Inhaled Nitric Oxide Reduces Brain Damage by Collateral Recruitment in a Neonatal Stroke Model

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Background and Purpose—We recently demonstrated that endogenous nitric oxide (NO) modulates collateral blood flow in a neonatal stroke model in rats. The inhalation of NO (iNO) has been found to be neuroprotective after ischemic brain damage in adults. Our objective was to examine whether iNO could modify cerebral blood flow during ischemia–reperfusion and reduce lesions in the developing brain.

Methods—In vivo variations in cortical NO concentrations occurring after 20-ppm iNO exposure were analyzed using the voltammetric method in P7 rat pups. Inhaled NO-mediated blood flow velocities were measured by ultrasound imaging with sequential Doppler recordings in both internal carotid arteries and the basilar trunk under basal conditions and in a neonatal model of ischemia–reperfusion. The hemodynamic effects of iNO (5 to 80 ppm) were correlated with brain injury 48 hours after reperfusion.

Results—Inhaled NO (20 ppm) significantly increased NO concentrations in the P7 rat cortex and compensated for the blockade of endogenous NO synthesis under normal conditions. Inhaled NO (20 ppm) during ischemia increased blood flow velocities and significantly reduced lesion volumes by 43% and cellular damage. In contrast, both 80 ppm iNO given during ischemia and 5 or 20 ppm iNO given 30 minutes after reperfusion were detrimental.

Conclusions—Our findings strongly indicate that, with the appropriate timing, 20 ppm iNO can be transported into the P7 rat brain and mediated blood flow redistribution during ischemia leading to reduced infarct volume and cell injury. (Stroke. 2012;43:3078-3084.)

Key Words: Doppler ultrasound imaging • inhaled NO • neonatal brain • neuroprotection • stroke

Neonatal hypoxia–ischemia is a common cause of neonatal brain injury and results in cerebral palsy, learning disabilities, and epilepsy.1 In addition to global cerebral ischemia arising from systemic asphyxia, recent data suggest a higher incidence of focal ischemia–reperfusion leading to stroke in near-term neonates.2 Tissue-type plasminogen activator is the only approved agent capable of improving reperfusion after ischemia in the adult brain.3 However, no safe neuroprotective molecule is currently available to protect the immature brain.

Among vasoactive molecules, nitric oxide (NO) is a small, highly diffusible and reactive molecule produced by the NO synthases and released from endothelial cells4 and perivascular nitriergic neurons.5,6 Endogenous NO is widely recognized as an important messenger and effector molecule for vascular tone and tolerance to damage as well as a mediator in a variety of acute and chronic inflammatory systems.7 NO is also involved in several critical processes in the developing brain; indeed, exposure to inhaled NO (iNO) during the first week of postnatal life has recently been shown to play a key role in myelination in the developing brain8 and to significantly reduce the size of excitotoxic lesions in neonatal rat pups.9

We recently demonstrated, by using 2-dimensional color-coded pulsed ultrasound imaging, that collateral recruitment or failure during ischemia, as revealed by changes in blood flow velocities in the basilar trunk (BT), determined the extent of the ischemic lesion in P7 rats10 and was
NO-dependent. Blood flow increases in the BT mirror the efficiency of collateral support through the circle of Willis and the opening of cortical arterial anastomoses among anterior, middle, and posterior vascular territories. However, the magnitude of collateral circulation and/or autoregulatory mechanisms was more prominent in immature than mature animals. In addition, iNO was effective in preventing brain ischemia in the adult with the selective improvement of blood flow in areas undergoing ischemia.

Here, we hypothesized that iNO would increase arterial and regional cerebral blood flow (CBF) during ischemia–reperfusion and reduce lesion size in the developing brain. Using a rat model of neonatal cerebral artery occlusion, we provide evidence that iNO significantly improves CBF during ischemia but not after reperfusion and reduces the infarct volume in P7 rats.

**Methods**

An expanded version of the methods is available in the online-only Data Supplement.

**Neonatal Ischemia–Reperfusion**

All animal procedures complied with the ethical guidelines of the Robert Debré Hospital Research Council Review Board (A75-19-01), and INSERM. Focal ischemia was induced in P7 Wistar rats (16–18 g; Janvier, Le Genest St-Irène, France) by left middle cerebral artery electrocoagulation combined with a transient (50 minutes) and concomitant occlusion of both common carotid arteries. Carotid blood flow restoration was verified with the aid of a microscope. Animals were euthanized after 48 hours.

**Gas Exposure and Drug Treatments**

Animals were randomly assigned to air, iNO, NO synthase inhibitor, and vehicle groups. For air and iNO exposure, animals were placed in a normoxic and normocapnic gas chamber (Biospherix, Redfield, NY) containing 0 (air); 5, 20, or 80 ppm NO during ischemia (50 minutes); or 5 or 20 ppm NO 30 minutes after reperfusion for 12 hours. NO, NO, and NO concentrations (<1 ppm) were monitored using the iNOvent system (Ikaria, Clinton, NJ). NG-nitro-L-arginine methyl ester (L-NAME, 20 mg/kg) or phosphate-buffered saline was injected intraperitoneally 1 hour before ischemia (n=6 for each condition). Two investigators blind to the treatment group determined the size of the lesion in each animal (n=64).

**Physiological Parameters**

Arterial blood was drawn by intracardiac puncture and gases (PaO2, PaCO2) were measured under air (n=4) and 20 ppm iNO exposure (n=6).

**Cortical NO Detection**

Rat pups were premedicated with buprenorphine (0.1 mg/kg intraperitoneally) and sodium pentobarbital (25 mg/kg intraperitoneally) and given additional injections of sodium pentobarbital (15 mg/kg) every 90 minutes. The oxidation current from NO was monitored with carbon fiber microelectrodes imпланted into the brain using the iNOvent system (Tacussel, France) and computerized (analog/digital interface; National Instruments). A NO donor, diethylamine NONOate (50 mg/kg), was administered intraperitoneally.

**Ultrasonographic Brain Imaging**

Thermoregulated rats were subjected to ultrasound measurements under 0.5% isoflurane anesthesia using an echocardiograph (Vivid 7; GE Medical Systems ultrasound, Horten, Norway) equipped with a 12-MHz linear transducer (12L). Heart rate and time-average mean blood-flow velocities (mBFVs) were measured in both intracranial internal carotid arteries and BT in basal, during ischemia (at 45 minutes), and after reperfusion.

**Regional CBF Monitoring**

Cortical regional CBF was monitored in the middle cerebral artery territory by laser Doppler flowmetry (Moor Instruments Ltd, Axminster, UK) in thermoregulated rats under isoflurane anesthesia (1% in O2/N2O [1:3]).

**Immunohistochemistry and Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Assay**

Detections of nitrotyrosine and microglia using tomatolectin in combination with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling were performed on coronal sections.
Inhaled nitric oxide (iNO) mediates the redistribution of blood flow during ischemia. A–B, Representative blood flow velocity waveforms in the basilar trunk (BT) during ischemia under air (A) and iNO at 20 ppm (B). C, During ischemia and iNO exposure, mBFV in the BT was increased to 161%±59% with 5 ppm iNO (n=11) and to 196%±48% with 20 ppm iNO (n=10) as compared with 119%±28% in air-exposed animals (n=13). D, Under basal conditions, l-NAME (20 mg/kg) induced a decrease in mBFV in the BT when compared with PBS-treated animals (*P<0.05); iNO (20 ppm) was able to reverse this effect. During ischemia, mBFV remained lower in l-NAME-treated animals than in PBS-treated controls (**P<0.01), whereas iNO delivered during ischemia reversed this effect (**P<0.001). mBFV indicates mean blood flow velocity; l-NAME, NG-nitro-l-arginine methyl ester; PBS, phosphate-buffered saline.

**Results**

**Inhaled NO Increases Cortical NO Concentrations**

Using in vivo voltammetric detection, we found that exposure to 20 ppm iNO was associated with a moderate, progressive, and significant increase in cortical NO concentration of up to 140%±10% of the basal NO concentration in P7 rats (Figure 1A). Similarly, diethylamine NONOate (50 mg/kg), an NO donor, induced a rapid and very significant increase of the concentration to 147%±9% of the basal concentration (Figure 1B). Under iNO at 20 ppm, pH and PacO₂ did not differ from those obtained under air; a significantly increase in Pao₂ was observed under iNO exposure (online-only Data Supplement Table 1).

**Inhaled NO Increases Collateral Recruitment During Ischemia in the Developing Brain**

We exposed 21 animals to iNO during the entire 50-minute period of ischemia (5 ppm iNO; n=11; 20 ppm iNO; n=10) and compared them with 13 animals subjected to the same procedure and exposed to air. A significantly increase in mBFVs in the BT was evidenced during ischemia in iNO-exposed pups (9.9±4.0 cm·s⁻¹ with 5 ppm iNO, P<0.01; 10.9±2.3 cm·s⁻¹ with 20 ppm iNO, P<0.001; 8.1±2.3 cm·s⁻¹ in air-exposed animals; Figure 2A–C). At reperfusion, mBFV returned to basal values in the 3 groups of animals (Figure 2C). Heart rates were slightly increased during exposure to 5 or 20 ppm iNO in response to...
NO-mediated arterial dilation (online-only Data Supplement Table I). Regional CBF in the cortex during ischemia was also significantly higher under exposure to 20 ppm iNO as compared with air exposure (37%±11% versus 29.1%±5.9%, respectively; P<0.01).

In another set of experiments, animals received either phosphate-buffered saline or l-NAME at a dose of 20 mg/kg. As previously reported,11 l-NAME, as compared with phosphate-buffered saline, induced a significant reduction in mBFV in both internal carotid arteries as well as in the BT (5.2±1.1 cm · s⁻¹ versus 6.7±1.3 cm · s⁻¹; P<0.01; Figure 2B). Inhaled NO (20 ppm) delivered during l-NAME treatment or during ischemia under l-NAME treatment was able to reverse the decrease in mBFV observed in the BT and to restore it to values comparable to those measured in the phosphate-buffered saline-treated group (Figure 2B).

Infarct volumes of animals in the previous 5-ppm iNO group were similar (4%–24%; median, 14%; one animal died) to those found in the air-exposed group (4%–23%; median, 15.5%; one animal died). In contrast, infarct volumes were significantly decreased in the 20-ppm iNO group (4%–17%; median, 8.5%, zero animal died; Figures 3A, 3C, and 3D). An inverse exponential regression between infarct volume and mBFV in the BT during ischemia was found in air-exposed animals (r=0.89, P<0.001; Figure 3B). The 5- and 20-ppm iNO groups also displayed right-shifted inverse exponential regressions (5 ppm iNO: r=0.95, P<0.001; 20 ppm iNO: r=0.81, P<0.01; Figure 3B).

**Impact of Excessive Blood Supply on Blood Flow and Infarct Volume**

The administration of iNO at high concentrations (80 ppm, n=5) during ischemia, in contrast, induced a dramatic increase in mBFV in the BT to 12.0±2.7 cm · s⁻¹ (P<0.01), and the mBFV remained persistently higher during reperfusion at 8.1±1.6 cm · s⁻¹ than in air-exposed pups (P<0.05; see online-only Data Supplement Figure II). These animals surprisingly exhibited significantly larger infarct volumes at 48 hours (18%–30.5%; median, 22%; P<0.05 versus air).

We then subjected 20 animals to ischemia and subsequently exposed them to iNO after reperfusion for 12 hours (5 ppm iNO: n=10, 20 ppm iNO: n=10). During ischemia and before iNO exposure, both groups displayed a significant increase in mBFV in the BT, suggesting that these animals would have small or moderate lesions according to previous reports.10 Inhaled NO given 30 minutes after the onset of reperfusion induced a significant increase in mBFV in the BT to 8.0±2.8 cm · s⁻¹ (5 ppm iNO) and 8.5±2.5 cm · s⁻¹ (20 ppm iNO), respectively, whereas mBFV in the BT was lower in air-exposed animals (6.2±1.1 cm · s⁻¹, P<0.01 versus 5 ppm iNO and P>0.001 versus 20 ppm iNO; Figure 4A). The right and left internal carotid arteries also showed a similar increase in mBFV (not shown). The 5- and 20-ppm iNO groups displayed a significant increase in infarct volumes (5 ppm: 10%–29%; median, 22.5%; 20 ppm: 6%–36%; median, 26%, respectively; Figure 4B; 4 animals died in the 20-ppm iNO group). Similar to the inverse exponential regression seen in air-exposed animals, the 5- and 20-ppm iNO groups also displayed upshifted inverse exponential regressions (5 ppm iNO: r=0.93, P<0.001; 20 ppm iNO: r=0.89, P<0.005; Figure 4C).

**Inhaled NO During Ischemia Prevents Oxidative Stress and Inflammation**

NO is capable of crossreacting with oxygen-derived free radicals to generate peroxynitrates and induce the nitrosylation of...
proteins, an indicator of cell damage. We observed that exposure to 20 ppm iNO during ischemia was associated with a significant, 43% decrease in the density of nitrotyrosine-positive cells in both the cortex and the external capsule around the cortical lesion. In contrast, exposure to 20 ppm iNO during reperfusion significantly increased the density of nitrotyrosine-positive cells around the cortical lesion 48 hours after ischemia when compared with air-exposed animals (Figures 5A and 5C, upper panel). Similar increased of nitrotyrosine-positive cells were shown in the brain of animals subjected to iNO at 80 ppm during ischemia (not shown). Similarly, exposure to 20 ppm iNO during ischemia significantly decreased the density of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive cells (45.6±21.0/0.065 mm² versus 92.1±35.5 in air-exposed animals, P<0.001) and the density of tomatolectin-positive microglial cells (44.8±13.1/0.065 mm² versus 73.0±19.4, P<0.01). In contrast, 20 ppm iNO administered during reperfusion significantly increased the density of both terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling-positive cells (143.4±49.6/0.065 mm², P<0.001) and tomatolectin-positive cells (92.1±21.5/0.065 mm², P<0.05; Figure 5B–C, lower panel) around the cortical lesion.

Discussion

The present study demonstrates that 20 ppm iNO was delivered to the brain and when administered during ischemia markedly reduced lesion size in a neonatal stroke model. The effect of iNO was associated with increased arterial blood flow velocities and cortical CBF in the ischemic penumbra during ischemia. In contrast, iNO exposure during reperfusion was detrimental to the P7 rat brain.

Using the same voltammetric device, we first observed that in control P7 rat pups, iNO was able to increase NO concentrations in the cerebral cortex, where significant amounts of NO seem to be produced continuously even under basal conditions. This increase in NO availability, despite long-lasting inhalation (over 90 minutes), reached a steady state, whereas an NO donor continually increases NO concentrations during its administration. These data confirmed previous data regarding the electrochemical detection of NO and its transport to the brain. They were consistent with a similar increase in serum concentrations of nitrites and nitrates observed in response to iNO (data not shown). These data suggested that exogenous NO could be converted to relatively stable serum NO metabolites (eg, nitrites or S-nitrosothiols) that could regenerate NO in peripheral organs such as the brain. Our findings confirmed previous reports in the adult rat, showing an increase in the diameter of small vessels and bioactive NO metabolites in the microcirculation with iNO exposure.

Inhaled NO (20 ppm) was able to increase blood flow in the large arteries after the blockade of NO synthesis under basal conditions, suggesting that iNO supplied the intravascular NO needed to maintain normal vascular function, as reported in humans. Again, iNO given during ischemia was capable of reversing the detrimental effects of l-NAME, a nonspecific NO synthase inhibitor that we have demonstrated as preventing collateral recruitment and increasing infarct volume. Inhaled NO may exert systemic effects through soluble guanylate-cyclase-dependent and/or -independent mechanisms. Inhaled NO has been shown to increase the concentration of cyclic guanosine monophosphate in the aortic wall. Very recently, iNO was demonstrated to reach the brain vasculature and induce cerebral venodilation, and
NO production was shown to be blocked by the topical application of the soluble guanylyl cyclase blocker ODQ (1H-[1,2,4]Oxadiazolo[4,3-d]quinoxalin-1-one) in the adult mouse brain. Similarly, soluble guanylate-cyclase α1 deficiency abolished the ability of iNO to inhibit the induction of inflammatory cytokines in the brain and to improve neurological function and 10-day survival rates after cardiac arrest. Together, these observations suggested that iNO during ischemia might confer organ protection, at least in part, through soluble guanylate-cyclase-dependent mechanisms.

The timing and duration of exposure to iNO could strongly affect the response to exogenous NO therapy, depending on the developmental stage of experimental model. Indeed, in adult mice and sheep, iNO (50 ppm for 60 minutes) administered after permanent middle cerebral artery occlusion has been found to be beneficial after ischemia. Similarly, iNO (40 ppm for 23 hours) administered after cardiac arrest and cardiopulmonary resuscitation in mice has been shown to prevent water diffusion abnormalities and neuronal damage and to improve the survival rate and neurological outcome. In contrast, in neonatal or juvenile rats, iNO appeared to be beneficial only when given during ischemia but was deleterious when administered later. When the Rice-Vannucci model is applied to P9 mouse pups, iNO (50 ppm) reduced neuronal damage when given during hypoxia. Conversely, iNO has been reported to increase lesion size when given after hypoxia–ischemia at 10 or 40 ppm. In agreement with these data, we found here that 5 and 20 ppm of iNO administered after ischemia–reperfusion in rat pups increased mBFVs and appeared to be harmful, increasing oxidative stress, inflammation, and infarct volumes. One reason for the discrepancy between findings in adults and newborns may be the establishment of collateral recruitment, which occurred within 10 minutes in the P7 brain but required time to develop in the adult brain.

NO appeared to be a double-edged sword, either neurototoxic or neuroprotective depending on the dose used and various spatial and temporal factors. High concentrations of NO (80 ppm) and/or excessive NO and O₂ given 30 minutes after reperfusion (our study) were accompanied by increased blood flow at the reperfusion but the worsening effect mainly depends on peroxynitrite formation according to our previous data demonstrating increased oxidative stress after endothelial NO synthase inhibition. Conversely, the infusion of l-arginine, an NO precursor, has been shown to improve CBF after ischemia in adults. In addition to its effects on collateral recruitment and the facilitation of penumbral blood flow, iNO could also induce neuroprotection through several other mechanisms including the regulation of circulating leukocytes, the inhibition of N-methyl-D-aspartate receptor activity, and the reduction of apoptosis. Finally, the putative S-nitrosoglutathione pathway has also been shown to be an important system for the protection of neurons and other brain cells against oxidative stress.

Taken together, our experiments strongly suggest that iNO could induce neuroprotection to ischemia in the developing brain depending on its concentration and the timing of exposure after the insult. iNO is not associated with any detectable deleterious hemodynamic effects or bleeding, but does improve CBF selectively in and around ischemic brain tissue. Several points need to be addressed further before these experimental data can be transferred to the clinic, but the fact that this agent is already widely used in neonates should facilitate the clinical evaluation of iNO.

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Disclosures

None.

References

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Inhaled NO improves brain damage by collateral recruitment in a neonatal stroke model.

Supplemental Methods

Neonatal ischemia-reperfusion. All experiments complied with ethical guidelines of Robert Debré Hospital Research Council Review Board (A75-19-01), INSERM and the ARRIVE guidelines (http://www.nc3rs.org/ARRIVE). Ischemia was induced in Wistar P7 rat pups (16-18 g; Janvier, Le Genest St-Isle, France; both sexes) and was adapted to isoflurane anesthesia. Briefly, thermoregulated (37.0±0.5°C) and anesthetized pups [1% isoflurane in O₂/N₂O (1:3)] were exposed to left middle cerebral artery electocoagulation (MCAo) combined with a transient (50 min) and concomitant occlusion of both common carotid arteries (CCA) by using vascular clips (18055-04, Fine Science Tools, Heidelberg, Germany). Carotid blood-flow restoration was verified with the aid of a microscope. During ischemia and recovery the pups were placed in a humidified incubator at 32-34°C. After recovery, pups were transferred to their mothers.

Ultrasound brain imaging. Thermoregulated rats were subjected to ultrasound measurements under 0.5% isoflurane anesthesia using an echocardiograph (Vivid 7, GE Medical Systems ultrasound®, Horten, Norway) equipped with a 12-MHz linear transducer (12L). Time-average mean blood-flow velocities (mBFVs) were measured in both intra cranial internal carotid arteries (ICA) and basilar trunk (BT) (supplemental Fig. 1) before surgery, during ischemia (at 40 min) and at 15 min after removal of the CCA occlusion. Heart rates were measured and reflected changes in cardiac output, as ventricular volume is quite invariable in newborns.
Regional cerebral blood-flow monitoring. Cortical regional cerebral blood flow (rCBF) was monitored in the MCA territory by laser Doppler flowmetry (Moor Instruments Ltd, Axminster, UK) in thermoregulated rats under isoflurane anesthesia [1% in O₂/N₂O (1:3), n=6, each condition]. Doppler probe was placed on the skull (~2 mm posterior and ~3 mm lateral to the bregma). Relative changes in rCBF were recorded in 3 regions of interest (penumbra) over a period of 5 min in basal and at the end of ischemia, and averaged; rCBF measurements were normalized to baseline in each animal.

Cortical NO detection. Rat pups were premedicated with buprenorphine (0.1 mg/kg i.p.), pentobarbital sodium (25 mg/kg i.p.) and supplemented by additional injections of pentobarbital sodium (15 mg/kg) every 90 min. Three-electrode potentiostatic system allowing, by use of differential normal pulse voltametry, detection and quantification of an oxidation current (+650 mV) specific for NO. Reference (Ag/AgCl wire, 250 µm in diameter) and auxiliary (Tungsten wire, 250 µm in diameter) electrodes were implanted in contact with the dura. The NO sensor was prepared on the basis of a carbon fiber (diameter: 30 µm, length: 500 µm) coated with porphyrin-nickel (Interchim, Montlucçon, France) and Nafion as previously described ³,⁴. The NO sensor was stereotaxically implanted into the frontal cortex (P: 2 mm, L: 1 mm/bregma, and 500 µm in depth) according to Paxinos and Watson’s atlas. Variations occurring in the NO peak height (oxidation current) were continuously monitored (1 measurement/2 min) with a Biopulse (Tacussel, France) and computerized (analogical/digital interface, National Instrument, USA) throughout 40-60 min consecutive baselines condition for each animal. The changes occurring in the oxidation current in all other conditions (iNO 20-ppm given using a nasal mask, injection of a NO donor, diethylamine NONoate, 50 mg/kg) were expressed in percentage versus the basal mean value of the NO release considered as reference.

Immunohistochemistry, immunofluorescence and TUNEL staining. Sections (n=6-8 each condition) from air- and iNO 20 ppm-exposed rat pups subjected to ischemia and sacrificed
at 48 hours were processed as previously described \(^5\) and incubated with a primary antibody against nitrotyrosine (NT, AB5411, 1:100, Millipore, St-Quentin-en-Yvelines, France)\(^6\). Sections were then incubated with a biotinylated anti-rabbit secondary antibody and immunolabeling was visualized using the streptavidin-biotin-peroxidase method. The specificity of the primary antibodies that we used was tested by their omission. Antibodies against tomatolectin (TL, 1:500, AbCys, Paris, France) to stain resident, activated macrophage/microglia \(^6\) were revealed using streptavidin coupled to the red fluorescent marker Cy3 (Jackson ImmunoRes laboratories, Interchim, Asnieres, France). These sections were then processed for TUNEL staining according to the manufacturer’s instructions (In situ Cell Death Detection Kit, AbCys, Paris, France). TUNEL-, NT- and TL-positive cells were counted (in a blind manner) in three to four coronal sections in the fronto-parietal cortex (at Bregma 0.24 mm) under an X20 objective using a 0.065 mm\(^2\)-grid.

*Physiological parameters.* Arterial blood was drawn by intracardiac puncture and gases (pH, \(\text{paO}_2\), \(\text{paCO}_2\)) were measured under air (n=4) and 20-ppm iNO exposure (n=6) by the means of blood-gas analyzer (Ciba-Corning 248).

**Supplemental Results**

**Supplemental Table**

*Table 1:* Physiological parameters in P7 rat pups.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>(\text{paO}_2), mm Hg</th>
<th>(\text{paCO}_2), mm Hg</th>
<th>Heart rate, bpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia (n=4)</td>
<td>7.377 ± 0.047</td>
<td>33.3 ± 5.9</td>
<td>51.9 ± 4.5</td>
<td>322 ± 29</td>
</tr>
<tr>
<td>Normoxia + iNO 20-ppm (n=6)</td>
<td>7.364 ± 0.041</td>
<td>41.5 ± 7.3*</td>
<td>51.3 ± 3.2</td>
<td>363 ± 31**</td>
</tr>
</tbody>
</table>

bpm indicates beat per min. Values are mean ± SD. *\(p<0.05\), **\(p<0.01\) versus normoxia.
Supplemental Figure-1: Representation of the collateral arterial recruitment during ischemia. **Left:** Circle of Willis is supplied by the two ICA and the BT. In rodents, in contrast with humans, the three cerebral arteries are branches of each ICA; each Pcom is developed between the BT and each PCA. Thus, circle of Willis is constituted, from behind to forward, by BT, the two Pcoms, the proximal segment of each PCA (P1), the two intracranial ICAs, the ACAs which flow into the median azygos ACA. **Right:** During ischemia (left MCAo and transient right and left CCAo, 50 min), only the BT supplied the two hemispheres through the Pcoms and cortical anastomosis from PCA and ACA to MCA territories. These blood-flow redistributions constitute the collateral arterial recruitment evidenced using US imaging and showed to be protective towards lesion extent in developing brain. Less effective is collateral blood flow supply more extensive is the brain lesion in the left MCA territory (Bonnin et al., 2011).
Supplemental Figure 2: Detrimental inhaled NO (iNO) during reperfusion. a: Effect of iNO at high concentration (80 ppm) during ischemia on rat pups submitted to ischemia. Time-average mBFVs (cm.s⁻¹) in basilar trunk (BT) were increased during ischemia (to 203±55%, n=5, **p<0.01 vs untreated rat pups) and remained elevated after reperfusion (to 138±47%, *p<0.05 vs untreated (n=13), whereas untreated ischemic rat pups returned to basal values. b: Infarct volumes at 48 hours was higher in iNO per-ischemia treated compared to untreated rat pups (21.4±6.2%, n=5 and 14.6±5.8%, n=12, respectively, *p<0.05 iNO vs untreated animals).

Supplemental Figure 3: Effect of iNO on oxidative stress at 48 hours after ischemia-reperfusion. Quantification of the number of 3 NT- positive cells in air- and iNO-exposed animals (n=5 each condition) with iNO given either during ischemia (iNO per) or after reperfusion (iNO post). Data are given in mean ± SD positive cells/per 0.065 mm². **p<0.01 versus air.
Supplemental References
MS ID#: STROKE/2012/664243


