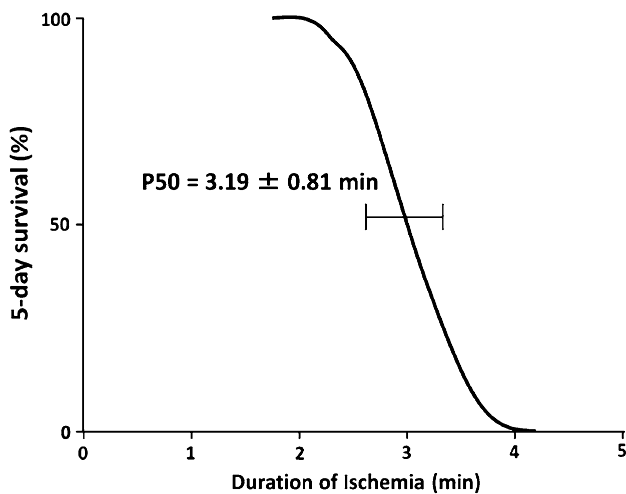

Fig. 2 The 50 % probability of 5-day survival, representing the relationship between the duration of global cerebral ischemia and mortality, was 3.19 ± 0.81 min

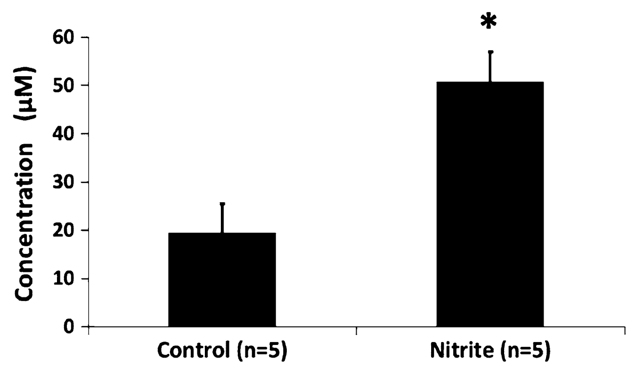

Fig. 3 Chronic sodium nitrite therapy alters blood nitrite levels. Concentrations of NOx in plasma at day 5 for MilliQ control and sodium nitrite (2 mM)

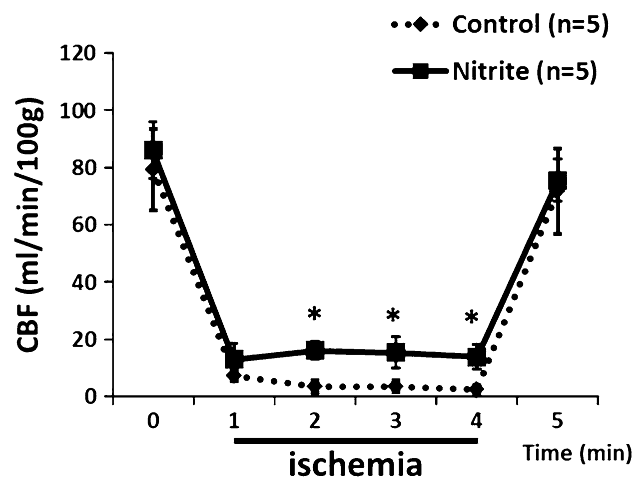

Fig. 4 Effects of nitrite on cerebral blood flow and blood pressure. Laser-Doppler flowmeter values are presented. The values are compared within the group at the indicated timepoints, and the cerebral blood flow data are shown as mean ± SD. Significant differences are denoted by *$p < 0.01$ ($n = 5$, multiple comparison by Bonferroni and Tukey–Kramer tests)

Plasma NOx concentration

As shown in Fig. 3, sodium nitrite supplementation for 5 days significantly increased plasma NOx levels compared with the control group.

Measurement of MABP and CBF

Before GCI, there were no significant differences in MABP and CBF between the groups. MABP was increased immediately after onset of GCI and remained constant during the ischemic period in all mice. CBF in the control group was immediately reduced to less than 10 ml/min/100 g (10 % of baseline) after the major vessels were clipped and it remained constant during GCI. Although CBF in the nitrite group also decreased immediately after onset of GCI, it was significantly higher during GCI in the nitrite group than in the control group, while MABP did not differ significantly between groups (Fig. 4; Table 1).

Table 1 Effects of nitrite on blood pressure

	Control ($n = 3$)	Nitrite ($n = 4$)
Blood pressure (mmHg)		
Pre-ischemic	80.7 ± 6.0	84.8 ± 10.8
Ischemic 0 min	89.0 ± 10.1	92.0 ± 12.6
Ischemic 1 min	125.0 ± 2.5	120.0 ± 7.1
Ischemic 2 min	130.3 ± 25.5	130.3 ± 4.5
Ischemic 3 min	130.0 ± 20.0	116.5 ± 19.8
Postischemic	87.3 ± 11.7	70.3 ± 9.0*

Data are presented as mean ± SD

* $p < 0.05$ compared with control

Nitrite improves long-term survival rate and neurological function after GCI

Although only 57 % of mice (17/30) in the control group survived 5 days after GCI, 83 % of mice (25/30) in the nitrite group survived 5 days after GCI ($p < 0.05$). Neurological function of the surviving mice in the control group ($n = 4$) gradually worsened starting at approximately 4 days after GCI, and at 5 days after GCI was significantly higher neuroscore in the control group than in the nitrite group ($n = 4$), suggesting that even the surviving mice in the control group had worsened neurological function at 5 days after GCI as compared with the nitrite group (Fig. 5).

Nitrite therapy is associated with cGMP

We evaluated the effect of nitrite on the levels of cGMP in mouse brain during GCI. The activity of cGMP during GCI was significantly higher in the nitrite group than in the control group (Fig. 6).

Neuronal injury in the hippocampal CA1 sector

In the control group, although few neurons in the CA1 sector showed degeneration at 2 days after GCI, most of the pyramidal neurons exhibited pyknotic, shrunken nuclei at 5 days after GCI, which was observed consistently. In contrast, 3 min of GCI in the nitrite group did not affect the appearance of neurons in the CA1 sector at 2 days and 5 days of GCI (Fig. 7a).

Immunohistological analysis revealed that neurons containing cleaved caspase-3 in the CA1 sector of the hippocampus appeared at 3 days post-GCI in the control group. On the other hand, sodium nitrite supplementation for 5 days before GCI inhibited the activation of caspase-3 in the CA1 region throughout this experiment (Fig. 7b).

Enhancement of cerebral blood flow by nitrite is dependent on NO

To investigate whether NO production from nitrite would be associated with neuroprotection after GCI, c-PTIO was administered intraperitoneally 30 min before GCI. Figure 8 shows that pretreatment with c-PTIO completely abolished the neuroprotective effects of sodium nitrite therapy.

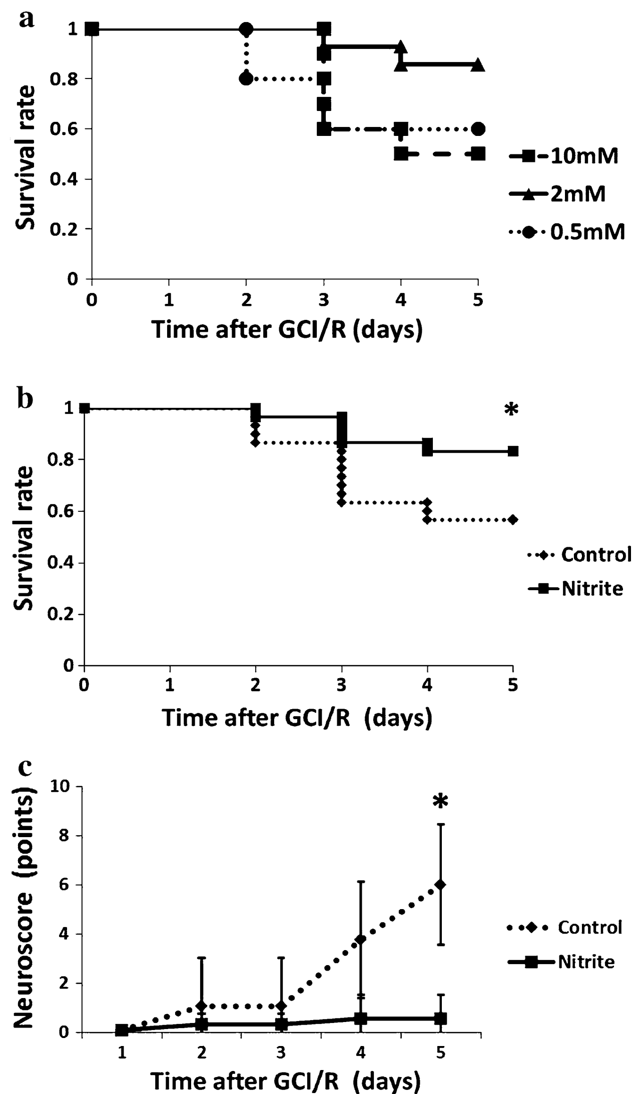

Fig. 5 **a** Survival rate during the first 5 days after global cerebral ischemia–reperfusion. *Control* mice subjected to global cerebral ischemia–reperfusion. *Nitrite* mice subjected to global cerebral ischemia–reperfusion treated with nitrite. *$p < 0.05$ vs. control. **b** Neurological function was determined on post-operative days 1–5. Only animals surviving the whole experimental observation period of 5 days were included in the analysis. Neurological function scores in control mice were significantly increased in comparison to the scores in global cerebral ischemia–reperfusion mice subjected to nitrite treatment (mean ± SD: Significant differences are denoted by *$p < 0.05$; $n = 14$, multiple comparison by Bonferroni and Tukey–Kramer tests)

Discussion

In the present study, using a new mouse model of GCI we have demonstrated that sodium nitrite therapy reduced mortality after 3-min GCI, and attenuated neurological dysfunction and histopathological changes in the CA1 sector of the hippocampus after reperfusion. In the group that received sodium nitrite therapy, CBF was significantly higher than that in the control group, and cGMP levels in the cerebrum were also significantly higher in the nitrite group during GCI, indicating that cerebral vascular dilatation and a subsequent increase in collateral blood flow may be associated with the attenuation of neurological

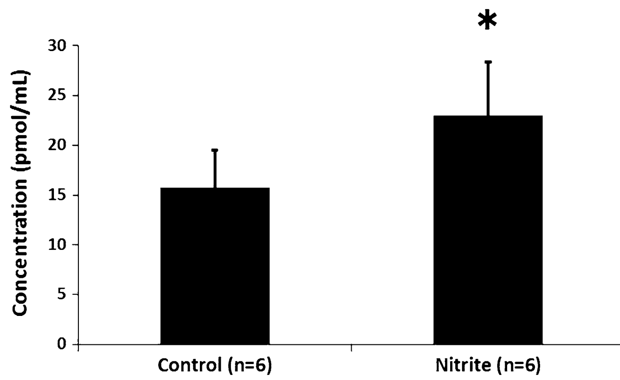

Fig. 6 Neuroprotection mediated by nitrite-derived NO. Nitrite treatment increases cGMP levels in the brain after global cerebral ischemia. $*p < 0.05$ vs. the control global cerebral ischemia group (Student's *t* test and Mann–Whitney *U* test)

impairment. Additionally, the neuroprotective effect of sodium nitrite therapy and its ability to attenuate mortality was abolished by intraperitoneal pretreatment with c-PTIO, suggesting that cerebral vasodilation by NO might be related, in part, to neuroprotective effects of sodium nitrite therapy after GCI in mice.

Several models of GCI have been developed in different species in order to investigate circulatory arrest-induced brain damage under laboratory conditions. However, techniques previously employed in rats and other rodents did not yield sufficiently robust results in mice because of the high variability of collateral flow in that species. One of the most widely used approaches to induce GCI is bilateral carotid occlusion with hypotension. Despite thorough surgical techniques and excellent physiological monitoring, these approaches resulted in inconsistent reduction of CBF due to residual collateral blood flow through the basilar artery. Another approach used to induce GCI is the three-vessel occlusion model, where the occlusion of carotid arteries is combined with clamping of the basilar artery; however, this method has major limitations and is surgically challenging. Alternatively, several authors have investigated the induction of global cerebral ischemia in mice by cardiac arrest. However, in the cardiac arrest model, blood flow to the brain as well as to other organs, including heart, lung, liver, kidney, etc., is compromised. In our new GCI model, clamping of three major vessels (brachiocephalic, left carotid, and left subclavian artery) originating from the aortic arch produced substantial reduction of CBF (less than 10 % of baseline), but did not reduce blood flow to other organs. Our study using this model revealed that GCI for longer than 3 min was associated with increased mortality at 5 days after GCI (Fig. 1). Mice that underwent GCI for 3 min also consistently showed gradual development of neurological dysfunction starting at approximately 4 days after GCI, as well as evidence of neurodegeneration in the CA1 sector of the hippocampus 5 days after reperfusion. It is now well known that injury to the brain initiated during ischemia continues, and is magnified, during the post-ischemic period. This reperfusion injury is caused in part by such processes as excitotoxicity, oxidative stress, metabolic

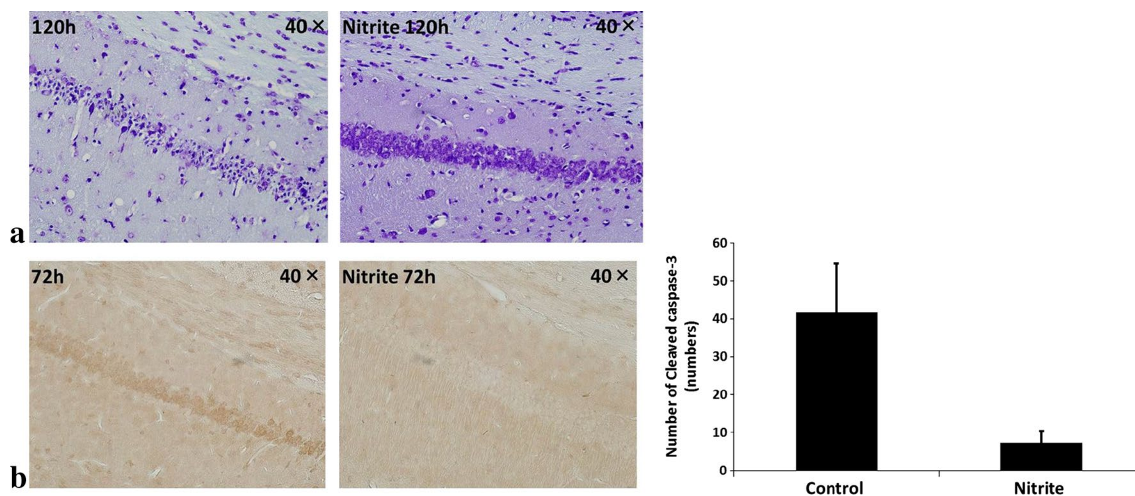

Fig. 7 **a** Neuronal cell death in the hippocampus after global cerebral ischemia–reperfusion. Photomicrographs showing histopathological changes in the hippocampal CA1 region after 3-min global cerebral ischemia–reperfusion (Nissl staining). **b** Representative photomicrographs of the hippocampal CA1 region of mice subjected to 3-min global cerebral ischemia–reperfusion showing cleaved caspase-3–immunoreactive neurons. Representative sections of approximately the same portion of the hippocampal CA1 region are shown for the control and nitrite-treated groups. Global cerebral ischemia–reperfusion resulted in significant cell death compared to control, which was to a large extent reversed by nitrite therapy

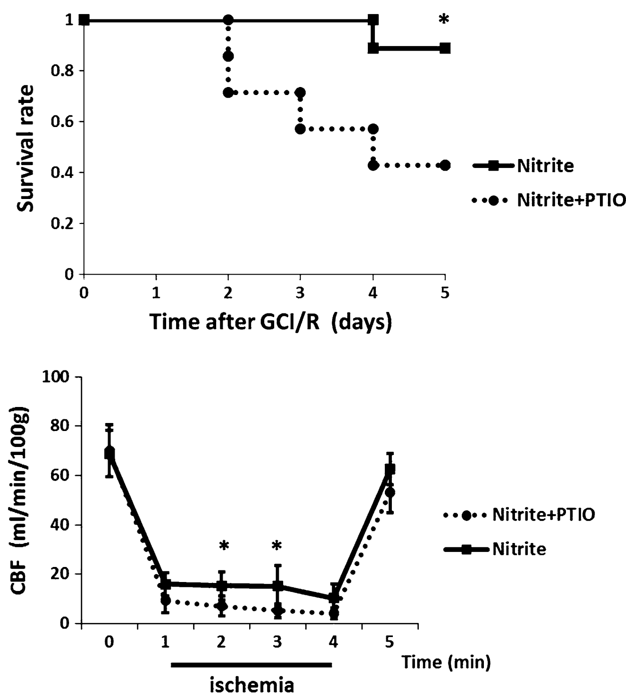

Fig. 8 Evaluation of corrected survival rate and cerebral blood flow demonstrated that carboxy-PTIO completely inhibited the neuroprotective effects of nitrite

failure, inflammation, apoptosis, and other mechanisms. Apoptotic processes are known to be particularly important in the development of delayed neuronal death in the hippocampus after GCI. It is likely that in our model, and likely other models as well, neurodegeneration was preceded by activation of caspase-3 in hippocampal neurons, which might also be capable of inducing apoptotic processes leading to delayed death of hippocampal neurons. We therefore believe that this new model could be a viable alternative to traditional GCI models.

Nitrite is an oxidative breakdown product of NO that has been shown to serve as an acute marker of NO flux/formation [15]. Recently, nitrite has moved to the forefront of NO biology with the discovery that it represents a critical NO storage form in both blood and tissues [16]. Interestingly, a report by Kleinbongard and colleagues [17] demonstrated that plasma nitrite levels progressively decrease with increasing cardiovascular risk. A number of studies in different animal species have confirmed the protective effects of low-dose nitrite in various settings of ischemia/reperfusion injury, including models of kidney ischemia [11], hepatic injury [18], lung injury [19], acute myocardial infarction [10, 20], cardiac arrest [12], and chronic limb ischemia [21]. With respect to the central nervous system, a study by Jung and associates also demonstrated neuroprotective effects of nitrite therapy against focal cerebral ischemia in mice, consistent with the present results [5].

In the present study, the attenuation of neurological impairment by sodium nitrite therapy was abolished by intraperitoneal pretreatment with c-PTIO, suggesting that NO might be related, in part, to the beneficial effects of sodium nitrite therapy in GCI mice. It has recently been established that, during hypoxia, nitrite can be converted into NO by acidosis, deoxyhemoglobin, xanthine oxidoreductase, complexes of the mitochondrial electron transport chain, cytochrome P450, the enzyme nitric oxide synthase, and other factors [22]. Since GCI should rapidly progress to a severe hypoxic condition and acidosis in the brain, we speculated that dietary sodium nitrite therapy increased the systemic level of nitrite and NO was generated from nitrite so efficiently in the brain after the onset of GCI that it resulted in neuroprotection.

In general, the neuroprotective effect evoked by NO can be caused by vascular and/or non-vascular mechanisms. NO receptors possess intrinsic guanylyl cyclase activity and thus cGMP accumulates in the cells when they are stimulated. Regarding the vascular mechanism responsible for the neuroprotective effect of NO, selective arteriolar vasodilatation in hypoperfused areas would be mediated via this NO/guanylyl cyclase/cGMP pathway without concomitant systemic hypotension. Our measurements of cGMP in the brain showed significantly higher levels during GCI in the nitrite group than in the control group. Correspondingly, CBF in the nitrite group also remained roughly above 18 ml/min/100 g, but it was less than 10 ml/min/100 g in the control group. Based on these data, it can be speculated that the neuroprotective effect of nitrite could be mediated, in part, via a vascular mechanism. In contrast, post-translational protein modification represents a non-vascular mechanism that may play a role in the neuroprotective effects of NO. For example, *S*-nitrosylation by NO is a post-translational regulatory mechanism that usually decreases the activity of the target protein. The present data suggest that activation of caspase-3 in the hippocampal area after GCI in this model might be one of the crucial factors responsible for the onset of neurodegeneration and subsequent increase in mortality rates. Since the main cytoprotective effects of NO have been attributed to the *S*-nitrosylation of thiol groups from cysteine residues, which can result in inhibition of caspase-3 and caspase-9, *S*-nitrosylation by NO of caspases including caspase-3 might suppress apoptosis in the brain after GCI, resulting in better neurological outcomes and attenuation of mortality.

The major health concern with inorganic nitrite is known for a risk for development of methemoglobinemia. In an animal study with intravenous infusion of high-dose sodium nitrite (1.8 μmol/min for 14 days), the blood methemoglobin level remained at clinically acceptable limits (<2.1 % of total hemoglobin level) even under a much higher plasma concentration of nitrite than our study [23].

We therefore speculated that sodium nitrite administration in this study produced much less blood methemoglobin level than toxicity.

Our data suggested that NO derived from nitrite under ischemic condition might be involved in neuroprotection after GCI, in part, by the vascular mechanism. It is, however, thought that NO derived from neuronal NOS in the ischemic brain produces cytotoxic effects, including peroxynitrite [24] and glutamate neurotoxicity [25]. In contrast, NO generated from endothelial NOS (eNOS) is known for potential neuroprotective effects through inducing vasodilation, which in turn decreases the severity of ischemia. In eNOS-deficient mice with cardiomyocyte-restricted overexpression of eNOS, which should have a higher level of blood nitrite concentration than eNOS-deficient mice, the neurological function score after cardiac arrest model was higher compared with eNOS-deficient mice [26]. This data suggested that intravascular nitrite might be associated with better neurological function after global cerebral ischemia, which is consistent with our data.

In conclusion, we demonstrated that supplementation with sodium nitrite could attenuate mortality and neurological impairment after 3-min GCI in mice. Although the precise mechanism should be elucidated in future studies, it can be speculated that this neuroprotective effect of sodium nitrite could be mediated, in part, via vascular mechanisms involving by NO.

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Conflict of interest Takasuke Fukuda, Manabu Kakinohana, Chitoshi Takayama, Masayuki Matsushita, and Kazuhiro Sugahara have no conflicts of interest to disclose.

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