Prothrombolytic action of normobaric oxygen given alone or in combination with recombinant tissue-plasminogen activator in a rat model of thromboembolic stroke

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ACUTE ISCHEMIC STROKE is a major cause of mortality and long-term neurologic morbidity in the adult population. The primary cause of acute ischemic stroke is a disruption of cerebral blood flow through thromboembolism that leads to an oxygen and glucose deprivation for the cell, and subsequent secondary excitotoxic mechanisms (8, 27). Limitation of the primary vascular insult by early reperfusion still remains the only approved therapy of acute ischemic stroke to date, since blockade of the excitotoxic cascade has failed to be efficient in humans. Although new techniques and devices for mechanical removal of the thrombus are being developed (13), the only approved therapy of acute ischemic stroke to date is thrombolysis by recombinant human tissue plasminogen activator (rtPA). Although adverse hemorrhagic effects have been reported, early reperfusion by rtPA has benefited ischemic stroke patients if given within 3–6 h of symptoms onset (14, 25, 42).

Unfortunately, although single centers have reported higher rates of rtPA administration, US national and population-based estimates of the use of rtPA therapy have been estimated and recently confirmed to be substantially less than 5% of acute ischemic stroke patients mainly due to some contraindications to treatment and most importantly to the narrow therapeutic time window of rtPA (23, 34).

Alternatively, as tissue hypoxia plays a critical role in the primary and secondary events that lead to ischemia-induced neuronal death (46), tissue oxygenation with oxygen-enriched medical air or 100% normobaric oxygen (NBO) is generally thought to be a logical therapeutic stroke strategy. Accordingly, experimental studies have demonstrated beneficial effects of NBO if started early after cerebral ischemia (9, 19, 22, 37, 38). However, in contrast, clinical studies performed with oxygen-enriched medical air or NBO have led to more puzzling conclusions. While one half of these studies has led to negative results (33, 34), the other half has concluded that NBO may benefit the ischemic patient (4, 36) possibly by increasing reperfusion (36). In line with this latter study, recent magnetic resonance imaging (MRI) data from a mechanical model of acute ischemic stroke have shown that NBO increases cerebral blood flow (CBF) in the ischemic penumbra (2). However, the only two studies performed in rats subjected to thromboembolic middle cerebral artery occlusion (MCAO) have failed to show significant NBO-induced changes in CBF (10, 20). However, it is noteworthy that these latter studies were performed in rats that were shifted to NBO immediately after nitrous oxide, a condition that could have biased results since nitrous oxide has been shown to inhibit potently thrombolysis (17).

To advance the critical question of the use of NBO in acute ischemic stroke, we studied the effects of NBO administered alone or in combination with rtPA on CBF and ischemic brain damage in rats exposed to thromboembolic MCAO-induced brain ischemia, a clinically relevant model of acute ischemic stroke in rodents.

MATERIALS AND METHODS

Animals. All animal-use procedures were examined by a local ethic committee (Cyceron, Caen, France) in accordance with the European Communities Council Directive of 24 November 1986 for the use of animals in biomedical experimentation (86/609/EEC, also known as the Declaration of Helsinki), and approved with permit number 14–27. Before surgery, all animals were housed at 21 ± 0.5°C with lights on from 8:00 pm to 8:00 am in a group of four in Perspex home cages with free access to food and water. After surgery, all animals were housed individually in similar environmental conditions.
Middle cerebral artery occlusion-induced ischemia. Male Sprague-Dawley rats weighing 250–275 g were used to assess the effects of NBO given alone or in combination with rtPA on CBF and MCAO-induced brain damage. Rats were subjected to MCAO-induced ischemia by administration of an autologous blood clot as described in detail previously (6). Briefly, 24 h before the animals were subjected to ischemia a whole caudal blood sample of 200 μl was withdrawn and allowed to clot at 37°C for 2 h. Then, 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. On the day of surgery, the rats were anesthetized with 2 vol% isoflurane in medical air composed of 25 vol% oxygen and 75 vol% nitrogen, intubated, and ventilated artificially. Catheters were inserted into the femoral vein to allow injection of rtPA 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. Rats were placed prone in a stereotaxic frame, and a laser-Doppler flowmetry probe was positioned onto the right parietal bone, previously thinned with a saline-cooled dental drill (coordinates 1.7 mm posterior, 5.5 mm lateral from the bregma), to assess successful induction of cerebral ischemia and to monitor changes in CBF continuously. Changes in CBF were expressed as a percentage from CBF control 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 microtubing directed into the internal carotid artery up to 2 mm after the pterygopalatin-internal carotid artery bifurcation. Following a 45-min period of occlusion during which all rats were given medical air, microtubing was removed from the internal carotid artery to the external carotid artery and the rats were given rtPA + medical air, NBO + saline, or NBO + rtPA. rtPA alone or in combination with NBO was given over a 45-min period at the dose of 0.9 mg/kg in 1 ml saline solution (10% bolus and 90% perfusion), a dose shown to be more clinically relevant particularly in gas pharmacology studies than that of 10 mg/kg often used in rodents studies (18). NBO alone was also given for 45 min. Then all rats were given medical air again. Rats were maintained normothermic throughout the surgery and ischemia-reperfusion protocol. Then the rats were returned back to their home cage and allowed to move freely with food and water ad libitum. To mimic clinical recommendations (31), rats showing less than 50% (controls, n = 1; rtPA, n = 1; NBO, n = 2; NBO + rtPA, n = 2) or more than 85% reduction (rtPA, n = 2; NBO, n = 1) in CBF as calculated over a 15-min period before treatment were excluded from the study design. Also, rats that died during MCAO-induced ischemia (n = 7) and those that died before being used for histologic analysis (n = 3) were excluded from statistical data. The final number of animals taken into account for statistical analysis was n = 5 per group. Brain damage was assessed as described below.

NMDA-induced neuronal death in vivo. On the day of surgery, rats were anesthetized with 1.5% halothane in medical air and mounted on a stereotaxic apparatus with the incisor bar set at 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 N-methyl-D-aspartate (NMDA) alone or in combination with 3 μg rtPA in the form of Actilyse in 1 μl of PBS (pH 7.4) over a 2-min period. After an additional 5-min period, the micropipette was removed and the animals were returned to their home cage with free access to food and water. Sixty minutes after administration of NMDA or NMDA plus rtPA, rats were treated for 3 h with medical air or 100 vol% oxygen as described above. The number of animals was as follows: NMDA + air, n = 12; NMDA + rtPA + air, n = 8; NMDA + rtPA + NBO, n = 8. No rat was excluded from statistical analysis. Brain damage was assessed as described below.

Assessment of brain damage, brain hemorrhages, and disruption of the blood-brain barrier. Rats were killed by decapitation under isoflurane anesthesia 24 h after the onset of ischemia or injection of saline, NMDA or NMDA plus rtPA. Studies with excitotoxic, transient, or permanent ischemic models of brain injury have shown using magnetic resonance imaging, triphenyltetrazolium chloride, thionin, and neuronal nuclei immunochemical staining techniques that assessment of infarct size at 24 h is a time condition sufficient to obtain consolidated neuronal death, i.e., whose assessment would be similar if performed one or several days later (11, 15, 21). Before being killed, rats were given Evans blue intravenously (4% in 1 ml saline solution). Then 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 ImageJ software analyzer (Scion, MD), corrected for tissue edema when needed (ischemia) by calculating and dividing volume by the ipsilateral/contralateral brain hemispheres ratio, and expressed in cubic millimeters. While cutting the unstained brain mounted on the cryostat, macrophotographs were taken, digitized on a computer, and analyzed using the ImageJ software analyzer to assess brain hemorrhages and disruption of the blood-brain barrier as detailed previously (6). Evans blue extravasation was delineated, and multiplied by the extravasation’s optical density. Brain hemorrhages were delineated as blood evident at the macroscopic level, and expressed in square millimeters. The experiments were performed in a blinded manner according to the Stroke Therapy Academy Industry Roundtable (STAIR) recommendations as follows: the investigators who performed the surgery and treatment gave each group of rats a secret code that remained unknown to the experimenters in charge of assessing brain tissue damage, brain hemorrhages, and disruption of the blood-brain barrier until the end of the study.

rtPA and reteplase catalytic efficiency assay. The effects of oxygen at 0, 25, 50, 75, and 100 vol% on the catalytic efficiency of tPA (alteplase) and reteplase, a tPA-derived drug that lacks the fibronectin type I, EGf-like, and kringle 2 and catalytic domains, were assessed as detailed previously (6). Briefly, the recombinant form of human tPA in the form of Actilyse, reteplase in the form of Rapilysin (Actavis, Le Plessis Robinson, France), and their specific chromogenic substrate methyl-sulfonyl-t-phenyl-glycyl-arginine-7-amino-4-methylcoumarin acetate (spectrozyme XF, ref. 444; American Diagnostica, Stamford, CT) were separately diluted in 1 ml of distilled water in 1.5-ml sterile tubes. Each tube containing 0.4 μM of tPA, 0.09 unit of reteplase, or 10 μM of the tPA substrate was saturated for 20 min with oxygen of 0 to 100 vol% (with the remainder being nitrogen when necessary). The catalytic efficiency of human tPA and reteplase (for each, n = 12 per oxygen concentration) was assessed using the initial rate method by incubating 50 μl of tPA with 50 μl of substrate at 37°C in a spectrofluorometer microplate reader.

In vitro thrombolysis experiments. In vitro thrombolysis experiments were performed as detailed previously (6). Briefly, whole blood samples of 500 μl volume drawn from male mature Sprague-Dawley rats weighing 600–650 g (n = 4) were transferred in preweighed tubes of 1.5 ml and incubated at 37°C for 3 h. After clot formation and total serum removal, each tube was weighed to determine the clot weight. Blood clots were selected in the same weight range (0.296 ± 0.009 g) to reduce variability. Each tube was filled with saline solution containing 1 μg/ml of tPA in the form of Actilyse saturated with 100 vol% oxygen (n = 13) or air (n = 12), and incubated at 37°C for a 90-min period. Then the fluid was removed, and the tubes were weighed again to assess the percentage of clot lysis induced by rtPA in the presence of medical air or 100 vol% oxygen.

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Treatments were performed after 14 days in vitro. Intracellular free Ca\(^{2+}\) changed twice a week in a humidified atmosphere containing 5% CO\(_2\). NBO, normobaric oxygen at 100 vol%. *

NMDA-induced Ca\(^{2+}\) influx in neuronal cell cultures. Cerebral cortices from 14- to 15-day-old embryos were dissected in Dulbecco's modified Eagle medium containing 5% fetal bovine serum (FBS), 5% horse serum, and 2 mM glutamine (all from Sigma-Aldrich, Lyon, France). Cortical cells were dissociated for trituration, plated in glass petri dishes coated with 0.1 mg/ml poly-o-lysine (Sigma-Aldrich, Lyon, France) and 0.02 mg/ml laminin (Invitrogen, Villebon-sur-Yvette, France), and cultured in Dulbecco's modified Eagle medium changed twice a week in a humidified atmosphere containing 5% CO\(_2\). Treatments were performed after 14 days in vitro. Intracellular free Ca\(^{2+}\) in neurons was measured using fura-2 fluorescence videomicroscopy on the stage of a Nikon Eclipse inverted microscope equipped with a 100-W Xenon lamp and oil immersion objective (Nikon 40\(\times\), NA 1.3). Cultures on glass bottom dish were loaded for 45 min with 10 \(\mu\)M fura-2-acetoxymethyl ester (AM; F-1201, Invitrogen, Villebon-sur-Yvette, France) and 0.2% pluronic acid and 0.02% horse serum, and 2 mM glutamine (all from Sigma-Aldrich, Lyon, France) and 0.2% pluronic acid and incubated for an additional 15 min at room temperature in a HEPES-buffered saline solution. Fura-2 (ex: 340 nm/380 nm) ratio images were acquired and digitized through a CCD camera in a computer at a resolution time of 2 s per successive measurement using Metafluor 6.3 software (Universal Imaging, Chester, PA). Changes in intraneuronal free Ca\(^{2+}\) were induced by a stimulation of 30-s duration induced by 25 \(\mu\)M NMDA given alone or after exposure of cortical neurons to 20 \(\mu\)g/ml rtPA (and medical air) for a 15-min period in the presence or absence of NBO. During the experiments, the cells were continuously perfused at a rate of 2 ml/min using a peristaltic pump with a HEPES-buffered saline solution previously saturated with medical air or NBO. Size of samples was \(n = 105-120\) per condition.

Gas pharmacology. Oxygen and nitrogen of medical grade were bought to Air Liquide Santé (Paris, France). Gas mixtures containing oxygen at 0 to 100 vol%, with the remainder being nitrogen when necessary, were obtained using computer-driven flowmeters and gas analyzers.

Statistical analysis. Data were analyzed using the Statview software (SAS Institute, Cary, NC). In vitro data, given as means ± standard error of the mean (SE), were analyzed using parametric ANOVA and Student’s t-test. In vivo data, given as median value ± 25th–75th percentiles, were analyzed using the Kruskal-Wallis non-parametric ANOVA and Mann-Whitney nonparametric unpaired U-test. The level of significance was set at \(P < 0.05\). The number of animals per group for the in vivo ischemic studies was assessed by performing power analysis through an online software (www.dssresearch.com/toolkit/sscalc/size_a2.asp) with the following variables taken from a previous study with xenon at 37.5 vol%, based on the fact that NBO increased the catalytic activity of tPA by approximately the same amplitude as 37.5 vol% xenon did decrease it (7): mean ± SD for rtPA = 163 ± 59 mm\(^3\); mean ± SD for xenon at 37.5 vol% = 317 ± 62 mm\(^3\); α error level (confidence level) = 0.05; β error level (or statistical power, 1 – β) = 0.2 (or 0.8); minimum number of animals required, \(n = 3\). RESULTS

NBO has dual effects depending on if given alone or in combination with rtPA. First, we studied the effects of NBO given alone or in combination with rtPA in male adult Sprague-Dawley rats subjected to thromboembolic brain ischemia. Upon clot injection, CBF dropped to about 30% of preischemic baseline, and remained stable throughout the 2-h duration of the study in control animals but not in rats treated with rtPA and/or NBO. Physiological parameters remained within normal range in all groups (Table 1). Before NBO, arterial Po2 levels were between 75 and 85 mmHg in all rats. NBO given alone or in combination with rtPA rapidly and markedly elevated arterial Po2 levels to above 400 mmHg (Table 2).

Control rats treated with saline solution and medical air showed no CBF restoration (Fig. 1, A and B), had ischemic brain damage and swelling, respectively, of 395 mm\(^3\) (25–75 percentiles: 370–402 mm\(^3\); Fig. 1C) and 21.9% (16.7–23.7%; Fig. 1D), and further exhibited brain hemorrhages and disruption of the blood-brain barrier (Fig. 1, E and F), respectively, in the form of discrete hematomas and dark and discrete areas of Evans blue extravasation (Fig. 1, G and H).

As expected, rats treated with rtPA (and medical air) had sustained CBF restoration (Fig. 1, A and B), and reduced ischemic brain damage and swelling, respectively, of 167 mm\(^3\) (25–75 percentiles: 160–209 mm\(^3\); \(P < 0.01\); Fig. 1C) and 10.1% (25–75 percentiles: 7.3–12%; \(P < 0.01\); Fig. 1D) compared with control animals. However, despite these beneficial effects and in line with the well-known adverse properties of rtPA therapy, rtPA-treated rats showed no reduction of brain hemorrhages (Fig. 1, E, G, and H) and disruption of the blood-brain barrier (Fig. 1, F–H) compared with control rats. Likewise, rats treated with NBO (and saline solution) showed sustained CBF restoration (Fig. 1, A and B), and exhibited reduced ischemic brain damage and swelling, respectively, of 93 mm\(^3\).

### Table 1. Values of PaCO2, arterial pH, and temperature

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<tr>
<td>PaCO2 mmHg</td>
<td>40 ± 1</td>
<td>38 ± 1</td>
<td>39 ± 1</td>
<td>7.45 ± 0.01</td>
<td>7.43 ± 0.01</td>
<td>7.39 ± 0.01</td>
<td>37.4 ± 0.2</td>
<td>37.5 ± 0.1</td>
<td>37.4 ± 0.2</td>
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<td>NBO</td>
<td>38 ± 2</td>
<td>38 ± 2</td>
<td>39 ± 1</td>
<td>7.49 ± 0.01</td>
<td>7.47 ± 0.01</td>
<td>7.45 ± 0.01</td>
<td>37.8 ± 0.1</td>
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Data are expressed as means ± SE. PaCO2, arterial carbon dioxide partial pressure; pH, arterial pH; Temp, temperature; Ctrl, control values recorded during a 20-min period of control before the onset of ischemia; Isch, mean values recorded during the 45-min period of ischemia; Reperf, mean values recorded during the 45-min period of treatment; NBO, normobaric oxygen at 100 vol%. *\(P < 0.02\) vs. medical air.

### Table 2. Values of PaO2, SaO2, and MAP

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<tr>
<td>PaO2 mmHg</td>
<td>88 ± 12</td>
<td>85 ± 14</td>
<td>93 ± 2</td>
<td>96 ± 1</td>
<td>96 ± 2</td>
<td>97 ± 1</td>
<td>90 ± 11</td>
<td>85 ± 11</td>
<td>77 ± 10</td>
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<tr>
<td>NBO</td>
<td>84 ± 3</td>
<td>76 ± 3</td>
<td>506 ± 28*</td>
<td>97 ± 1</td>
<td>96 ± 1</td>
<td>100 ± 0*</td>
<td>96 ± 3</td>
<td>96 ± 3</td>
<td>105 ± 2*</td>
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Data are expressed as means ± SE. PaO2, arterial oxygen partial pressure; SaO2, arterial hemoglobin saturation of oxygen; MAP, mean arterial pressure. *\(P < 0.02\) vs. medical air.
Rats treated with NBO and rtPA in combination had sustained CBF restoration similar to that of rtPA- and NBO-treated rats (Fig. 1A). Although the lack of synergistic effect of combined NBO and rtPA on CBF restoration could appear surprising at first view, this result is in excellent agreement with previous data that have shown that CBF restoration produced by 10 mg/kg rtPA is only twice that produced by the clinical dose of 0.9 mg/kg rtPA (18) used in the present study. Despite showing CBF restoration, rats treated with combined NBO and rtPA had ischemic brain damage and swelling, respectively, of 435 mm³ (25–75 percentiles: 239–466 mm³; Fig. 1C) and 26.7% (25–75 percentiles: 21.4–32.6%; Fig. 1D) that were not different from those of control animals and greater than those of rtPA and NBO-treated rats (P < 0.01–0.05; Fig. 1, C and D). Also, rats treated with combined NBO and rtPA showed a trend toward increase of brain hemorrhages that reached statistical difference compared with NBO-treated rats (P < 0.02; Fig. 1, E, G, and H), and further exhibited lower (P < 0.05) and higher (P < 0.02) disruption of the blood-brain barrier, in the form of light and diffuse Evans blue extravasation that often goes beyond the ischemic brain damage areas, compared with rtPA- and NBO-treated rats, respectively (Fig. 1, F–H).

NBO facilitates the catalytic and thrombolytic efficiency of rtPA in vitro. Next, to examine whether NBO could have actually promoted thrombolysis in vivo by interacting directly with endogenous tPA, we characterized in vitro by the initial rate method the effects of various concentrations of oxygen on the catalytic efficiency of rtPA and reteplase, a tPA-derived drug that lacks the fibronectin type I, EGF-like, and/or kringle 1 domains and only possesses the kringle 2 and catalytic domains. We found that the addition of oxygen into the medium increases the catalytic efficiency of rtPA in a concentration-dependent manner (P < 0.001). Interestingly, the absence of oxygen into the medium led to a decrease of the catalytic activity of rtPA compared with control values obtained with medical air, a result that indicates as a novel concept that the lack of oxygen in an ischemic brain area would play a key role in the thrombosis process. Contrasting with the strong facilitating effect of oxygen on the catalytic efficiency of rtPA, we found that oxygen at the higher concentrations of 75 vol% and 100 vol% induced a modest decrease of the catalytic efficiency of reteplase (P < 0.05), indicating that NBO would produce thrombolysis in vivo by interacting with endogenous tPA through the fibronectin type I, EGF-like, and/or kringle 1 domains (Fig. 2A). To demonstrate this possibility, we studied the effect of NBO on the thrombolytic efficiency of rtPA and reteplase in vitro in whole blood samples drawn from male mature rats. NBO increased rtPA-induced thrombolysis by about 40% compared with whole blood samples treated with rtPA and medical air (P < 0.005) but failed to alter the thrombolytic efficiency of the tPA-derived drug reteplase (Fig. 2B).

**DISCUSSION**

**Neuroprotective effects of NBO administered alone.** We showed that NBO achieved clot lysis similar to 0.9 mg/kg rtPA, and thereby reduced ischemia-induced brain damage, swelling, and hemorrhages. Because we showed that NBO facilitates the catalytic and thrombolytic efficiency of rtPA through direct interaction with the fibronectin type I, EGF-like, and/or kringle 1 tPA domains, we assume that the in vivo thrombolytic effects of NBO occur through facilitation of the fibrinolytic properties of endogenous tPA, which activity in contrast with what is generally thought is significantly increased within the ischemic brain (44). Also, in line with previous data that have reported that NBO given before rtPA did reduce rtPA-induced brain hemorrhages (42), we found that NBO-treated rats had reduced brain hemorrhages compared with control rats. In light of our findings that NBO has a facilitating action on the catalytic and thrombolytic efficiency of tPA, it is likely that the reduction of brain hemorrhages in this latter study could result from the fact that NBO yet has produced recanalization or partial recanalization through activation of endogenous tPA before rtPA injection. Taken together, these results provide experimental evidence for a small clinical pilot study in patients ineligible for rtPA therapy that has first hypothesized that NBO could reduce ischemic brain damage by increasing reperfusion (36), as well as basic mechanisms for previous data that have shown in animal models that NBO allows delaying the transition of ischemia to infarction (“buying time”; 22). Taken together with previous findings that have shown that NBO decreases ischemia-induced acidosis and disruption of energy metabolism (40), in-
creases tissue oxygenation (26), and reduces NMDA-mediated excitotoxicity (16), our results highlight the mechanisms by which intraischemic NBO has beneficial action on brain damage, swelling, and hemorrhages.

**Lack of neuroprotective action of combined NBO and rtPA.** In contrast with the beneficial effects of NBO (and rtPA) administered alone, we found that rats treated with combined NBO and rtPA despite showing CBF restoration had ischemic
brain damage and swelling equivalent to those of control animals, and greater than those of rtPA- and NBO-treated rats. Because NBO alone induces a facilitation of the catalytic activity of tPA, we hypothesized that the adverse effects of combined NBO and rtPA on brain damage and swelling could have resulted from a NBO-induced potentiation of the well-known rtPA-induced facilitation of ischemia- and NMDA-mediated excitotoxic processes (32, 43, 44). However, such a mechanism is unlikely to be true since as shown in this study NBO reduces the facilitating effects of rtPA on NMDA-induced intraneuronal calcium influxes and neuronal death, a result in agreement with previous data that have reported redox modulation of the NMDA receptor with reduction producing a potentiation and oxidation an inhibition of the NMDA receptor response and glutamate-induced neuronal death (25, 39). These contrasting effects of combined NBO and rtPA on ischemia- and NMDA-induced brain damage clearly support that the basic mechanisms involved in the lack of neuroprotective action of combined NBO and rtPA are likely to occur at the neurovascular rather than the parenchymal side of the central nervous system. Strong support for this and our findings are recent data that have shown on one hand that rtPA and NBO, respectively, increases and decreases serum concentrations of matrix metalloproteinase-9, whose expression is known to be critically associated with ischemia-induced disruption of the blood-brain barrier, and on the other hand that combining oxygen and rtPA suppresses the beneficial effects of NBO at decreasing matrix metalloproteinase-9 (30). Based on these findings, the authors suggest as found in the present report that combining NBO and rtPA could not be a safe strategy (30).

Comparison with previous data. Our findings that NBO restores CBF oppose previous investigations that have reported no effect of NBO alone on CBF and ischemic brain damage in rats subjected to a thromboembolic model of acute ischemic stroke (10, 20) or showed that NBO could be coadministered safely with rtPA (20). Differences in the duration of NBO treatment—less than 1 h until reperfusion in the present study vs. 3 h or 3.5 h from before to after reperfusion in these latter studies—could account for these discrepancies since postischemic NBO is known to exacerbate brain tissue damage and deteriorate neurologic outcome (16, 31). Also, such discrepancies could mainly result from the fact that the above mentioned studies were performed in rats anesthetized with 70 vol% nitrous oxide that were shifted to NBO immediately after

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**Fig. 2.** A: concentration-dependent effects of oxygen of 0 to 100 vol% on the catalytic efficiency of human rtPA (black square) and the tPA-derived drug reteplase (RPA) that lacks the fibronectin type I, EGF-like, and/or kringle 1 tPA domain (open square) compared with their own controls performed in medical air composed of 25 vol% oxygen and 75 vol% nitrogen taken as a 100% value (gray square). NBO at concentration of 50–100 vol% increases the catalytic efficiency of tPA. In contrast, the absence of oxygen reduces the catalytic efficiency of tPA, thereby suggesting as a novel concept that the lack of oxygen in an ischemic brain area would play a key role in the thrombosis process. Size of samples is \( n = 12 \) per condition. * \( P < 0.001 \) vs. rtPA own controls; ‡ \( P < 0.05 \) vs. RPA own controls. B: effects of NBO (100 vol% oxygen) on the thrombolytic efficiency of rtPA and reteplase (RPA) in vitro on blood clots obtained from whole blood samples. NBO increases the thrombolytic activity of rtPA but not of reteplase. Size of samples is \( n = 6 \) per condition. * \( P < 0.005 \) vs. rtPA own controls. Data are expressed as means ± SE. Taken together, these data indicate that NBO decreases the catalytic and thrombolytic activity of rtPA through direct interaction with the fibronectin type I, EGF-like, and/or kringle 1 tPA domain.

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**Fig. 1.** A: time-course effects of recombinant tissue-plasminogen activator (rtPA) and normobaric oxygen (NBO) given alone or in combination on cerebral blood flow (CBF) in a rat model of thromboembolic stroke. Rats treated with rtPA, NBO, or coadministration of rtPA and NBO showed similar CBF restoration. Untreated control rats exhibited no reperfusion. B: individual data of cumulative CBF during treatment from T to T′ in control rats (black), and rats treated with NBO (yellow), rtPA (blue), or combined NBO–rtPA (green). To plot data, because NBO and rtPA at 0.9 mg/kg had similar effects on CBF restoration, we assume that NBO may be considered equivalent to 0.9 mg/kg rtPA, and the combination of NBO plus rtPA equivalent to 1.8 mg/kg. Values with 10 mg/kg rtPA (red) were obtained from a previous study (17). Data analysis shows a high and significant correlation coefficient (\( R = 0.9074, df = 23, P < 0.001 \)) that indicates that the lack of significant effect of combined NBO and rtPA at increasing cerebral reperfusion compared with NBO or rtPA alone is in good agreement with the fact that CBF restoration produced by 10 mg/kg rtPA is only twice that produced by 0.9 mg/kg rtPA. C–F: scores of brain damage (C), brain swelling (D), brain hemorrhages (E), and Evans blue extravasation (F) in rats treated with rtPA, NBO, or coadministration of rtPA and NBO. Top part and bottom part of the histograms in C represent subcortical and cortical brain damage, respectively. Rats treated with rtPA and NBO alone, but not in combination, had decreased brain damage, swelling, and hemorrhages compared with middle cerebral artery occlusion (MCAO) control rats. No significant difference was found between rtPA- and NBO-treated rats. G: typical examples of brain slices from control rats and rats treated with rtPA and NBO alone or in combination. Left: Evans blue extravasation; right: Evans blue extravasation and comparison with neuronal death. Data are expressed as the median value and 25th–75th percentiles. The number of animals is \( n = 5 \) per group. ** \( P < 0.01 \), * \( P < 0.05 \) vs. MCAO control rats; \( SP < 0.01 \), ‡ \( P < 0.05 \) vs. rtPA-treated rats; ‡‡ \( P < 0.05 \) vs. NBO-treated rats.
nitrous oxide (10, 20), a condition that could have biased results and altered NBO-induced facilitation of tPA catalytic and thrombolytic activity since nitrous oxide has been recently shown to inhibit potently the catalytic and thrombolytic activity of tPA (17). Indeed, inert gases and oxygen bind and compete for binding to proteins within hydrophobic cavities as a function of multiple factors among which affinity, as defined by the Hildebrand coefficient and the Meyer-Overton rule (with xenon > nitrous oxide > krypton > argon > oxygen > nitrogen > helium), play a critical role (5, 28, 45). Accordingly, once bound, inert gases with actually higher affinity than oxygen, such as xenon and nitrous oxide, do not remove out from the brain as soon as it is generally thought when administration is stopped. For instance, clinical trials have shown that the time to obtain full recovery from 8-h moderate sedation with xenon at 35 vol% [that is half the minimum anesthetic concentration (MAC) of xenon] is 35 min (3). Given the MAC value of nitrous oxide that is 1.5-fold higher that of xenon (7; which indicates that nitrous oxide given at equal concentration as xenon is less potent at producing narcosis/anesthesia) and the blood/gas partition coefficient of nitrous oxide that is 0.46 vs. 0.12 for xenon (12; which indicates that nitrous oxide has a longer wash-out than xenon), it can be estimated that the time to obtain full recovery from a similar nitrous oxide sedation would be ~90 min. Support for such long-lasting effect of nitrous oxide and our findings that NBO increases CBF are recent MRI data that have demonstrated in rats subjected to mechanical acute ischemic stroke that oxygen, given ~90 min after nitrous oxide and MCAO procedure (with medical air in between), increases CBF in the ischemic penumbra in a concentration-dependent manner (2). Other examples for long-lasting effects of nitrous oxide are also available, for instance in pain research. In that way, particularly, and in line with our findings that nitrous oxide inhibits thrombolysis (17), clinical studies in patients anesthetized with or without nitrous oxide for noncardiac surgery have shown that the use of nitrous oxide is associated with an increased risk of postoperative myocardial ischemia (1). Therefore, it can be concluded with little doubt that administration of nitrous oxide at 70 vol% before NBO treatment may have actually altered NBO-induced facilitation of tPA thrombolytic activity and thereby NBO-tPA interactions in the above mentioned studies (10, 20). Also, it is possible that the dose of 10 mg/kg rtPA used in these studies could have masked the effect of NBO on rtPA, since the use of such a high dose of rtPA could have allowed the presence of rtPA molecules free of oxygen (18) in contrast with the dose of 0.9 mg/kg used in the present report and in previous studies on inert gas modulation of rtPA thrombolytic and proteolytic properties (6, 17).

Clinical perspectives. From a clinical perspective, our results provide experimental evidence that the use of NBO alone may be an efficient alternative therapeutic strategy to rtPA, particularly for the vast majority of patients who cannot be treated with rtPA, and/or a primary care therapeutic intervention for those eligible for rtPA therapy. If such, since delayed NBO has no beneficial effect and can even deteriorate outcome, it is likely that the use of NBO should be restricted to the ischemic period to promote reperfusion and then stopped once reperfusion has been assessed using CBF monitoring. Also and in contrast with the beneficial effect of NBO alone, our results further suggest that NBO should not be administered concomitantly with rtPA therapy in acute ischemic stroke patients.

We believe that our findings of 1) prothrombolytic action of NBO should be tested and compared with rtPA in carefully designed clinical trials in acute ischemic stroke patients, and 2) adverse interactions between NBO and rtPA should be used as a principle of caution to avoid combining NBO and rtPA until our results will or will not be confirmed in acute ischemic stroke patients.

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AUTHOR CONTRIBUTIONS


REFERENCES


