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Early Intravenous Infusion of Sodium Nitrite Protects Brain Against In Vivo Ischemia-Reperfusion Injury

Keun-Hwa Jung, MD; Kon Chu, MD; Song-Yi Ko, MS; Soon-Tae Lee, MD; Dong-In Sinn, MD; Dong-Kyu Park, BS; Jeong-Min Kim, MD; Eun-Cheol Song, MD; Manho Kim, MD, PhD; Jae-Kyu Roh, MD, PhD

Background and Purpose—The rate of nitric oxide (NO) generation from nitrite is linearly dependent on reductions in oxygen and pH levels. Recently, nitrite-derived NO has been reported to exert a profound protection against liver and heart ischemia-reperfusion injury. In this study, we hypothesized that nitrite would be reduced to NO in the ischemic brain and exert NO-dependent neuroprotective effects.

Methods—Cerebral ischemia-reperfusion injury was induced by intraluminal thread occlusion of middle cerebral artery in the adult male rats. Solutions of sodium nitrite were infused intravenously at the time of reperfusion. Sodium nitrate and carboxy-PTIO (30 minutes before ischemic surgery), a direct NO scavenger, were infused for comparisons.

Results—Nitrite reduced infarction volume and enhanced local cerebral blood flow and functional recovery. The effects were observed at concentrations of 48 nmol and 480 nmol, but not at 4800 nmol nitrite and 480 nmol nitrate. The neuroprotective effects of nitrite were inhibited completely by the carboxy-PTIO. The 480 nmol nitrite attenuated dihydroethidium activity, 3-nitrotyrosine formation, and lipid peroxidation in the ischemic brain.

Conclusions—Nitrite exerted profound neuroprotective effects with antioxidant properties in the ischemic brains. These results suggest that nitrite, as a biological storage reserve of NO, may be a novel therapeutic agent in the setting of acute stroke. **(*Stroke*. 2006;37:2744-2750.)**

Key Words: cerebral ischemia-reperfusion injury ■ neuroprotection ■ nitric oxide ■ nitrite ■ oxidative stress

Recent insight into the basic mechanism involved in ischemic stroke indicates that endothelial dysfunctions along with the oxidative stress and inflammation represent a key step in the cerebral ischemia/reperfusion (I/R) injury.[1] Nitric oxide (NO) is primarily known for an endothelial survival factor maintaining the endothelial integrity and a vasodilator regulating the blood flow.[2] In addition to its major role, as a potentially protective agent, NO can improve neuronal survival, inhibit platelet aggregation and neutrophil adhesion, and scavenge reactive free radicals, thus reducing the ischemic injury.[3,4] However, a concomitant surge in production of superoxide and NO after reperfusion may lead to formation of peroxynitrite, a powerful oxidant.[5] So far, evidences have indicated that NO may be linked both to protective and toxic effects after I/R, depending on the level, the location, the source, and the environment.[6–8]

NO synthase (NOS) is a dominant physiological source of NO. However, the enzymatic activity of NOS requires oxygen and is blocked under hypoxia. Therefore, alternative pathways for hypoxic release of NO have high physiological relevance. The agents that liberate NO have been recognized as highly critical for therapeutic purposes especially in ischemic disorders. A variety of structurally different NO donors have been shown to limit infarct size by improving blood flow in the penumbra areas and reducing the oxidative stress in an NO-dependent fashion.[9–11] Recent works support the application of nitrite as a direct NO donor for treatment of ischemic disorders. The anion nitrite is reduced to form NO as a result of reduction by deoxyhemoglobin, myoglobin, tissue heme proteins, and nonenzymatic disproportionating.[12,13] The NO release from nitrite, and in parallel the vasodilatory effect, are increased under conditions of acidosis, hypoxia, and tissue I/R.[14] This improved understanding of the biochemical conversion of nitrite to NO has resulted in a great deal of interest in the potential beneficial effects of nitrite therapy in animal models of ischemia.[13]

The ischemic cerebral environment might allow for the acidic and hypoxic reduction of nitrite to NO. In this study, we investigated whether hypoxia-dependent NO production from nitrite in ischemic brain might limit I/R injury.

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Materials and Methods

Focal Transient Cerebral I/R

All the procedures were performed following an institutional approval in accordance with *NIH Guide for the Care and Use of Laboratory Animals*. Male Sprague-Dawley rats (12 weeks old; Genomics, Republic of Korea), weighing 200 to 220 grams, were randomly assigned to 3 experimental groups: drug-treated I/R group (n=87); saline-treated I/R group (n=30); and normal control group (n=9). Focal cerebral ischemia was induced by performing intraluminal filamentous occlusion of the middle cerebral artery for 90 minutes, according to methods that have been described.[15] After 90 minutes of middle cerebral artery (MCA) occlusion, the reperfusion of the MCA was initiated by removing the MCA occlusive filament. The right femoral artery was cannulated to monitor mean arterial blood pressure, arterial blood gases, and pH.

Experimental Protocols

The drug-treated rats were randomly assigned to nitrite-treated, nitrate-treated, and carboxy-PTIO-nitrite groups. Sodium nitrite and sodium nitrate (Sigma-Aldrich) were dissolved in phosphate-buffered saline and the pH was adjusted to 7.4. In all experiments, a final volume of 500 μL containing sodium nitrite (48 to 4800 nanomoles) or sodium nitrate (480 nmol) was administered intravenously to the rats for 1 minute via intravenous cannula situated in the tail vein at the time of reperfusion. Carboxy-PTIO [2-(4-carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt], a direct intravascular NO scavenger, was used to inhibit NO-dependent effects. Carboxy-PTIO (Alexis Biochemicals) was dissolved in phosphate-buffered saline and was administered intravenously at a dose of 1 mg/kg in a volume of 500 μL, 30 minutes before ischemia.

Evaluation of Infarct Volume

We measured infarction volumes (at 1 day after I/R, n=12 per group) using 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) staining, as described elsewhere.[15] The infarcted and total hemispheric areas of each section, at intervals of 1-mm thickness, were traced and measured using an image analysis system (Image-Pro Plus; Media Cybernetics). Two investigators blinded to the study protocol measured the infarct sizes with a computerized image analyzer. To compensate for the effect of brain edema, the corrected infarct volume was calculated: Corrected infarct area=Measured infarct area$\times${1$-$[(Ipsilateral hemisphere area$-$Contralateral hemisphere area)/Contralateral hemisphere]}.[15]

Local Cerebral Blood Flow Measurement

Laser-Doppler flowmetry (Perimed; Probe 403, Sweden) was performed to monitor local cerebral blood flow (LCBF) of ischemic cortical area supplied by the MCA throughout the I/R process. The animals were placed with the head immobilized in a stereotaxic frame and the probe was placed in a burr hole over the parietal cortex distal to the site of core ischemic damage. The LCBF was determined before ischemia, during ischemia, and then for 3 hours after injection of drug at the reperfusion. Flow values shown were expressed as percent of baseline.

Neurological Scores

To examine the effects of the nitrite (480 nmol) on the neurological deficits of rats after cerebral I/R, behavioral tests were performed by 2 investigators blinded to the treatment status of the rats with modified limb placing tests (MLPT) and Rotorod test.[15,16] The animals were monitored during the period of postoperative recovery starting at 2 hours, or 1, 3, or 7 days after I/R (n=6 per group).

Cyclic Guanosine Monophosphate Measurement in Brain Tissue

Levels of cyclic guanosine monophosphate (cGMP) in the ipsilateral hemisphere of ischemic brain with saline, nitrite (480 nmol), or nitrite (480 nmol) treatment after stroke in rats were measured (n=3 per group). The animals were killed at 1 day after I/R and the ipsilateral hemisphere cortical tissue was rapidly dissected. Levels of cGMP were measured using a commercially available low pH Immunoassay kit (R&D Systems Inc) according to the manufacturer's instruction. Values for cGMP were standardized by total protein (picomolars per milligrams tissue).

Analysis of Superoxide and Peroxynitrite Formation

Three hours after reperfusion, the rat brains were processed for the analysis of oxidative and nitrative stress (n=6 per group). The oxidative fluorescent dye dihydroethidium (DHE; Sigma-Aldrich) was used to evaluate in situ production of superoxide.[17] Frozen enzymatically intact brains were cut into 30-μm-thick sections and placed on a glass slide. The sections were simultaneously incubated with DHE (10 μmol/L) in phosphate-buffered saline for 30 minutes at 37°C, in a humidified chamber that was shielded from light. We also monitored peroxynitrite formation by detecting nitrosylated tyrosine residues on proteins. We performed immunostaining with anti-3-nitrotyrosine (3-NT) antibody (1:1000; Upstate Biotechnology). Coronal sections cut through the striatum (3 sections per brain, 1 mm in width) were imaged in parallel. Superoxide or 3-NT–positive cells were manually counted in the eight regions around the striatum under $\times$100 magnification using a laser scanning confocal microscopy with a Bio-Rad MRC 1024 (argon and krypton).

Measurement of Lipid Peroxidation

Malondialdehyde (MDA) was estimated as an indicator of lipid peroxidation (n=3 per group). The brain tissues were homogenized with sodium phosphate buffer (pH 7.4). The reagents (1.5 mL acetic acid, 1.5 mL thiobarbituric acid, and 0.2 mL sodium dodecyl sulfate) were added to 0.1 mL of processed tissue sample. The mixture was then heated at 100°C for 60 minutes. The mixture was cooled with tap water and 5 mL of n-butanol: pyridine (15:1) and 1 mL of distilled water was added. After centrifugation at 4000 rpm for 10 minutes, the organic layer was withdrawn and absorbance was measured at 532 nm using a spectrophotometer.

Statistical Analysis

All data in this study are expressed as means$\pm$standard deviations. Data were analyzed by 1-way analysis of variance (ANOVA) followed by Tukey test (when appropriate) or Student *t* test if they were normally distributed. In the case of LCBF measurement, values of different time points were compared by 2-way repeated measures ANOVA. Without normal distribution, we used the Mann–Whitney *U* test. $P<0.05$ was regarded as statistically significant.

Results

Nitrite Exerted Dose-Dependent Neuroprotective Effects in Ischemic Brains

In the ischemia-control group, I/R produced a well-defined infarct involving both the neocortex and the striatum (Figure 1A). We investigated the effects of nitrite (48 to 4800 nmol) or nitrate (480 nmol) on cerebral infarct size after I/R (Figure 1B to 1E). The nitrite-treated (48 and 480 nmol) group exhibited an infarct 33% and 77% smaller than that of saline-treated group (Figure 1F, n=12, $P<0.05$, ANOVA and Tukey's test). The administration of 4800 nmol nitrite and 480 nmol nitrate failed to limit cerebral infarct volume.

Nitrite Treatment Enhanced Local Cerebral Blood Flow Without Blood Pressure Changes

The experimental groups did not differ with respect to the pre-ischemic, intra-ischemic, or postischemic rectal temperature, arterial blood gases, and pH values. Mean arterial blood pressure also showed no significant differences between

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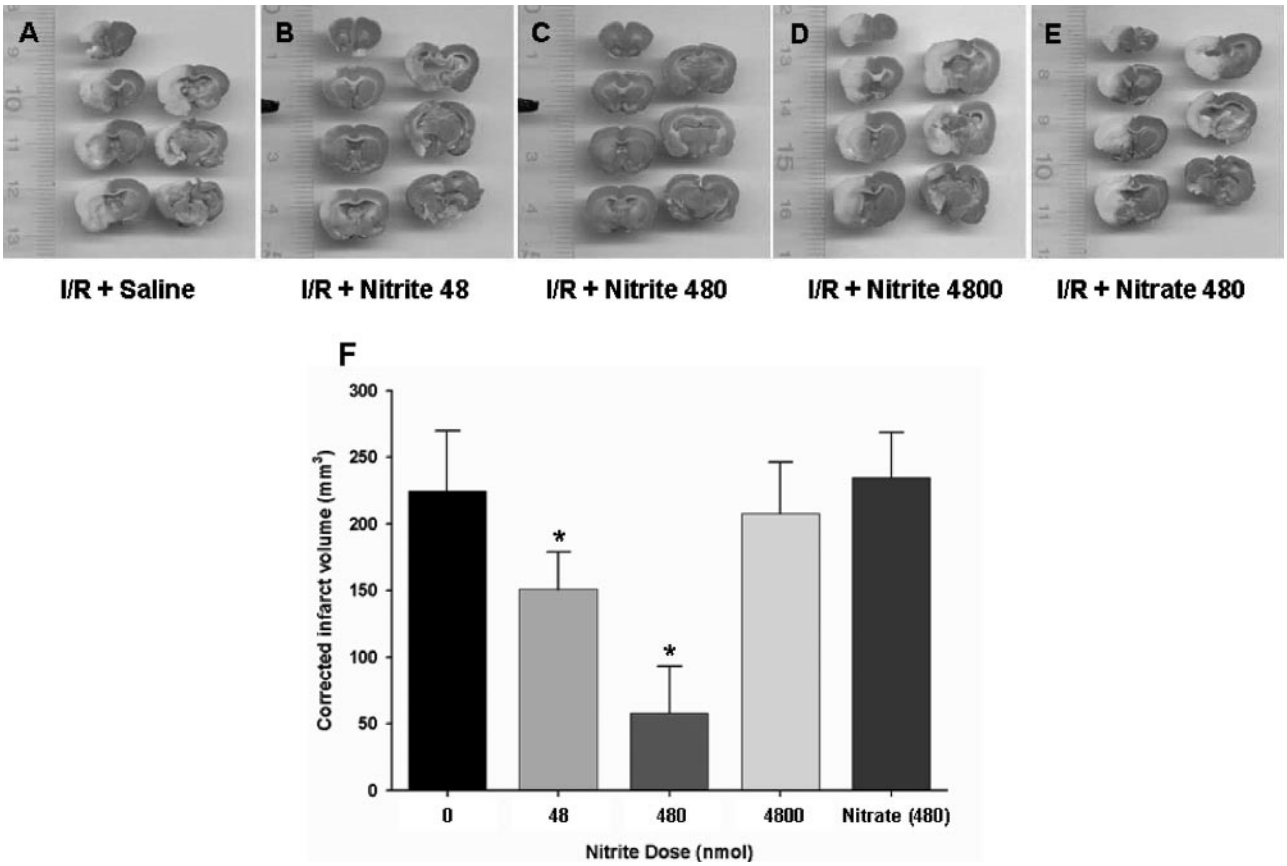

Figure 1. Nitrite-mediated protection against cerebral I/R injury. A to E, Representative photomicrographs of TTC staining 24 hours after I/R show the effect of saline, nitrite, or nitrate on the infarct volume. F, Evaluation of corrected infarction volume demonstrates that nitrite (48 and 480 nmol) has induced a reduction in infarction volumes as compared with the saline-treated or nitrate-treated groups. Bars represent mean±SD. *$P<0.05$ vs the saline-treated I/R group (n=12, ANOVA and Tukey test).

saline-treated and nitrite-treated (480 nmol) animals until 60 minutes after drug administration (see Table I, available at http://stroke.ahajournals.org). Dynamic changes in ipsilateral LCBF measured by laser-Doppler flowmetry were measured starting 30 minutes before induction of ischemia until 3 hours after reperfusion. In all groups, MCA occlusion resulted in an immediate reduction of LCBF to ≈10% of baseline. After reperfusion, a short period of postischemic hyperemia was followed by a decrease below normal values. The LCBF significantly increased in the ischemic hemisphere in intermediate-dose nitrite treatment (480 nmol; range, 158±12% to 102±10%), but not in nitrate treatment (480 nmol; range, 140±18% to 63±9%), immediately after drug administration at the reperfusion (Figure 2A; n=3, $P<0.05$, ANOVA). The increase in LCBF was independent of changes in arterial pressure and blood gases.

Nitrite Treatment Promoted Functional Recovery

The reduction in infarct volume by nitrite (480 nmol) was associated with a better performance on the MLPT and Rotorod test. Both the nitrite-treated group and the ischemia-control group exhibited equal deficits in neurological scale at 2 hours after I/R. The nitrite-treated group showed less neurological deficit and exhibited better recovery characteristics, with significant difference from day 1 after I/R (Figure 2B, 2C; n=6, $P<0.01$, t test). At day 7, the group of rats which had been treated with nitrite scored <2 points on the MLPT and exhibited a 25% better recovery on the rotarod test.

Neuroprotective Effects of Nitrite Are Dependent on NO

Although the carboxy-PTIO alone did not aggravate the infarct volume, pretreatment with carboxy-PTIO completely abolished the protective effects of nitrite therapy (Figure 3E; n=12, ANOVA and Tukey's test). In addition, we evaluated the effect of nitrite (480 nmol) or nitrate (480 nmol) on the levels of cGMP in ipsilateral cortex of rats with I/R injury. Saline-treated rats after I/R exhibited a significantly higher level of cortical cGMP compared with normal control rats. Administration of nitrite significantly increased cGMP (2.0±0.4 pmol/mg, $P<0.05$) compared with the saline-treated (1.4±0.3 pmol/mg) or nitrate-treated (1.4±0.3 pmol/mg) group in the ipsilateral hemisphere at 1 day after I/R (Figure 3F; n=3, $P<0.05$, ANOVA and Tukey test).

Adequate Dose of Nitrite Reduced the Oxidative Stress in the Ischemic Brain

In the normal rats, few evidence of DHE-induced EtBr fluorescence or 3-NT immunoreactivity was observed in the cortex and striatum. Superoxide-generating cells and 3-NT–

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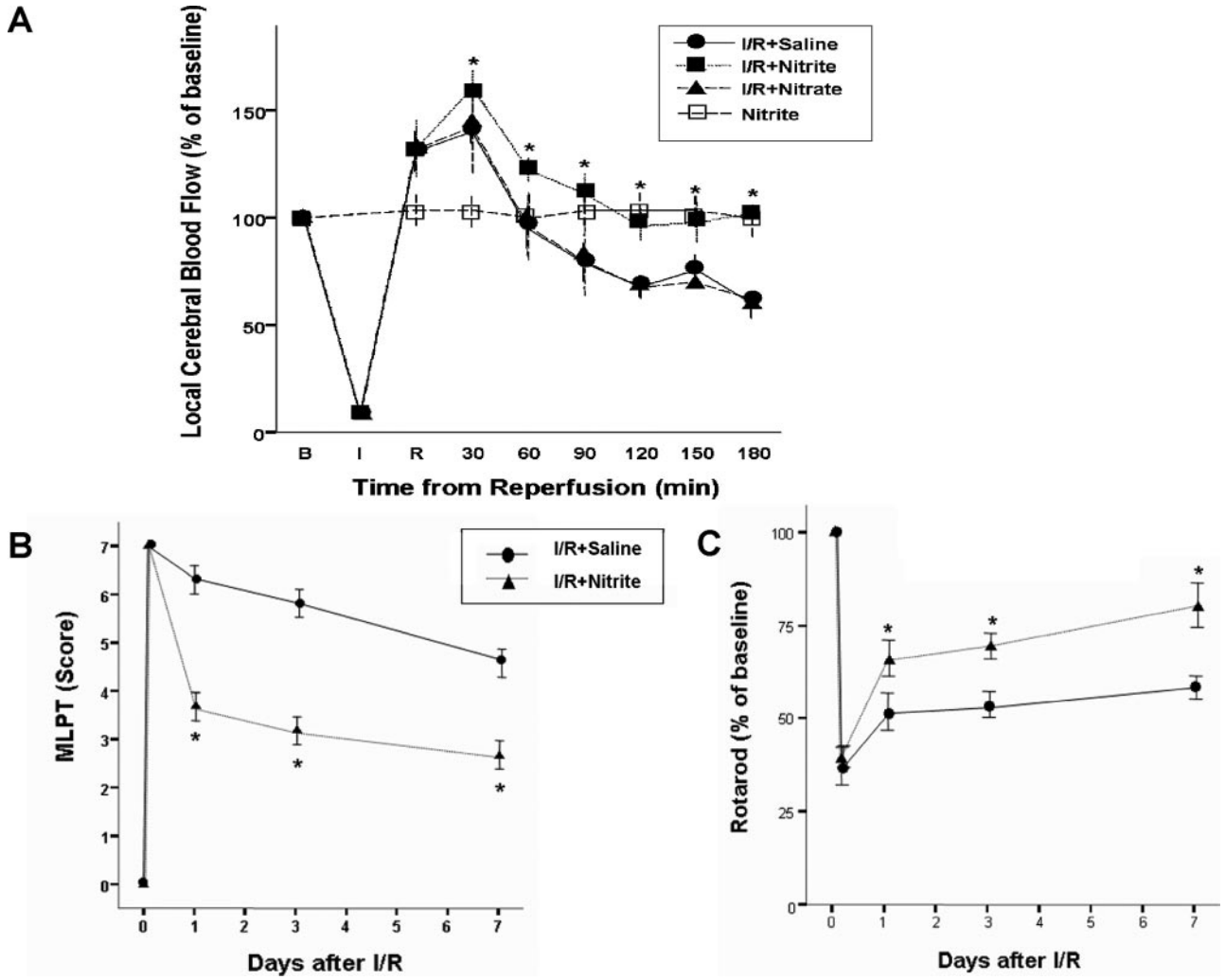

Figure 2. Effects of nitrite on LCBF and functional deficits. A, The laser Doppler flow values are presented as percent of combined averaged baseline values. Baseline, ischemia, and reperfusion points are represented by B, I, and R, respectively. The values are compared within the group at indicated time, and the LCBF data are shown as means±SD. Significant differences are denoted by *$P<0.05$ (n=3, ANOVA). Serial data from (B) MLPT and (C) rotarod test showed a clear effect of 480 nmol nitrite on the motor functions after I/R injury. In the MLPT and rotarod test at 2 hours, and 1, 3, and 7 days after I/R, nitrite-treated group exhibited less pronounced initial deficits, and recovered better than did the saline-treated I/R group. Bars represent mean±SD. *$P<0.05$ vs the saline-treated I/R group (n=6, *t* test).

positive cells were increased in the ischemic hemisphere (192±20, 103±23 cells per section, respectively), whereas only a few labeled cells were detected in the nonischemic hemisphere (Figure 4). Data from image analysis indicated decreases of superoxide (51±18 cells per section) and 3-NT production (44±19 cells per section) in the 480 nmol nitrite-treated group, compared with the ischemia-control group (Figure 4; n=6, $P<0.05$, ANOVA and Tukey test). In contrast, higher concentration of nitrite (4800 nmol) failed to attenuate the superoxide production (169±23 cells per section), and rather increased the 3-NT formation (138±12 cells per section) in the ischemic brain. These histological results were further supported by biochemical assay for MDA, a lipid peroxidation product. Nitrite (480 nmol) significantly decreased the levels of MDA (0.31±0.06 μg/mg protein) as compared with the saline-treated group (0.42±0.04 μg/mg protein) (Figure 4H; n=3, $P<0.05$, Mann–Whitney *U* test).

Discussion

The effective NO synthesis may be particularly important in protection against I/R injury. The NO release from nitrite is increased rapidly under conditions of acidosis or hypoxia. This concept has led to studies testing nitrite as a direct NO donor in experimental ischemia models, such as hypoxia-induced pulmonary vasoconstriction,[18] hemorrhagic stroke,[19] hepatic and cardiac I/R injury,[13] and renal I/R injury.[20] Nitrite exerted profound dose-dependent protective effects against hepatic and myocardial I/R, although it did not provide protection in renal I/R injury, suggesting a unique metabolism of nitrite in each tissue.[15,20] Cerebral ischemia also decreases the extracellular pH and oxygen level in the brain. Therefore, NO can be generated efficiently in the ischemic brain by direct reduction of nitrite. In our experiments, nitrite treatment conferred significant neuroprotection that was associated with a marked improvement in clinical recovery.

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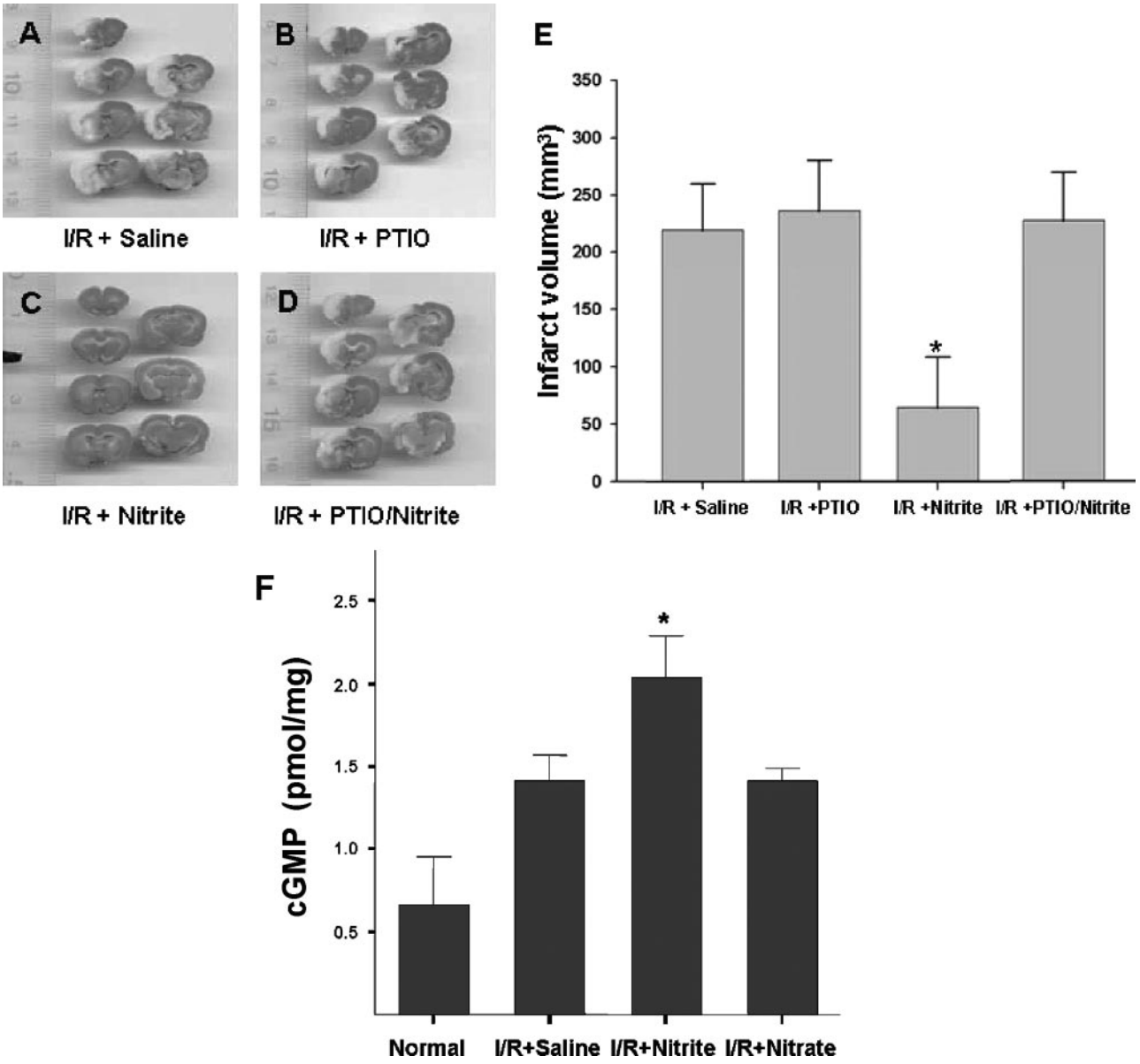

Figure 3. Neuroprotection mediated by nitrite-derived NO. A to D, Representative photomicrographs of TTC staining 24 hours after I/R showed the effect of the carboxy-PTIO on the infarct volume. E, Evaluation of corrected infarction volume demonstrated that carboxy-PTIO completely inhibited the neuroprotective effects of nitrite (n=12, ANOVA and Tukey test). F, Nitrite treatment increases the cGMP level in the ipsilateral cortex after I/R. *$P<0.05$ vs the saline-treated I/R group (n=3, ANOVA and Tukey test).

The vascular mechanism is responsible for the neuroprotective effect of nitrite. Low and nontoxic doses of sodium nitrite were able to increase LCBF rapidly in the ischemic brain without concomitant hypotension. These results support the concept that the biological activity of NO can exist in the local circulation around the ischemic brain. To further clarify a mechanism involving the hypoxic reduction of nitrite to NO, we preceded nitrite with carboxy-PTIO. The carboxy-PTIO has been reported to react with NO to generate NO_2 and PTI derivative in a stoichiometric manner.[21] In this study, the carboxy-PTIO completely inhibited protective effects of nitrite in the ischemic brain, suggesting the NO-dependent action of nitrite. In addition, our data that carboxy-PTIO alone did not affect the infarct size suggested that the carboxy-PTIO might preferentially quench the biological action of excess NO without affecting NOS activity or endogenous nitrite. Because the formation of carboxy-PTI and/or NO_2 may complicate the interpretation of data, the possibility that its reaction products contribute to the antagonism should be also considered.

The indirect way of demonstrating the NO-dependent mechanism of nitrite is to measure the degree to which NO receptors in the tissue are active. NO receptors possess intrinsic guanylyl cyclase activity, and so when they are stimulated, cGMP accumulates in the cells.[22] Our data that nitrite treatment increased the cGMP level in the cerebral I/R supported that nitrite effect was mediated via a NO/guanylyl cyclase/cGMP pathway. However, *S*-nitrosothiols and *N*-nitrosamines formed via reactions of nitrite with deoxyhemoglobin and possibly tissue heme proteins may have critical effects on protection against I/R injury.[23] To further demonstrate the pharmacological profiles of

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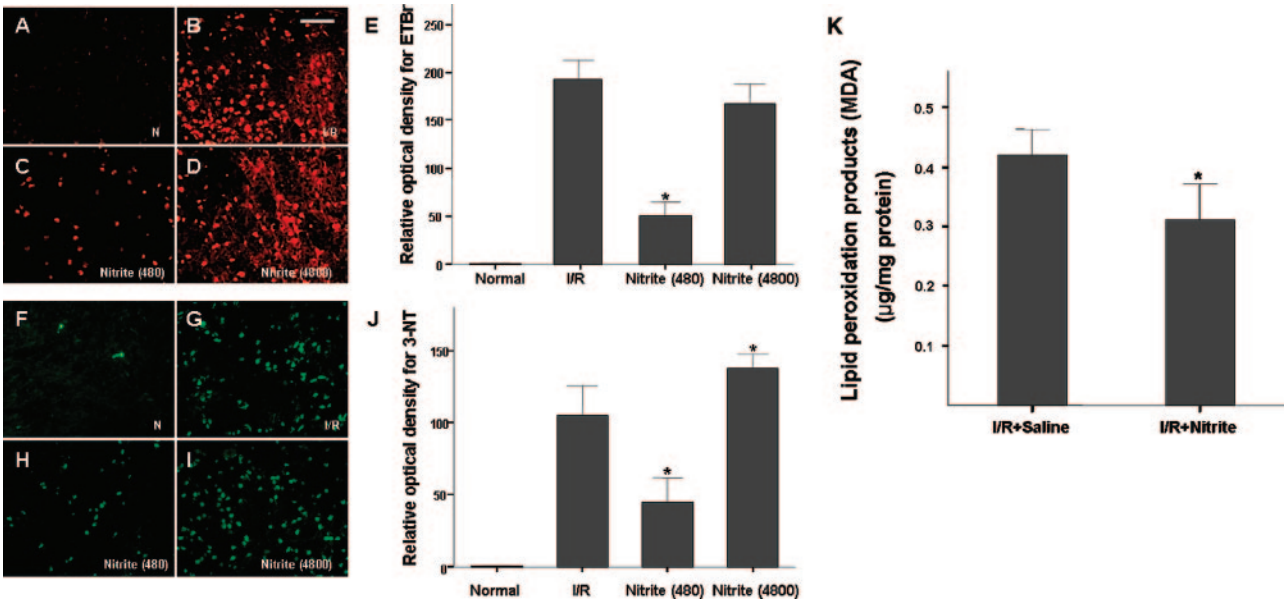

Figure 4. Antioxidant effects of nitrite. A to D, Fluorescent images after incubation with DHE exhibited superoxide levels in the ischemic brain (red). E, Quantitative measurements of the fluorescence indicated a significant decrease in the 480 nmol nitrite-treated group, as compared with the saline-treated I/R group. F to I, Representative photomicrographs showed 3-NT formation in the ischemic brain (green). J, Data from image analysis also indicated a significant decrease in the 480 nmol nitrite-treated group, as compared with the saline-treated I/R group. Bar=50 μm. *$P<0.05$ vs the saline-treated I/R group (n=6, ANOVA and Tukey test). K, Nitrite (480 nmol) significantly decreased the elevated MDA content in rat brain after I/R. *$P<0.05$ vs the saline-treated I/R group (n=3, Mann–Whitney *U* test).

nitrite in the cerebral I/R paradigm, the new analyzing techniques need to be developed to discriminate between the various NO-related compounds.

Another important finding in this study is that the nitrite effect is dose-dependent. NO is highly reactive with other free radicals, and the reaction between NO and superoxide can promote protection by reducing superoxide toxicity.[3,4] However, NO produced in massive outbursts may form a stronger oxidant peroxynitrite with resultant protein nitration, DNA damage, and energy failure.[24] It is now well appreciated that very high, nonphysiological levels of NO actually promote cellular necrosis and apoptosis, whereas the demonstrated protective effects of NO typically involve nanomolar or low micromolar concentrations of NO.[8] In our study, the 480 nmol nitrite could confer the antioxidant effects in the ischemic brain, whereas the detrimental effects of high dose of nitrite after I/R were most likely secondary to nitrite-mediated generation of superoxide or peroxynitrite. Therefore, the strategies simply increasing NO in ischemic cortex may even exacerbate the I/R injury.

Targeting neurovascular protection by structurally suitable NO donor in the ischemic brain might be effective candidates for the treatment of acute stroke. Our observations not only expand our understanding of the physiological roles of NO in the brain but also offer novel treatment strategies targeting the efficient NO donation.

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Disclosures

None.

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