Interactions between Nitrous Oxide and Tissue Plasminogen Activator in a Rat Model of Thromboembolic Stroke


ABSTRACT

Background: Preclinical evidence in rodents has suggested that inert gases, such as xenon or nitrous oxide, may be promising neuroprotective agents for treating acute ischemic stroke. This has led to many thinking that clinical trials could be initiated in the near future. However, a recent study has shown that xenon interacts with tissue-type plasminogen activator (tPA), a well-recognized approved therapy of acute ischemic stroke. Although intras ischemic xenon inhibits tPA-induced thrombolysis and subsequent reduction of brain damage, postischemic xenon virtually suppresses both ischemic brain damage and tPA-induced brain hemorrhages and disruption of the blood–brain barrier. The authors investigated whether nitrous oxide could also interact with tPA.

Methods: The authors performed molecular modeling of nitrous oxide binding on tPA, characterized the concentration-dependent effects of nitrous oxide on tPA enzymatic and thrombolytic activity in vitro, and investigated the effects of intras ischemic and postischemic nitrous oxide in a rat model of thromboembolic acute ischemic stroke.

Results: The authors demonstrate nitrous oxide is a tPA inhibitor, intras ischemic nitrous oxide dose-dependently inhibits tPA-induced thrombolysis and subsequent reduction of ischemic brain damage, and postischemic nitrous oxide reduces ischemic brain damage, but in contrast with xenon, it increases brain hemorrhages and disruption of the blood–brain barrier.

Conclusions: In contrast with previous studies using mechanical acute stroke models, these data obtained in a clinically relevant rat model of thromboembolic stroke indicate that nitrous oxide should not be considered a good candidate agent for treating acute ischemic stroke compared with xenon.

A CUTE ischemic stroke through thromboembolism remains a major cause of acute mortality and chronic morbidity. The most common approved therapy of acute ischemic stroke is thrombolysis by the recombinant form of the serine protease human tissue-type plasminogen activator (tPA).¹ Although recombinant tPA has benefited ischemic
stroke patients if given within 4.5 h of symptoms,2–4 adverse effects, primarily brain hemorrhages and disruption of the blood–brain barrier integrity through tPA-mediated proteolytic processes, have been reported.3–7 In contrast, strategies of neuroprotection by the use of glutamatergic receptor antagonists to counteract ischemia-induced overstimulation of the N-methyl-D-aspartate (NMDA) glutamatergic receptors, whose postsynaptic activation is known as a critical event in neuronal death induced by acute ischemic stroke,8–10 have not been proven efficient in patients experiencing ischemic insults, primarily because these compounds produced intolerable neurotoxic and psychotomimetic side effects.11,12 Taken together, this has led to the conclusion that methods of neuroprotection that could prevent tPA toxicity are needed.13

During the past decade, much attention has been paid to the potentially neuroprotective properties of the remarkably safe anesthetic gases xenon and nitrous oxide. Both gases share many pharmacologic properties as antagonists of the NMDA and nicotinic acetylcholine receptor,14–17 activators of the TREK-1 two-pore–domain K+ channel,18 and enzyme inhibitors.19,20 Preclinical evidence in rodents has proven that these gases may have effective neuroprotective properties by reducing excitotoxic neuronal death,14 mechanical middle cerebral artery occlusion (MCAO)-induced brain damage,21–25 neonatal hypoxic–ischemic brain damage,26,27 and cardiopulmonary bypass-induced neurologic and neurocognitive dysfunction,28 with no adverse side effects when used at subanesthetic concentrations. This has led to many thinking that clinical trials could be initiated in acute stroke patients in the near future. However, a recent study performed in a rat model of thromboembolic stroke has shown that xenon inhibits tPA by binding directly within its catalytic site, thereby producing adverse effects by reducing tPA-induced thrombolyis and, as a consequence, thrombolysis-induced reduction of brain damage when administered during the intraischemic period and beneficial effects by suppressing both ischemic brain damage and recombinant tPA-induced brain hemorrhages and disruption of the blood–brain barrier when given after reperfusion.29 Because nitrous oxide shares pharmacologic and neuroprotective properties similar to that of xenon in excitotoxic-ischemic models,14,21–23 we hypothesized that nitrous oxide may modulate the catalytic efficiency of tPA and thereby alter the beneficial and/or adverse effects of recombinant tPA therapy in a rat model of thromboembolic ischemia.

Materials and Methods

Animals
We used male Sprague-Dawley rats. All animal-use experiments were approved by a local ethics committee (Caen, France) in accordance with the French legislation for biomedical experimentation and the European Communities Council Directive of November 24, 1986 (86/609/EEC). Rats were housed at 21 ± 0.5°C in acrylic home cages with free access to food and water in groups of four. After being used for surgery, all rats were housed individually. Light was maintained on a light–dark reverse cycle, with lights on from 8:00 PM to 8:00 AM.

Modeling of the Binding Site of Nitrous Oxide within the S1 Pocket of tPA
We performed the structural superposition of the catalytic domain of tPA (Protein Data Bank entry: 1A5H)30 to elastase in complex with nitrous oxide with the software PyMOL (DeLano Scientific, San Carlos, CA). The root mean square deviation between the catalytic domain of tPA and elastase was 1.5 Å for 221 aligned carbon α (Cα).

IPA Catalytic Activity Assay with Nitrous Oxide
We assessed the effects of nitrous oxide on the catalytic efficiency of tPA by using the initial rate method. The recombinant form of human tPA (Actilyse®; Boehringer Ingelheim, Ingelheim am Rhein, Germany) and murine tPA (ref. IRTPA; Innovative Research, Novi, MI) and their specific chromogenic substrate methylsulfonyl-d-phenyl-glycil-arginine-7-amino-4-methylcoumarin acetate (Spectrozyme® XF, product 444; American Diagnostica, Stamford, CT) were separately diluted in 1 ml distilled water in 1.5-ml sterile tubes. Each tube containing 0.4 μM tPA or 10 μM tPA substrate was saturated for 20 min at a flow rate of 60–80 ml/min with nitrous oxide of 25–75 vol% or medical air composed of 25% oxygen and 75% nitrogen as described previously.29 We assessed the catalytic efficiency of human and murine tPA (N = 3, n = 12, per concentration) in the presence of air or nitrous oxide by incubating 50 μl tPA with 50 μl substrate at 37°C using a spectrofluorometer microplate reader.

In vitro Thrombolysis Experiments with Nitrous Oxide
We used male Sprague-Dawley mature rats weighing 600–650 g (n = 6). Whole blood samples of 500 μl volume were transferred in preweighed 1.5-ml tubes and incubated at 37°C for 3 h. Saline solution was saturated for 30 min with nitrous oxide of 25–75 vol% (with the remainder being oxygen at 25 vol% and nitrogen as needed) or with medical air as described previously.29 Briefly, after clot formation and total serum removal, each tube was weighed to determine the clot weight. We selected blood clots in the same weight range (0.260 ± 0.056 g) to reduce variability. Each tube was filled with saline solution containing 1 μg/ml tPA in the form of Actilyse® saturated with 25 vol% nitrous oxide (n = 16) or air (n = 12), 37.5 vol% nitrous oxide (n = 16) or air (n = 15), 50 vol% nitrous oxide (n = 12) or air (n = 8), or 75 vol% nitrous oxide (n = 16) or air (n = 15), and incubated at 37°C for a 90-min period. The fluid was removed, and the tubes were weighed again to assess the percentage of clot lysis induced by tPA in the presence of medical air or nitrous oxide of 25–75 vol%.

MCAO Experiments with Intraischemic Nitrous Oxide
Male Sprague-Dawley rats weighing 250–275 g were used to assess the effects of nitrous oxide on cerebral blood flow
reperfusion induced by tPA and subsequent reduction of ischemic brain damage. Nitrous oxide was used at 75 vol%, a concentration shown in a recent dose-effect study to provide maximal neuroprotection in rats subjected to MCAO-induced ischemia.23 Rats were subjected to MCAO-induced ischemia by administration of an autologous blood clot by the intraluminal method. Twenty-four hours before the animals were subjected to MCAO-induced ischemia, a whole caudal blood sample of 200 μl was withdrawn and allowed to clot at 37°C for 2 h. The clot was extruded from the catheter into a saline-filled petri dish and stored at 4°C for 22 h before being used the day after to induce thromboembolic ischemia.

On the day of surgery, the rats were anesthetized with 2 vol% isoflurane in medical air, intubated, and ventilated artificially. Catheters were inserted into the femoral vein to allow injection of tPA or saline solution and in the femoral artery for continuous monitoring of heart rate, diastolic, systolic, and mean arterial pressures and for the periodic analysis of blood gases and pH. A midline incision was performed, and the right common carotid artery was exposed to perform coagulation of the proximal branches of the external carotid artery. A laser-doppler flowmetry probe was positioned onto the right parietal bone (previously thinned) to assess successful induction of cerebral ischemia and monitor changes in cerebral blood flow while the rat was being placed prone. Changes in cerebral blood flow were monitored continuously and expressed as a percentage from the cerebral blood flow values recorded during a 20-min preocclusion period. A single clot measuring 40 mm in length was injected in a volume of 50 μl saline solution through a polyethylene-10 catheter directed into the internal carotid artery up to 2 mm after the pterygopalatine-internal carotid artery bifurcation, as detailed previously.29 After a 45-min period of occlusion during which all rats were given medical air, the catheter was removed from the internal carotid artery to the external carotid artery, and the rats were given tPA in the form of Actilyse® at 0.9 mg/kg (a dose shown to be clinically relevant as that of 10 mg/kg, which is often used in rodents31) in 1 ml saline solution (10% bolus plus 90% perfusion over a 45-min period) with either medical air (n = 5) or nitrous oxide (n = 5). Then, all animals were given medical air again. Sham-treated rats (n = 5) were given medical air and saline solution. Rats were maintained in a normothermic state. After surgery, the polyethylene-10 catheter was removed, and the rats were returned to their home cages and allowed to move freely and provided food and water ad libitum. Brain damage was assessed 24 h after the onset of ischemia. To mimic clinical recommendations, rats showing less than 50% reduction in cerebral perfusion [sham rats (saline + medical air), n = 1; control rats (tPA + medical air), n = 2] were excluded from the study design.

**MCAO Experiments with Postischemic Nitrous Oxide**

Male Sprague-Dawley rats weighing 250–275 g were used to assess the effects of nitrous oxide at 75 vol% on ischemic brain damage. Rats were anesthetized with isoflurane in medical air and subjected to thromboembolic MCAO-induced ischemia by administration of an autologous blood clot, as described above, while breathing spontaneously throughout the surgical intervention. After injection of the blood clot, the animals were awakened and allowed to move freely in their home cages. After a 45-min period of ischemia, the ligature of the common carotid artery was removed, and the rats were given tPA in the form of Actilyse®, as described above, through a catheter that had been inserted into the femoral vein and exteriorized at the neck on the day before the main surgical protocol. After tPA infusion, the rats were treated for 3 h in a closed chamber with either medical air (n = 5) or nitrous oxide at 75 vol% (n = 4) at a flow rate of one volume chamber per minute. Such a flow rate allows maintaining carbon dioxide concentrations at 0.03 vol%. Sham rats (n = 4) were treated with saline solution and medical air. After treatment, the rats were allowed to move freely in their home cages and provided food and water ad libitum. Brain damage was assessed 48 h after the onset of ischemia. To mimic clinical recommendations, rats showing less than 50% reduction in cerebral perfusion [sham rats (saline + medical air), n = 1; control rats (tPA + medical air), n = 2] were excluded from the study design.

**NMDA and NMDA Plus tPA-induced Neuronal Death with Postinsult Nitrous Oxide**

On the day of surgery, rats were anesthetized with halothane (1.5%) in medical air and mounted on a stereotactic apparatus with the incisor bar set 3.9 mm below the horizontal zero. During surgery, body temperature was kept at 37 ± 0.5°C. A burr hole was drilled and a micropipette (~10 μm at the tip) was lowered into the right striatum (anterior, 0.6 mm; lateral, 3.0 mm; ventral, 5.8 mm, from bregma) to allow injection of 50 nmol of NMDA alone or in combination with 3 μg tPA in the form of Actilyse® in 1 μl phosphate buffered saline solution (pH 7.4) over a 2-min period. After an additional 5-min period, the micropipette was removed, and the rats were returned to their home cages with free access to food and water. Sixty minutes after administration of NMDA or NMDA plus tPA, the rats were treated for 3 h with medical air or nitrous oxide as described above for rats subjected to ischemia. The number of animals was n = 8 per group. Brain damage was assessed 24 h after NMDA or NMDA plus tPA injection.

**Gas Pharmacology**

Oxygen, nitrogen, and nitrous oxide of medical grade were purchased from Air Liquide Santé (Paris, France). Gas mixtures containing 75 vol% nitrogen and 25 vol% oxygen (medical air) or nitrous oxide at 25–75 vol%, with the remainder being oxygen at 25 vol% completed with nitrogen when necessary, were obtained using computer-driven calibrated flowmeters and gas
analyzers, and used or published as described above according to a blinded and randomized procedure.

**Assessment of Brain Damage**

Rats were killed by decapitation under isoflurane anesthesia. Brain damage was assessed 24 h after injection of NMDA or NMDA plus tPA or after the onset of ischemia for intraischemic experiments and 48 h after the onset of brain ischemia for postischemic experiments to allow “better” comparison with previous mechanical and thromboembolic studies. Comparative studies using multiple staining techniques have shown that assessment of infarct size at 24 h is a time condition sufficient to obtain consolidated infarct volumes (i.e., whose assessment would be similar if performed one or several days later), thereby allowing reliable comparison between the data obtained at 24 h or 48 h after the onset of brain ischemia. The brain was removed and frozen in isopentane. Coronal brain sections (20 μm) were cryostat-cut, mounted on slides, stained with thionin, and digitized on a computer. The lesion areas were delineated by the pallor of staining in the necrotic tissue compared with the surrounding healthy tissue; then volumes of brain damage were estimated by integration over the whole brain of the infarct surfaces calculated with the Image® software analyzer (Scion Corp., Frederick, MD), corrected for tissue edema and expressed in cubic millimeters.

**Assessment of Brain Hemorrhages and Disruption of the Blood–Brain Barrier Integrity**

Forty-eight hours after the onset of brain ischemia, rats were given Evans blue intravenously (4% in 1-ml saline solution over a 30-s period). The brain was removed and sliced as described above. While the unstained brain still mounted on the cryostat was cut, macrophotographs were made and analyzed with a computer using the Image® software analyzer. We assessed brain hemorrhages and disruption of the blood–brain barrier integrity as described previously. Evans blue extravasation was delineated and multiplied by the extravasation optical density; the ratio of extravasation was calculated for each rat by dividing the volume of the brain showing extravasation as obtained by integration over the whole brain of the surfaces showing Evans blue extravasation by the total infarction volume, corrected for tissue edema. Brain hemorrhages were delineated as blood evident at the macroscopic level. For each rat, hemorrhagic transformation was calculated as the sum of the hemorrhagic ratio obtained by dividing the total surface of hemorrhages by the total surface of infarction in the slices that showed hemorrhages. Finally, coronal sections were used to assess infarct size as described above.

**Statistical Analysis**

Data were analyzed using Statview software (SAS Institute, Cary, NC). In vitro data, given as mean ± the SE of the mean (SEM), were analyzed using one-way parametric analysis of variance and unpaired Student t test with two-tailed testing. In addition, the inhibitory concentration of nitrous oxide producing half of the maximal effect (IC50) was also calculated. In vivo data, given as median value and the twenty-fifth to seventy-fifth percentiles, were analyzed using the Kruskal-Wallis nonparametric analysis of variance and Mann–Whitney nonparametric unpaired U-test with two-tailed testing.

The level of significance was set at P < 0.05. The number of animals per group for in vivo studies was assessed by performing power analysis through an online software** with the following variables taken from a previous study with xenon at 50 vol% (based on the fact that the minimum anesthetic concentration ratio of the effects of xenon and nitrous oxide indicates that nitrous oxide at 75 vol% is as potent as xenon at 50 vol%); mean ± SD for tPA = 166 ± 52 mm3; mean ± SD for xenon at 50 vol% = 330 ± 140 mm3; α error level (confidence level) = 0.05; β error level (statistical power) = 0.25. Rats that died during MCAO-induced ischemia and those that died before being used for histologic analysis [intraischemic experiments: sham rats (saline + medical air), n = 1; control rats (tPA + medical air), n = 2; nitrous oxide-treated rats (tPA + nitrous oxide), n = 1; postischemic experiments: sham rats (saline + medical air), n = 2; control rats (tPA + medical air), n = 4; nitrous oxide-treated rats (tPA + nitrous oxide), n = 3] were excluded from statistical data. No data were excluded from statistical analysis from in vitro studies or in vivo NMDA and NMDA + tPA studies.

**Results**

**Modeling of the Binding Site of Nitrous Oxide within the S1 Pocket of tPA**

First, we investigated whether nitrous oxide could bind to tPA. Xenon binds within the S1 pocket of elastase, and nitrous oxide binds to elastase at the same location. Because all serine proteases share a similar structure around their identical catalytic triad and thus can be easily structurally superposed, we performed modeling of the possible binding of nitrous oxide to the catalytic domain of tPA by superposing the crystallographic structure of elastase in complex with nitrous oxide to the crystallographic structure of the human tPA catalytic domain in complex with the bis-benzamidine inhibitor tPA Stop. As shown in figure 1A, the structure of the catalytic domain of tPA in complex with tPA Stop reveals that an extremity of this inhibitor binds within the specificity pocket S1. The nitrous oxide atom fits within the S1 pocket of tPA at the position of one extremity of the tPA inhibitor tPA Stop. This suggests that nitrous oxide could reduce the catalytic efficiency of tPA by binding within the S1 specificity pocket.

**Nitrous Oxide Dose-dependently Inhibits the Catalytic Efficiency of tPA in vitro**

Next, we investigated the effects of nitrous oxide on the catalytic efficiency of the recombinant form of human and murine tPA...
In vitro Thrombolysis

Murine tPA: Thrombolytic Efficiency of tPA

Nitrous Oxide Dose-dependently Inhibits the Thrombolytic Efficiency of tPA

Next, to examine whether nitrous oxide may inhibit tPA-induced thrombolysis, we studied the effect of this inert gas at concentrations of 25–75 vol% on the thrombolytic efficiency of tPA in whole blood samples drawn from male mature rats. After clot formation and total serum removal, each tube was filled with saline solution containing a clinically relevant concentration of 1 µl/ml tPA in the form of Actilyse® previously saturated with nitrous oxide or medical air. We found that nitrous oxide at concentrations of 25–75 vol% reduced tPA-induced thrombolysis in a concentration-dependent manner (P < 0.001; IC50 = 27.13 ± 1.04 vol%; fig. 1B). This suggests that nitrous oxide could reduce the thrombolytic efficiency of tPA.

**Nitrous Oxide Dose-dependently Inhibits the Thrombolytic Efficiency of tPA in vivo**

To examine whether nitrous oxide may actually decrease thrombolysis in vivo, we investigated the effects of intraischemic nitrous oxide on tPA-induced cerebral blood flow reperfusion and subsequent reduction of brain damage in rats subjected to thromboembolic brain ischemia. Nitrous oxide was used at 75 vol%, a concentration shown in a recent dose-effect study to provide maximal neuroprotection in rats subjected to MCAO-induced ischemia. At 45 min after the onset of brain ischemia, rats were administered tPA in the form of Actilyse®. In line with the findings of clinical studies of tPA-treated patients, we found that rats injected with tPA exhibited full cerebral reperfusion 40 min after tPA injection. All physiologic parameters, including arterial pressure, decreased to within normal range values (table 1). Sham rats treated with saline solution and medical air showed no cerebral blood flow reperfusion and had a volume of ischemic brain damage of 395 mm³ (twenty-fifth to seventy-fifth percentiles: 370–402 mm³). As expected, control rats treated with tPA and medical air exhibited full cerebral blood flow reperfusion (P < 0.01) and a reduced volume of brain damage of 167 mm³ (twenty-fifth to seventy-fifth percentiles: 160–209 mm³) compared with sham-treated rats (P < 0.001, IC50 = 27.13 ± 1.04 vol%; fig. 1C). No evidence of clot lysis was found in whole blood samples treated with saline solution and medical air.

**In vivo Intraischemic Nitrous Oxide Inhibits tPA-induced Thrombolysis and Subsequent Reduction of Brain Damage**

Table 1. Range of Physiologic Values of Arterial PaO2, SaO2, PaCO2, Arterial pH, and Body Temperature during Ischemia in Rats Treated with Medical Air and Rats Subjected to Intraischemic Nitrous Oxide at 75 vol%

<table>
<thead>
<tr>
<th></th>
<th>PaO2 (mmHg)</th>
<th>PaCO2 (mmHg)</th>
<th>SaO2 (%)</th>
<th>Arterial pH</th>
<th>Temp. (°C)</th>
</tr>
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<tbody>
<tr>
<td>Air</td>
<td>75–100</td>
<td>36–40</td>
<td>95–97</td>
<td>7.37–7.46</td>
<td>37.1–37.6</td>
</tr>
<tr>
<td>N2O 75</td>
<td>75–90</td>
<td>35–41</td>
<td>95–97</td>
<td>7.39–7.52</td>
<td>37.4–37.6</td>
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PaO2 = arterial carbon dioxide partial pressure; PaCO2 = arterial oxygen partial pressure; SaO2 = arterial hemoglobin saturation of oxygen; Temp = body temperature.
In contrast to this latter finding, we found that rats treated with tPA and intraischemic nitrous oxide showed a reduction in cerebral blood flow reperfusion of 42% (twenty-fifth to seventy-fifth percentiles: 35–48%; $P < 0.05$) compared with control animals treated with tPA and medical air (fig. 2A–B). As might be expected from this effect, we found that rats treated with tPA and intraischemic nitrous oxide at 75 vol% had greater volumes of brain damage (308 mm$^3$; twenty-fifth to seventy-fifth percentiles: 291–457 mm$^3$; $P < 0.05$) than did control animals treated with tPA and medical air (fig. 2C).

In vivo Postischemic Nitrous Oxide Reduces Ischemic Brain Damage but Increases Brain Hemorrhages

We then studied the effects of postischemic nitrous oxide on brain hemorrhages, disruption of the blood–brain barrier integrity, and ischemic brain damage in rats subjected to thromboembolic brain ischemia. Forty-five minutes after the onset of brain ischemia, rats were given intravenous tPA in the form of Actilyse®. Fifteen minutes after tPA infusion ended, rats were treated with medical air (controls) or nitrous oxide at 75 vol%. Sham rats treated with saline solution and medical air had a mean volume of ischemic brain damage of 383 mm$^3$ (twenty-fifth to seventy-fifth percentiles: 350–420 mm$^3$) and exhibited brain hemorrhages and disruption of the blood–brain barrier. Compared with sham-treated rats, control rats treated with tPA and medical air had similar ratios of brain hemorrhages and disruption of the blood–brain barrier integrity, despite a reduced volume of brain damage (99 mm$^3$; twenty-fifth to seventy-fifth percentiles: 92–134 mm$^3$; $P < 0.01$; fig. 3A–D). Rats treated with tPA and postischemic nitrous oxide at 75 vol% had reduced brain damage (49 mm$^3$; twenty-fifth to seventy-fifth percentiles: 40–59 mm$^3$; $P < 0.02$) but exhibited greater ratios of brain hemorrhages ($P < 0.01$) and disruption of the blood–brain barrier ($P < 0.05$) compared with control rats treated with tPA and medical air (fig. 3A–D).

In vivo Postischemic Nitrous Oxide Reduces NMDA- and NMDA Plus tPA-induced Neuronal Death

Finally, we investigated the effects of nitrous oxide on NMDA-induced neuronal death when given 1 h after NMDA or NMDA plus tPA injection. Administration of NMDA in the striatum led to a volume of excitotoxic neuronal death of 18.6 mm$^3$ (twenty-fifth to seventy-fifth percentiles: 17.3–19.5 mm$^3$). As expected from previous data, rats injected with NMDA plus tPA showed greater brain damage than did those injected with NMDA alone, with a volume of neuronal death of 33.2 mm$^3$ (twenty-fifth to seventy-fifth percentiles: 29.6–36.8 mm$^3$; $P < 0.001$). We found that nitrous oxide reduced NMDA-induced brain damage by approximately 25%, so that rats treated with nitrous oxide and NMDA had a lower volume of brain damage (13.8 mm$^3$; twenty-fifth to seventy-fifth percentiles: 12.6–16.4 mm$^3$; $P < 0.05$) than did rats that were given NMDA and medical air. Nitrous oxide further decreased neuronal death produced by coinjection of NMDA plus tPA by more than 40% so that rats treated with nitrous oxide and NMDA plus tPA had a lower volume of brain damage (19.2 mm$^3$; twenty-fifth to seventy-fifth percentiles: 16.3–21.9 mm$^3$; $P < 0.001$) than did rats that were given NMDA plus tPA in medical air (fig. 4).

Discussion

During the past decade, preclinical studies have proven that inert gases such as xenon and nitrous oxide at subanesthetic
concentrations may have effective neuroprotective properties with no adverse side effects. However, a recent study performed in a rat model of thromboembolic stroke has shown that xenon interacts directly with tPA, the most common approved therapy of acute ischemic stroke. Interactions of xenon with tPA produce adverse effects by reducing tPA-induced thrombolysis and, as a consequence, thrombolysis-induced reduction of brain damage when administered during the intraischemic period and beneficial effects by suppressing ischemic brain damage and tPA-induced brain hemorrhages and disruption of the blood–brain barrier when given after reperfusion. Because nitrous oxide has pharmacologic and neuroprotective properties similar to that of xenon in excitotoxic-ischemic models, we hypothesized that nitrous oxide may modulate the thrombolytic and proteolytic effects of tPA.

First, we found that nitrous oxide dose-dependently inhibits the catalytic (enzymatic) and thrombolytic efficiency of tPA in vitro. As expected from these results, we showed that nitrous oxide, when administered with tPA during the intraischemic period, reduces tPA-induced thrombolysis and thereby decreases and even suppresses the benefits of tPA therapy in terms of reduction of ischemic brain damage. The lack of neuroprotective action of intraischemic nitrous oxide almost certainly can be attributed to nitrous oxide inhibiting the enzymatic activity of tPA—that is the basic mechanism responsible for its thrombolytic action—by binding within the S1 pocket of tPA at the position of the specific tPA inhibitor tPA Stop, which is shown in the current study.

Next, in contrast to the adverse effect of intraischemic nitrous oxide and in agreement with previous reports in rats subjected to mechanical ischemia, we found that nitrous oxide given after ischemia reduces ischemic brain damage at the cortical but not the subcortical level. Such a dichotomic effect of postischemic nitrous oxide at the cortical and subcortical level could result at least in part from nitrous oxide increasing the cerebral metabolic rate in subcortical areas, such as the striatum and the thalamus, a condition that could “prolong” ischemia-induced disruption in oxygen and glucose even after reperfusion. In addition, as

**Fig. 3.** Effect of postischemic nitrous oxide (N₂O) at 75 vol% on middle cerebral artery occlusion (MCAO)-induced brain damage (A). Effects of postischemic nitrous oxide at 75 vol% on MCAO-induced disruption of the blood–brain barrier as assessed using Evans blue extravasation and MCAO- and tissue plasminogen activator (tPA)-induced brain hemorrhages (B). Typical examples of brain damage in rats treated with saline (Sal) and medical air, tPA and medical air, or tPA and nitrous oxide at 75 vol%. Slices in the background are those shown in D below (C). Examples of Evans blue extravasation and brain hemorrhages in brain slices (those shown in the background of B) treated with saline and medical air, tPA and medical air, or tPA and nitrous oxide at 75 vol% (D). Data are median value ± twenty-fifth to seventy-fifth percentiles. *P < 0.05 versus sham-treated rats; †P < 0.01–0.05 versus control rats (tPA + medical air). Sub-Cx = subcortex.
reported previously, we found that postinsult nitrous oxide decreased NMDA-induced neuronal death and postinsult nitrous oxide decreased to a greater extent the potentiation by tPA of NMDA-induced neuronal death, a finding that to our knowledge is reported here for the first time. Therefore, it is likely that inhibition of residual tPA by nitrous oxide after reperfusion played a major role in the ability of postischemic nitrous oxide to reduce ischemia-induced cortical brain damage in addition to its well known pharmacologic antagonistic action at the NMDA receptor, whose postsynaptic activation is known as a critical event in excitotoxic neuronal death. However, additional pharmacologic properties of nitrous oxide at other neuronal targets of potentially neuroprotective interest, such as the nicotinic acetylcholine receptor and the TREK-1 two-pore-domain K+ channel, could have contributed to the neuroprotective action of postischemic nitrous oxide at the cortical level. Alternatively, in contrast with its beneficial effects on ischemic cortical brain damage and NMDA- and NMDA-plus-tPA-induced neuronal death, we found that postischemic nitrous oxide increases tPA-induced brain hemorrhages and disruption of the blood–brain barrier. This effect, which is in good agreement with previous data showing that nitrous oxide increases cerebrovascular permeability to proteins and produces disruption of the blood–brain barrier, opposes that of xenon being shown to reduce dramatically ischemic brain damage and tPA-induced brain hemorrhages and disruption of the blood–brain barrier. Difference between the effects of postischemic xenon and postischemic nitrous oxide on tPA-induced brain hemorrhages and disruption of the blood–brain barrier are likely to occur at the vascular, rather than the parenchymal, level. This could be because xenon has no effect or even decreases cerebral blood flow in specific brain regions at concentrations to 1 minimum anesthetic concentration, whereas in contrast, nitrous oxide at concentrations as low as 0.5 minimum anesthetic concentration is well known to increase cerebral blood flow and cerebral blood flow velocity, conditions that could favor disruption of the blood–brain barrier, particularly during reperfusion.

The impact of isoflurane administration on the findings of the current study and the possibility that postischemic nitrous oxide at 75 vol% may have offered neuroprotection by reducing the rats’ brain temperature should be considered. During the intraischemic experiments, rats were maintained anesthetized after tPA injection for 45 min with a combination of isoflurane and nitrous oxide. In contrast, during postischemic experiments, rats were given isoflurane and nitrous oxide separately. Differences in neuroprotection against MCAO-induced brain damage between postischemic nitrous oxide alone and intraischemic nitrous oxide–isoflurane, as reported in this study and as can be concluded from independent studies, have been attributed to a proapoptotic interaction between isoflurane and nitrous oxide because neither nitrous oxide nor isoflurane produces apoptosis when given alone in the adult rat. In addition, it is possible that isoflurane could have contributed to neuroprotection in the postischemic experiments of this study because this volatile anesthetic has been shown to be a neuroprotectant preconditioning agent in the adult rat, although adverse apoptogenic effects have been reported in the developing brain. Alternatively, the possibility that 75 vol% postischemic nitrous oxide in freely moving animals may offer neuroprotection by reducing the rats’ brain temperature by 1°C has been ruled out previously because nitrous oxide at a lower concentration of 50 vol% has neuroprotective action without altering brain temperature, the brain temperature must be decreased to at least 34°C to provide neuroprotection in adult rats, and a reduction of brain temperature to 35°C is not sufficient to produce neuroprotection in rat pups.

Because compatibility between treatments is an inalienable condition for allowing combination therapeutic strategies, the interactions of nitrous oxide with tPA shown in this study clearly caution against using nitrous oxide for treating acute ischemic stroke patients; this is in contrast to our previous findings in rats subjected to mechanical brain ischemia. However, the current report has several possible limitations inherent in the study design and methodology. Intraischemic and postischemic rats were killed at different times after the onset of ischemia and exposed to different isoflurane-nitrous oxide protocols with tPA. However, as discussed in Materials and Methods and elsewhere in the text, each nitrous oxide group was compared with its own tPA control group, and we are confident, based on our in silico and in vitro catalytic and thrombolytic experiments, that differences between intraischemic and postischemic nitrous oxide result from the effect of nitrous oxide at inhibiting competitively the enzymatic
and thromboembolic properties of tPA. In addition, no investigation was performed to study neurologic outcome after treatment, mainly because a catheter had been inserted into the femoral vein to allow tPA injection, a condition that could have impaired assessment of neurologic outcome using behavioral motor parameters as performed in our previous mechanical MCAO-induced ischemia studies. Based on previous data showing that nitrous oxide provided behavioral motor neuroprotection despite producing no reduction of subcortical brain damage, a finding thought to be related to most ischemic episodes being free from the neurologic deficits (silent brain infarctions) known to occur in deep brain structures including the basal ganglia, it could be hypothesized that rats treated with postsischemic nitrous oxide that exhibited reduced cortical brain damage could have had positive neurologic outcome despite major brain hemorrhages. However, even if true, the occurrence of a significant hemorrhage in the brain parenchyma in humans is recognized as a critical event with consequences (depending on the brain anatomical area where it occurs) that can result in disability to fine specific functions, such as speech and language, that would be difficult or even impossible to detect in rodents.

In conclusion, because xenon in contrast to nitrous oxide has been demonstrated to suppress both ischemic brain damage and tPA-induced brain hemorrhages and disruption of the blood–brain barrier when given after reperfusion, we conclude that the findings of the current study indicate a clear advantage to the use of xenon as a neuroprotectant compared with the use of nitrous oxide. In addition, although our findings could not apply directly to heart attack-induced global brain ischemia per se, it is probable that nitrous oxide could inhibit the enzymatic-catalytic properties of the thrombolytic agents used in critical care cardiology because all serine proteases share a similar structure and identical catalytic triad. If such, it is likely that nitrous oxide also could interfere with global brain ischemia indirectly. This should be investigated if a clinically relevant model of thromboembolic cardiac stroke exists.

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