Normobaric Hyperoxia Reduces the Neurovascular Complications Associated With Delayed Tissue Plasminogen Activator Treatment in a Rat Model of Focal Cerebral Ischemia

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Background and Purpose—A major limitation of tissue plasminogen activator (tPA) thrombolysis for ischemic stroke is the narrow time window for safe and effective therapy. Delayed tPA thrombolysis increases the risk of cerebral hemorrhage and mortality, which, in part, is related to neurovascular proteolysis mediated by matrix metalloproteinases (MMPs). We recently showed that normobaric hyperoxia treatment reduces MMP-9 expression and blood–brain barrier disruption in the ischemic brain. Therefore, we hypothesized that normobaric hyperoxia could increase the safety of delayed tPA thrombolysis in stroke.

Methods—Male Sprague-Dawley rats were exposed to normobaric hyperoxia (95% O2) or normoxia (21% O2) during 5-hour filament occlusion of the middle cerebral artery followed by 19-hour reperfusion. Thirty minutes before reperfusion, saline or tPA was continuously infused to rats over 1 hour. Outcome parameters were neurological score, mortality rate, brain edema, hemorrhage volume, and MMP-9. Hemorrhage was quantified with a hemoglobin spectrophotometry method. Edema was evaluated as hemispheric enlargement. MMP-9 was measured by gelatin zymography.

Results—In normoxic rats, delayed tPA treatment at 4.5 hours after stroke onset resulted in high mortality, more severe neurological deficits, increased hemorrhage volumes, and augmented MMP-9 induction compared with saline. Rats treated with combined normobaric hyperoxia and tPA showed significantly reduced tPA-associated mortality, brain edema, hemorrhage, and MMP-9 augmentation as compared with tPA alone.

Conclusions—Our results suggest that early normobaric hyperoxia treatment may represent an important strategy to increase the safety of delayed tPA thrombolysis in ischemic stroke.

Key Words: cerebral hemorrhage • tPA • matrix metalloproteinases • oxygen • stroke

Thrombolytic reperfusion with tissue-type plasminogen activator (tPA) is now an established stroke treatment, but only for those patients presenting within 3 hours of ischemic stroke onset1 or within 4.5 hours if the initial stroke is less severe.2 Delayed tPA therapy is associated with increased risk of serious neurovascular complications involving cerebral hemorrhage and edema, leading to high mortality in patients with stroke.3–8 Any strategy that can safely extend the thrombolytic time window would allow more patients with stroke to benefit from tPA treatment.

Although the mechanisms underlying tPA’s neurovascular complications are not fully understood, it has been suggested that they occur as a result of blood–brain barrier (BBB) disruption.9 Matrix metalloproteinases (MMPs), a family of zinc-binding proteolytic enzymes, play an important role in mediating BBB disruption after cerebral ischemia by degrading the major components of basal laminar around the BBB microvasculature.10 Animal and human studies have provided strong evidence linking MMP-9 induction and tPA-induced hemorrhagic transformation in ischemic stroke. For example, in rodent experiments, tPA exacerbated ischemia-induced BBB damage by enhancing the proteolytic activity of MMP-9.11,12 MMP inhibitors significantly reduced the incidence of tPA-associated hemorrhage and mortality in ischemic stroke animal models.13,14 Human studies indicate that patients with stroke with higher pretreatment plasma levels of MMP-9 are more likely to experience cerebral hemorrhagic complications after tPA.15–17 These data indicate that inhibition of MMP-9 may represent an important strategy to increase the safety of tPA thrombolysis in ischemic stroke.

We and others have recently shown that normobaric hyperoxia (NBO) treatment significantly reduced infarction volumes and improved neurological function in animal stroke models.18–21 Furthermore, NBO treatment was demonstrated to attenuate MMP-9 induction and BBB disruption in the ischemic brain.18,24 Because NBO is readily available, non-

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invasive, and can be initiated within minutes after stroke symptom onset, these neurovascular protective effects of NBO may allow it to serve as an effective strategy to improve the safety and efficacy of tPA thrombolysis. In this study, we tested the hypothesis that NBO treatment could reduce the neurovascular complications associated with delayed tPA thrombolysis in a filament suture model of middle cerebral artery occlusion (MCAO) in rats.

Materials and Methods

Animal Model and Experimental Design

The Laboratory Animal Care and Use Committee at the University of New Mexico approved all experimental protocols. Male Sprague-Dawley rats (Charles River Laboratories; Wilmington, Mass) weighing 290 to 325 g were subjected to MCAO and reperfusion following the same surgical procedures as we previously described.24 Thirty-six rats were randomly divided into 4 experimental groups: (1) normoxia/saline (Normo/saline, n=8); (2) NBO/saline (NBO/saline, n=8); (3) normoxia/tPA (Normo/tPA, n=10); and (4) NBO/tPA (NBO/tPA, n=10). All rats were subjected to 5-hour MCAO with 19-hour reperfusion. Ten minutes after the onset of MCAO, anesthesia was discontinued, and rats were put into an anesthesia box, which was ventilated (3 L/min) with medical air (21% O2, normoxia) or a gas mixture of 95% O2, 5% CO2 (NBO) until 30 minutes before the end of 5-hour MCAO. Ninety-five percent O2 and 5% CO2 was chosen as the NBO treatment because our earlier study demonstrated that rats breathing this gas mixture were able to maintain the ischemic penumbra Po2 close to the preischemic level and showed a relatively normal blood pH and breathing rhythm compared with rats breathing 95% O2, 5% N2 or 100% O2.18 Physiological saline (2.5 mL/kg body weight) with or without recombinant tPA (10 mg/kg body weight; Genetech) was administered (10% bolus, 90% continuous infusion) to rats through the tail vein. The relatively high dose of tPA was necessary to achieve a fibrinolytic effect in rats similar to that of thrombolytic therapy in humans.25 The drug or saline treatment was started 30 minutes earlier than the suture. This regimen assured that the reperfused tissue was returned to their cages.

Analysis of Neurological Deficits and Confirmation of Successful MCAO

At the end of 19-hour reperfusion, the neurological deficit was assessed with Rogers’ 8-point neurological scale as described previously.18,26 The surviving rats (27 of 36 rats) were transcardially perfused under deep anesthesia with 250 mL cold phosphate-buffered saline to remove intravascular blood. Brains were removed and processed for assessing infarction, brain edema, hemorrhage, and MMP-9 expression as described subsequently. To verify successful MCAO, a 1-mm thick brain coronal section 6 mm away from the tip of the frontal lobe was stained with 2,3,5-triphenyltetrazolium chloride (TTC) as described in our recent study.24 Animals that died during reperfusion were excluded for measuring brain edema, hemorrhage, and MMP expression; however, mortality rate was calculated for each group and was used as an important parameter for the beneficial effect of NBO. No incidence of subarachnoid hemorrhage, which resulted from perforation of the meninges, was observed in all the rats, including those that died during reperfusion.

Postischemic Tissue Processing

Brains were sectioned into 5 2-mm thick and one 1-mm thick coronal slices from an 11-mm thick region 3 mm away from the tip of the frontal lobe. After digitally photographing the 2-mm thick brain slices, they were carefully cleaned of meninges. Nonischemic and ischemic hemispheric tissue were then collected and homogenized in 1.5 mL ice-cold phosphate-buffered saline. The resulting homogenates were divided into 2 parts: 150 µL for measuring hemoglobin contents and the rest for cerebral microvessel isolation. The 1-mm thick brain section was used for TTC staining as described previously.

Measurement of Brain Edema

Brain edema was assessed by measuring the hemispheric areas of each 2-mm thick brain slice on the digital photographs obtained as described previously using ImageJ software (National Institutes of Health). Edema was quantified as a relative increase of the brain area in the ischemic hemisphere versus the nonischemic hemisphere as described previously.24

Isolation of Cerebral Microvessels

Isolation of cerebral microvessels was performed exactly as described in our previous study.24 Briefly, the hemispheric homogenates were filtered through a 41-µm nylon mesh (Spectrum). Microvessels retained on the mesh were then purified with dextran T-500 and stored at −80°C until further analysis.

Spectrophotometric Assay of Cerebral Hemorrhage

Cerebral hemorrhage was quantified as previously described.27 Hemispheric brain tissue from normal rats was processed with exactly the same procedure as described previously. Incremental volumes of homologous blood were added to each hemispheric sample. After homogenization, an aliquot of 150 µL from each sample was sonicated on ice for 30 seconds followed by a 15-minute centrifugation at 14 000 g at 4°C. Then 80 µL of supernatant was added to 320 µL reaction reagent (QuantiChrom Hemoglobin Assay Kit; BioAssay Systems). After 5 minutes, optical density of each reaction was measured with a microplate reader at 400 nm. These procedures yielded a linear relationship between hemoglobin concentrations in hemispheric brain tissue and the volume of added blood. Measurements from 150 µL hemispheric tissue homogenate obtained from ischemic rats were compared with this standard curve to obtain data in terms of hemorrhage volume (in microliters).

Gelatin Zymography Analysis for MMP-9

Gelatin zymography was performed to evaluate MMP-9 levels in the isolated cerebral microvessels as we described.24 Briefly, microvessel lysates (30 µg protein) were loaded onto 10% SDS-polyacrylamide gels copolymerized with 1 mg/mL gelatin (Sigma) for electrophoresis. After electrophoresis, gels were washed in 2.5% Triton X-100 and then incubated for 72 hours at 37°C with a developing Tris buffer before staining with Coomassie blue R-250. Gels were then destained and MMP-9 band intensity was quantified. A mixture of human MMP-2/9 (Chemicon) was used as gelatinase standards.

Statistical Analysis

The data are presented as means±SEM. Statistical analysis was carried out using χ2 test, paired t test, or analysis of variance (ANOVA) as indicated in the text. A value of P<0.05 was considered statistically significant.

Results

TTC staining of the 1-mm thick brain section showed that 5-hour MCAO with 19-hour reperfusion induced significant infarction in the ischemic hemispheres of all surviving rats (n=27) except one NBO/tPA rat, which showed a very small infarct area (6% of the ischemic hemisphere), indicating unsuccessful MCAO, and was excluded from this study. As shown in the TTC-stained sections (Figure 1), regardless of saline or tPA treatment, NBO-treated rats showed a relatively smaller infarct area than normoxic rats. However, this reduction in lesion size by NBO was much less impressive than our
previous results obtained from rats subjected to less severe ischemia (90-minute MCAO).24

As shown in Figure 2A, the Normo/tPA group showed dramatically higher mortality (70% [7 of 10 rats]) than the Normo/saline (0% [zero of 8 rats]), NBO/saline (12.5% [one of 8 rats]), and NBO/tPA groups (11.1% [one of 9 rats]; P<0.05, \( \chi^2 \) test). Similar results were obtained for Rogers’ neurological score assessment, in which Normo/tPA rats showed more severe neurological deficits than the other 3 groups (P<0.05; ANOVA; Figure 2B). No significant difference in mortality and neurological score was noted among Normo/saline, NBO/saline, and NBO/tPA groups.

Brain edema and cerebral hemorrhage were assessed in surviving rats. As expected, brain swelling was observed in the ischemic hemispheres of all groups (Figure 3A). In both saline- and tPA-treated rats, NBO treatment significantly reduced hemispheric enlargement (P<0.05 versus normoxia; ANOVA). In the normoxic rats, tPA did not significantly increase brain edema compared with saline (P>0.05; Figure 3B). Hemorrhage, identified as blood evident at the macroscopic level (Figure 3A), was clearly visible in the ischemic hemispheres of all surviving rats except one Normo/saline and one NBO/saline rat. As expected, Normo/tPA rats showed larger hemorrhage size than the other 3 groups. None of the rats displayed any grossly visible hemorrhage in their nonischemic hemispheres (Figure 3A). Spectrophotometric measurement of whole blood showed a linear response between blood volume and hemoglobin absorbance (Figure 3C), which validated our method for quantifying hemorrhage. As shown in Figure 3D, a low value of hemorrhage volume was detected in the nonischemic hemispheres, which may reflect the residue blood left in the cerebral vasculature after transcardial perfusion with phosphate-buffered saline. Rats in all 4 groups had larger blood volumes in the ischemic hemisphere than Normo/saline rats (P<0.05; ANOVA). No significant difference in hemorrhage volume was observed for both ischemic and nonischemic hemispheric microvessels among Normo/saline, NBO/saline, and NBO/tPA rats.

Because the “tPA–MMP-9 hypothesis” has been proposed as an important mechanism underlying tPA’s neurovascular complications,13,15,16 we investigated whether 5-hour MCAO with 19-hour reperfusion would lead to increased MMP-9 expression in the cerebral microvessels of the ischemic brain and, more importantly, whether tPA treatment would further enhance MMP-9 expression. As shown in Figure 4, the proform of MMP-9 was dramatically upregulated in the ischemic hemispheric microvessels of all rats (Figure 4B). Furthermore, Normo/tPA rats showed significantly higher levels of MMP-9 in both ischemic and nonischemic hemispheric microvessels than the other 3 groups (P<0.05; ANOVA). No significant difference in MMP-9 levels was observed for both ischemic and nonischemic hemispheric microvessels among Normo/saline, NBO/saline, and NBO/tPA rats.

**Discussion**

Thrombolysis with tPA is currently the only US Food and Drug Administration-approved treatment for acute ischemic stroke.1 However, the clinical use of tPA is constrained to <5% of patients with ischemic stroke mainly due to its narrow therapeutic time window.28 Delayed tPA treatment is associated with elevated risks of edema and cerebral hemorrhage, leading to increased mortality in patients with stroke.3–8 tPA-augmented ischemic damage to the BBB through upregulating MMP-9 is implicated in these compli-
Therefore, inhibiting MMP-mediated BBB disruption is a potential strategy to prevent tPA’s neurovascular complications and safely extend its therapeutic time window. The present study demonstrates that NBO treatment reduced tPA-associated mortality, brain edema, and hemorrhage in a rat model of cerebral ischemia. Moreover, inhibition of tPA-augmented MMP-9 induction in the BBB microvasculature may be an important underlying mechanism for this protection.

Studies have demonstrated that NBO can effectively reduce infarction volume and improve neurological outcome after transient cerebral ischemia. These neuroprotective effects are important for slowing down the evolution of ischemic damage to the brain and may therefore “buy time” for reperfusion. The potential for combined NBO treatment with tPA thrombolysis was recognized several years ago. Recently, Henninger et al showed that early NBO treatment in conjunction with tPA at a later time point significantly reduced infarct volume and did not increase hemorrhage risk, providing important initial evidence to support the combination therapy with NBO and tPA in ischemic stroke. However, it remains unclear about NBO’s potential to decrease tPA-associated hemorrhage, a potentially fatal complication of tPA. Our present study was designed to address this critical issue by investigating the effects of NBO on mortality, brain edema, and hemorrhage associated with delayed tPA treatment.

To mimic delayed tPA thrombolysis in the clinical setting, we used a rat stroke model of 5-hour MCAO with 19 hours of reperfusion and administered tPA to rats after 4.5 hours of ischemia, which represented a time point outside the established 3-hour time window of tPA treatment. Although using a filament suture to mechanically occlude the middle cerebral artery was not directly relevant to thromboembolic ischemia in patients, this stable model would allow us to compare the different effects of NBO and normoxia on tPA’s complications under identical and controlled ischemia and reperfusion conditions. In contrast, the more clinically relevant stroke model by injection of a preformed clot into the middle cerebral artery is at present still problematic in terms of stability because we cannot predict how long and which part the clot will occlude the middle cerebral artery. Our results showed that delayed tPA treatment resulted in high mortality in the normoxic rats, which is consistent with previous studies with mechanical or embolic stroke animal models. Surprisingly, although NBO treatment reduced the mortality rate from 70% to 11% in tPA-treated rats, it did not decrease neurological deficits in the saline-treated rats. The lack of effect on neurological deficits in the saline-treated rats differs from our earlier report with rats subjected to a much shorter ischemic duration of 90 minutes. This discrepancy is most likely due to the significantly different severities of ischemia associated with 90-minute versus 5-hour MCAO. Consistent with this is the finding (Figure 1) that the reduction in infarct area by NBO was much less impressive in rats subjected to 5-hour than 90-minute MCAO (quantitative data of infarct size on those TTC-stained brain sections are not shown). Reports that the neuroprotective effects of short-term NBO alone cannot be sustained with permanent or prolonged ischemia further corroborate our results.

Thrombolysis with tPA can elevate the risk of intracranial hemorrhage, a major cause of death in delayed tPA treatment for ischemic stroke. Our results showed that tPA dramatically increased the amount of hemorrhage in the ischemic hemisphere of normoxic rats (Figure 3), which is likely responsible for the high mortality rate observed in the Normo/tPA group (Figure 2). As expected, NBO treatment significantly reduced tPA-exacerbated hemorrhage and mor-
decreased, infarct or edema indicates that there is no signif-

cytotoxic edema through a separate mechanism. In addition,
also contributes to hemispheric enlargement in ischemic
BBB disruption-triggered vasogenic edema, cytotoxic edema
but not hemorrhage, in saline-treated rats. Besides
ischemic stroke, our results show that NBO reduced brain
mon mechanism (ie, BBB disruption) for their occurrence in
rats after prolonged 5-hour ischemia. It is worth pointing out
hemispheric enlargement already developed in the normoxic
unlike its effects on cerebral hemorrhage, delayed tPA treat-
ment did not increase hemispheric enlargement in normoxic
rats, which differs from our previous report with a much shorter ischemic duration of 90 minutes.24 This discrepancy may also be due to the reduction in the neuroprotective effects of NBO under prolonged cerebral ischemia,20,23 The observation that NBO reduced MMP-9 induction in tPA-treated but not in saline-treated rats suggests that NBO may inhibit tPA-augmented MMP-9 induction but not ischemia-triggered MMP-9 induction under our experimental conditions. Several mechanisms such as Rho/Rock35 and low-density lipoprotein receptor-related protein pathways11 have been proposed to mediate tPA-induced MMP-9 expression. However, how NBO inhibits tPA-augmented MMP-9 induction remains to be elucidated. Our results support the idea that inhibition of tPA-augmented MMP-9 induction may be part of the mechanisms accounting for the reduction in hemorrhage and mortality by NBO. On the other hand, because MMP-9 assessment was only done at a single time point (24 hours after ischemia), and no specific experiments were carried out to investigate the causal relationship between MMP-9 and tPA-augmented hemorrhage and mortality rate, further studies are required to ascertain that NBO improves the safety of delayed tPA treatment through interfering with the tPA–MMP-9 mechanism. Because oxygen therapy is rarely initiated within the first minutes of stroke onset, it is also necessary to investigate the neurovascular protection of NBO at different time points after ischemia.

In conclusion, our results indicate that early NBO treat-
ment can reduce brain edema, cerebral hemorrhage, and
mortality in delayed tPA treatment for ischemic stroke. Inhibition of tPA-augmented MMP-9 increases in the ischemic BBB microvasculature may represent a mechanism for NBO’s protection. Our findings suggest that NBO may represent an important strategy to reduce hemorrhagic complications and mortality of tPA thrombolysis and safely extend its current narrow therapeutic time window.

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Disclosures
None.

References

Figure 4. Effects of NBO and tPA on MMP-9 induction in ischemic cerebral microvessels. A. Representative gelatin zymogram showing MMP-9 expression in the nonischemic (Non-I) and ischemic (I) hemispheric cerebral microvessels for each group. STD is a mixture of standard MMP-2 and MMP-9. B. Quantified MMP-9 band intensity for each group. A significant increase in MMP-9 levels was observed in the ischemic hemispheric microvessels in all animal groups (*P<0.05, versus nonischemic; paired t test). Normo/tPA rats showed higher levels of MMP-9 in both ischemic and nonischemic hemispheric microvessels than the other 3 groups (#P<0.05). Data are expressed as mean±SEM.
6 Stroke July 2009


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