Rapid Conditioning With Oxygen Oscillation
Neuroprotection by Intermittent Normobaric Hyperoxia After Transient Focal Cerebral Ischemia in Rats

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Background and Purpose—Normobaric hyperoxia (NBO) has been shown to exert neuroprotective effects against cerebral ischemia and to restore penumbral oxygenation. Inspired by recent reports on postconditioning with intermittent occlusions of cerebral artery, we tested the hypothesis that intermittent NBO (iNBO) may cause oscillation of cerebral oxygenation and thereby elicit repetitive interruptions to reperfusion, leading to attenuated ischemia/reperfusion damage after transient focal cerebral ischemia in rats.

Methods—Rats were subjected to 90 minutes of middle cerebral artery occlusion. During ischemia, animals received air, iNBO (4 cycles of 3 minutes of NBO and 2 minutes of air), continuous NBO (cNBO; 75 minutes), short NBO (18 minutes), or a combination of iNBO and cNBO. Infarct volume and neurological score were evaluated at 24 and 72 hours after ischemia. Production of superoxide was assessed by the hydroethidine method, and the expression of Akt and phosphorylated Akt was examined by Western blot.

Results—iNBO and cNBO had similar effects in reducing infarct volume and neurological deficit at 24 hours after ischemia, whereas at 72 hours the neuroprotection exerted by iNBO was greater than cNBO. Combining iNBO and cNBO produced no greater protection, and short NBO failed to provide neuroprotection. Both iNBO and cNBO attenuated superoxide production. Importantly, prolonged activation of Akt was observed in the iNBO group, and neuroprotection by iNBO was partly eliminated by inhibition of Akt activation.

Conclusions—iNBO may represent a novel form of postconditioning, and this neuroprotection is likely mediated by attenuating superoxide generation and activation of the Akt pathway.

Key Words: intermittent normobaric hyperoxia ■ neuroprotection ■ postconditioning

Stroke is a leading cause of mortality and adult disability.1,2 After an ischemic stroke occurs, brain cells are deprived of the glucose and oxygen they need to function. However, during reperfusion, restoration of oxygen and glucose supply can trigger a parallel cascade of deleterious biochemical processes.3 Therefore, better understanding of reperfusion injury processes will aid the development of more effective therapy for ischemic stroke. Recently, a concept in the field of cerebral ischemia has emerged by which modified reperfusion subsequent to a prolonged ischemic episode can confer neuroprotection, a phenomenon termed “postconditioning.” In 2006, it was first demonstrated that ischemic postconditioning with repetitive interruptions of reperfusion reduced brain infarct size in rats.4 Although evolved from preconditioning, ischemic postconditioning represents a relatively novel approach to cerebral protection and can be applied after the onset of ischemia.

It is widely accepted that improving brain tissue oxygenation is a logical and important stroke treatment strategy.5 Animal studies from our laboratory and others have documented that normobaric hyperoxia (NBO) has substantial neuroprotective effects in acute cerebral ischemia.6–8 Furthermore, our previous results demonstrated that NBO rapidly restored penumbral oxygenation status to the preischemic level.7,9 Inspired by the reported postconditioning phenomenon, restoration of oxygen during ischemia with NBO can be considered as prior conditioning to reperfusion. In this way, we speculated that intermittent NBO (iNBO) treatment may cause oscillation of cerebral tissue oxygenation during ischemia, and then can alter the patterns of reperfusion as a novel form of conditioning, thus rendering the brain cells more resistant to the subsequent reperfusion insult.

Reperfusion injury is mediated, in part, by the overproduction of reactive oxygen species.10 Although postconditioning is associated with reduced generation of reactive oxygen species, little is known about the protective mechanisms responsible for postconditioning. Some pilot studies have shown that the activation of phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway may mediate the protective effects of postconditioning in the brain.11 Akt activation

Received May 10, 2011; accepted August 23, 2011.
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© 2011 American Heart Association, Inc.
Stroke is available at http://stroke.ahajournals.org DOI: 10.1161/STROKEAHA.111.625756

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requires PI3K-dependent phosphorylation at 2 sites, threonine 308 (Thr308) and serine 473 (Ser473). It is generally agreed that Akt pathway contributes to neuronal survival after stroke and phosphorylated Akt (P-Akt) at Ser473 temporarily increases after reperfusion in focal ischemia. More importantly, postconditioning increases Akt phosphorylation, and Akt inhibition partially blocks the protective effects of postconditioning. In this study, a focal ischemia model in rats was used to test our hypothesis that iNBO treatment reduces reperfusion injury after ischemic stroke through activation of the Akt pathway.

**Materials and Methods**

**Focal Cerebral Ischemia and Reperfusion**

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA; n=198) weighing 290 to 320 g were used in these experiments. The University of New Mexico Laboratory Animal Care and Use Committee approved all experimental protocols. For all surgical procedures, rats were anesthetized with isoflurane (5% for induction, 2% for maintenance) in N2O:O2 (70%:30%). Temperature was maintained at 37°C ±0.5°C with a heating pad. Transient focal ischemia was induced by intraluminal middle cerebral artery occlusion (MCAO) as previously described. In brief, a 4.0 nylon monofilament suture coated with silicon rubber was inserted into the internal carotid artery to occlude middle cerebral artery. After 90-minute ischemia, the suture was gently withdrawn to allow for cerebral reperfusion. Sham animals were subjected to the equivalent surgical preparation, but the suture was not advanced beyond the internal carotid bifurcation.

**Experimental Protocol**

Rats were randomized into 5 groups, and treatment was performed according to 1 of the 5 protocols shown in Figure 1A. Air group was administered air during 90 minutes of MCAO; iNBO group received 4 cycles of 3 minutes of NBO (100% O2) and 2 minutes of air (21% O2), followed by air until the end of MCAO; in the continuous NBO (cNBO) group, rats was continuously administered NBO until reperfusion (maintained for 75 minutes). The duration of NBO/air exposure (3 minutes/2 minutes) was selected based on several considerations, including rapid response of cerebral tissue oxygenation to NBO, the ischemia/reperfusion duration cycles used for the postconditioning study, and our preliminary experimental testing with iNBO. In the short NBO (sNBO) group, rats received continuous NBO for 18 minutes (the same total duration of 4 cycles of NBO/air as in iNBO), followed by air. For the iNBO plus cNBO combination (sum) group, rats received 4 cycles of 3 minutes of NBO and 2 minutes of air, followed by continuous NBO until reperfusion. All NBO treatment (100% O2 at ambient pressure) was initiated 15 minutes after MCAO onset. After reperfusion, these animals received no further therapy and breathed room air.

**Evaluation of the Infarct Volume and Neurological Score**

The 2,3,5-triphenyltetrazolium chloride staining was performed to determine the infarct volume at 24 and 72 hours after MCAO, as previously described. Infarction volume was calculated using Image Pro Plus software (Media Cybernetics, Bethesda, MD) and expressed as the percent of the infarcted tissue as compared with the total brain. Neurological deficit scores were evaluated 24 and 72 hours after MCAO based on the 8-point scale of Rogers by a blinded observer, as previously described. Neurological deficient scores were analyzed by Mann-Whitney U test.

**In Situ Detection of Superoxide Anion Production**

Superoxide anion was detected with the use of hydroethidine (Het) as previously described, with some modifications. Rats were administered intravenously 0.2 mg Het (Molecular Probes) 10 minutes before sacrifice.

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**Figure 1.** Effects of various permutations of normobaric hyperoxia (NBO) treatment at 24 hours after 90 minutes of middle cerebral artery occlusion (MCAO). A, Schematic overview of the experimental protocols. B, Representative photographs of 2,3,5-triphenyltetrazolium chloride (TTC)-stained brain sections from each group of rats. C, Quantification of infarct volume expressed as a percent of infarcted tissue to total brain (n=8). D, Neurological scores that were assessed immediately before the animals were euthanized for infarct size measurement (n=5). *P<0.05, compared with air group.
before induction of ischemia, killed 1 hour after reperfusion, and
transcardially perfused with icy phosphate-buffered saline and 4% paraformaldehyde. Some animals with sham surgery were also injected with Het solution, which served as a control for baseline of superoxide radicals. Brains were removed and fixed in 4% paraformaldehyde for 24 hours and then sectioned into 50 µm sections with a vibratome. The sections were photographed with a fluorescent microscope at excitation of 510 nm and emission of 580 nm. Pictures were taken with the ×40 objective and regions of interest were placed on the ischemic penumbra. The intensity of fluorescence was semiquantitatively analyzed using ImageJ software and expressed as mean value per cell.

Western Blot Analysis of Akt and Phosphorylated Akt Expression
Brain tissues from the following 4 groups were prepared: (1) sham surgery without ischemia; (2) ischemia plus air; (3) ischemia plus iNBO; and (4) ischemia plus cNBO. In each experimental condition, samples corresponding to the ischemic penumbra from ipsilateral and contralateral hemispheres were harvested at 15 minutes, 1 hour, or 22.5 hours after reperfusion. Protein extraction was performed as previously described. Afterward, 40 µg protein was loaded per lane onto SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Blots were probed with antibodies to phosphorylated Akt (P-Akt, Ser473) and Akt (Cell Signaling), and difluoride membrane. Blots were probed with antibodies to phosphorylated Akt (P-Akt, Ser473) and Akt (Cell Signaling), and β-actin was used as the loading control. Finally, membranes were scanned with a LI-COR Odyssey Infrared Imager (LI-COR Biosciences). Optical densities of protein bands were measured by Photoshop CS2 (Adobe), and values were normalized to the β-actin content and expressed as relative intensity.

Protein Kinase Inhibitor Study
To assess the role of protein kinase Akt in NBO neuroprotection, 10 µL of the PI3K inhibitor LY294002 (10 mmol/L) or vehicle was infused into the ventricular space 1 hour before ischemia, according to procedures described previously. Infarct size was measured 24 hours after stroke as described.

Statistical Analysis
Data are expressed as mean±SD. One-way analysis of variance was used to compare the protective effects of NBO on infarct size and fluorescence intensity of Het, followed by Scheffe post hoc test. For the Akt inhibition study and optical densities of protein bands from Western blots, differences were analyzed using 2-way analysis of variance with Student-Newman-Keuls test. \( P<0.05 \) was considered statistically significant.

Results
Effects of Various Permutations of NBO and Air on Infarct Volume and Neurological Score After Stroke
Subjecting animals to 90 minutes of MCAO resulted in a severe infarction when measured at 24 hours after MCAO (Figure 1B, C). Four short cycles of iNBO (18 minutes total) reduced infarct volume by \( \approx 34\% \), which is equivalent to the degree of neuroprotection afforded by 75 minutes of continuous cNBO. However, 18 minutes of continuous NBO (ie, sNBO) failed to decrease infarct volume. These results suggest that how NBO is administered is more critical than duration in terms of neuroprotective outcome. Interestingly, combining iNBO and cNBO (sum group) did not produce greater protection than each treatment alone (Figure 1C). Similar results were obtained for the neurological function assessment: both iNBO and cNBO reduced the neurological scores, whereas sNBO did not, and combining iNBO and cNBO did not afford greater neuroprotection (Figure 1D).

Therefore, sNBO and sum groups were not included in the rest of the study.

To determine whether iNBO results in extended neuroprotection, some animals were subjected to iNBO or cNBO treatment and then recovered for 72 hours. As shown in Figure 2A, more profound reduction in infarct volume was observed in the iNBO group compared with the air group (\( P<0.01 \)). In agreement with our previous studies, cNBO treatment maintained the infarct reduction at 72 hours after MCAO. At this time point, the infarct size appeared to show an obvious decreasing trend in the iNBO group versus cNBO group, although it was not statistically significant (\( P=0.057 \)). Importantly, significant improvements (\( P<0.05 \)) in neurological function were still found in the iNBO group, but not in the cNBO group (Figure 2B), although the neurological scores for all groups improved considerably compared with those of 24 hours. These findings suggest that iNBO may provide better neuroprotection than cNBO with extended time.

To assess whether iNBO might cause any potential adverse effect, we also collected the mortality data for each treatment.
There were no differences in mortality among all groups on analysis: air, 3 of 24; iNBO, 3 of 28; cNBO, 2 of 24; sNBO, 0 of 8; and sum, 1 of 9. No rats in the sham group died. Additionally, using the concentration of thiobarbituric acid reactive substances in serum as an indicator of systemic oxidative stress, we found that there was no significant difference among the animal groups (data not shown). These results indicate that iNBO did not induce untoward stress to the animals.

Production of Superoxide at Early Stage of Reperfusion

Figure 3A shows representative results of Het staining for superoxide in the ischemic penumbra. Few signals for Het staining were observed in brains subjected to sham surgery. Compared with air-treated brains with ischemia, these Het signals were markedly decreased after iNBO or cNBO treatment (Figure 3A, B), suggesting both patterns of NBO can inhibit superoxide production after reperfusion.

Changes in P-Akt and Akt Expression After Ischemia/Reperfusion

To investigate the molecular mechanism of neuroprotection by iNBO, Western blot was used to analyze P-Akt and Akt expression. As shown in Figure 4A, P-Akt and Akt were constitutively expressed in the nonischemic sham brain. In the ischemic rats, P-Akt was significantly increased in the ipsilateral hemisphere at 1 hour after reperfusion, whereas it was decreased by 22.5 hours. In contrast, after iNBO or cNBO treatment, P-Akt was significantly upregulated as early as 15 minutes after reperfusion. More importantly, P-Akt remained at an elevated level 22.5 hours after reperfusion only after iNBO treatment, but not cNBO (Figure 4A, B), suggesting that iNBO and cNBO differentially modulated levels of P-Akt after ischemia, and that iNBO resulted in the prolonged phosphorylation of Akt. Unlike P-Akt, Akt level was found to be unchanged at 15 minutes or 1 hour after reperfusion for all groups of animals. Interestingly, Akt level was decreased by 22.5 hours, which was restored with both iNBO and cNBO treatment (Figure 4A, C).

In contrast to the ipsilateral hemisphere, we did not observe a significant change in Akt expression or Akt phosphorylation in the contralateral side after either ischemia or NBO treatment. Additionally, no changes in levels of P-Akt and Akt were observed in the sham rats subjected to each type of NBO treatment (data not shown).

Effect of Akt Inhibition on Neuroprotection Exerted by iNBO and cNBO

To further study the contribution of Akt activity to the protective effect by NBO, we examined the effects of inhibiting the activity of the upstream activator of Akt, PI3K, with the specific inhibitor LY294002, on phosphorylation levels of Akt and infarct size. As shown in Figure 4, a significant P-Akt upregulation was observed 1 hour after reperfusion in the ipsilateral hemisphere of all animals groups after MCAO, as well as at 22.5 hours in the iNBO treatment group. Pretreatment of LY294002 abolished the elevation of Akt phosphorylation in all these animal groups (Figure 5). These findings suggest that inhibition of Akt activity partly eliminated the elevation of P-Akt induced by NBO.

Next, we evaluated the effects of Akt inhibition on infarct size after ischemia under different NBO treatment conditions. Administration of LY294002 partially abolished the protective effect of iNBO (Figure 6). However, inhibition of Akt did not affect the neuroprotective effect by cNBO. Furthermore, the use of LY294002 was not able to induce any significant change in the infarct volume of rats subjected to air treatment. These results suggest that upregulation of P-Akt plays an important role in neuroprotective effect by iNBO, and that iNBO and cNBO may provide neuroprotection through different mechanisms.
Figure 4. Western blot analysis of phosphorylated Akt (P-Akt) and Akt in rats. A, Representative protein bands for P-Akt (Ser473), total Akt, and β-actin from both the contralateral and ipsilateral penumbra at 15 minutes, 1 hour, and 22.5 hours after reperfusion. B, Quantitative analysis of relative abundance of P-Akt in the ipsilateral hemisphere. C, Quantitative analysis of relative abundance of Akt in the ipsilateral hemisphere. The optical densities of all protein bands were analyzed using Photoshop. Relative optical densities of protein bands were analyzed using Photoshop. Relative optical densities of protein bands were analyzed using Photoshop. Relative optical densities of protein bands were analyzed using Photoshop. 

C indicates contralateral nonischemic hemisphere; I, ipsilateral ischemic hemisphere. n=4. *P<0.05, compared to sham group; #P<0.05, compared to air-treated group.
Discussion

It is now well-established that reperfusion after ischemia can cause injury; therefore, manipulation of the reperfusion process may produce neuroprotection. Inspired by the postconditioning concept, NBO delivered during ischemia could be viewed as a prior interruption to reperfusion, which, in turn, increases the conditioning capacity to reperfusion injury. Because NBO treatment during ischemia can quickly increase penumbra tissue oxygenation,7 4 short cycles of iNBO/air treatment (iNBO), which is expected to cause oscillation of cerebral tissue oxygenation before reperfusion could be viewed as 4 episodes of posts ischemia conditioning. This hypothesis is examined in the present study. Application of iNBO (4 cycles of 3 minutes of NBO and 2 minutes of air) provides effective neuroprotection against brain injury induced by transient MCAO, whereas sNBO with the same total duration (18 minutes as iNBO) fails to afford neuroprotection (Figure 1), suggesting that iNBO is not just simply providing oxygen to the oxygen-starved tissue, and that other mechanisms must be important, also.

We investigated the underlying protective mechanisms of iNBO. In our experimental stroke model, hyperoxia salvaged ischemic brain tissue with a concomitant decrease in the generation of superoxide at 1 hour after reperfusion (Figure 3), which is consistent with the results reported previously.7,8 In the reported postconditioning study, a series of interruptions of reperfusion attenuated the amount of superoxide during early reperfusion after stroke.4 If the concept of postconditioning is extended, then iNBO can be viewed as a process with brief episodes of interruptions that are moved from after reperfusion to before reperfusion. Thus, it is plausible that the protective effects of iNBO against reperfusion injury are likely to have some common mechanisms to postconditioning.

In the present study, a prolonged increase in P-Akt was observed after iNBO treatment, but not cNBO (Figure 4), which might explain why the improvement in infarct volume and neurological function is better for iNBO than cNBO at 72 hours after MCAO (Figure 2). Our study further demonstrated that inhibition of Akt activity with the selective PI3K inhibitor reduced the elevation of P-Akt in the groups treated with air, iNBO, or cNBO (Figure 5); however, only the neuroprotective effect induced by iNBO was partly eliminated by pretreatment with the selective PI3K inhibitor (Figure 6). In this regard, iNBO is not simply a variation of NBO, but may serve as an active process by activating prosurvival kinases such as PI3K/Akt pathway, induction of the antioxidant defenses, and elevation of various stress proteins and transcription factors, rendering the brain cells more resistant to the perturbation that occurs in the transition from ischemia to reperfusion.19–21 Alternatively, iNBO could induce intermittent hemodynamic changes that increases blood flow and volume to the ischemic region,22 thus affording direct neuroprotection per se against the ongoing ischemia. Several experimental studies have reported possible mechanisms of benefit from hyperoxia, including directly increasing tissue oxygen7 and improving aerobic metabolism and restoring mitochondrial function.23,24 Therefore, typical hyperoxia (for example, cNBO) may be considered a passive process that maintains neuronal survival and function by improving brain tissue oxygenation and regulating energy metabolism, which in turn decreases the deleterious effects caused by ischemia.

Akt activity is suggested to be upregulated by phosphorylation. Recent reports suggest that Akt is involved in the cell...
survival signaling pathway after transient cerebral ischemia. Moreover, postconditioning stimulus resulted in the prolonged activation of Akt and inhibition of Akt partly reversed the neuroprotective effect exerted by postconditioning, consistent with our present results. The common activation pathways existing between iNBO and postconditioning support the concept that iNBO could be viewed as another pattern of interruption to reperfusion. Furthermore, levels of total Akt decreased 24 hours after stroke in the ischemic penumbra, and this decrease was prevented by NBO (Figure 4) but not by postconditioning, suggesting that modulating the phosphorylation of Akt rather than its protein expression is implicated in the neuroprotection for ischemic stroke.

Results presented herein suggest that iNBO is a novel and attractive neuroprotective approach that has significant potential for clinical translation, especially because iNBO could be easily applied to stroke patients in a clinical setting. In the present study, the iNBO used here just included 4 cycles of 3 minutes of NBO and 2 minutes of air. It is conceivable that the pattern of iNBO could be optimized with different repetition number and duration to achieve even greater neuroprotection. However, judging by the recommendations from Stroke Therapy Academic Industry Roundtable (STAIR), the current study has several limitations, including lack of blinded randomization, lack of laser Doppler flowmetry monitoring to ensure similar degrees of ischemia, no physiological data obtained, and no power calculation performed. Further animal study is required to assess iNBO as a viable intervention strategy for clinical consideration.

Conclusions

In summary, iNBO can act as a novel form of conditioning through prior interruptions to reperfusion. The possible mechanism for the observed neuroprotection may involve reduction of superoxide and prolonged activation of Akt. Although triggered by the concept of ischemic postconditioning, iNBO may represent another form of active conditioning, which will lead to a greater understanding of the potential of NBO and endogenous protection.

Sources of Funding

This work was supported in part by grants from National Institutes of Health (P20RR15636, P30RR031156, and R01AG031725).

Disclosures

None.

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Stroke. 2012;43:220-226; originally published online October 20, 2011;
doi: 10.1161/STROKEAHA.111.625756
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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