Inhalation of Nitric Oxide Prevents Ischemic Brain Damage in Experimental Stroke by Selective Dilatation of Collateral Arterioles


Rationale: Stroke is the third most common cause of death in industrialized countries. The main therapeutic target is the ischemic penumbra, potentially salvageable brain tissue that dies within the first few hours after blood flow cessation. Hence, strategies to keep the penumbra alive until reperfusion occurs are needed.

Objective: To study the effect of inhaled nitric oxide on cerebral vessels and cerebral perfusion under physiological conditions and in different models of cerebral ischemia.

Methods and Results: This experimental study demonstrates that inhaled nitric oxide (applied in 30% oxygen/70% air mixture) leads to the formation of nitric oxide carriers in blood that distribute throughout the body. This was ascertained by in vivo microscopy in adult mice. Although under normal conditions inhaled nitric oxide does not affect cerebral blood flow, after experimental cerebral ischemia induced by transient middle cerebral artery occlusion it selectively dilates arterioles in the ischemic penumbra, thereby increasing collateral blood flow and significantly reducing ischemic brain damage. This translates into significantly improved neurological outcome. These findings were validated in independent laboratories using two different mouse models of cerebral ischemia and in a clinically relevant large animal model of stroke.

Conclusions: Inhaled nitric oxide thus may provide a completely novel strategy to improve penumbral blood flow and neuronal survival in stroke or other ischemic conditions. (Circ Res. 2012;110:727-738.)

Key Words: collateral blood flow ■ ischemic penumbra ■ ischemic stroke ■ nitric oxide inhalation

Every year stroke is responsible for the death of 5.5 million people. Despite its high incidence and mortality, clinical therapeutic options are still limited. Research efforts to find novel treatment strategies focus primarily on rescuing the ischemic penumbra, the viable tissue surrounding the nonviable infarct core. In the penumbra, blood flow is critically reduced but still sufficient to sustain neuronal integrity for several hours. The delayed nature of cell death in the penumbra leaves a unique window of opportunity for therapeutic interventions. If adequate cerebral perfusion is re-established sufficiently fast, then penumbral tissue can be effectively saved. Therefore, penumbral reperfusion at the earliest possible time is the most critical factor in determining neurological outcome and in preventing mortality after stroke.

In This Issue, see p 651
Editorial, see p 652

Currently, the only clinical treatment option to increase penumbral blood flow and, hence, to prevent the progression...
of ischemic brain damage is thrombolysis by local or systemic administration of recombinant tissue plasminogen activator (rtPA). A major drawback of rtPA, however, is that it may be fatal if administered in patients with hemorrhagic stroke who present with similar symptoms as ischemic stroke patients. Before hemorrhagic stroke, however, can be ruled out by advanced imaging techniques, the therapeutic window for rtPA, recently extended from 3 to 4.5 hours after the onset of ischemia, has often closed. As a result, less than 5% of all stroke patients are eligible for rtPA thrombolysis according to current protocols. The remaining 95% may only hope for spontaneous reperfusion, which in most cases occurs too late to prevent penumbral cell death and, hence, fails to restore neurological function. Accordingly, novel strategies are required to prolong neuronal survival in the ischemic penumbra.

Nitric oxide (NO), a potent endogenous vasodilator, was originally proposed to have an exclusively pulmonary effect when given by inhalation. Whereas few studies saw mild spontaneous reperfusion, which in most cases occurs too late to prevent penumbral cell death and, hence, fails to restore neurological function. Accordingly, novel strategies are required to prolong neuronal survival in the ischemic penumbra.

In the present study, we therefore investigated the effect of iNO on the cerebral microcirculation by in vivo fluorescence microscopy. Because our results indicated that iNO preferentially dilates arterioles in areas of low perfusion, we hypothesized that iNO may serve as a specific vasodilator of collateral vessels in the ischemic penumbra after experimental stroke. Therefore, we analyzed the effects of iNO on CBF, cerebral metabolism, neuronal cell death, and functional outcome in murine models of cerebral ischemia and validated these findings in a clinically relevant large animal model of ischemic stroke in sheep.

### Methods

#### Animals

Male 6-week-old to 10-week-old C57BL/6 mice (25–30 g) (provided by Charles River Laboratories, Kisslegg, Germany) were anesthetized, intubated, and mechanically ventilated as previously described. Anesthesia for all intravital microscopy experiments was induced by intraperitoneal injection of medetomidine, midazolam, and fentanyl.

#### Transient and Permanent Focal Cerebral Ischemia: Chronic Experiments (24 Hours/12 Hours/7 Days)

Experimental middle cerebral artery (MCA) occlusion was induced under 1.2% isoflurane in 70% N2O and 30% O2 administered by a facemask.

#### Perinatal Hypoxia

Pups were anesthetized with isoflurane (1.0%–1.5%) in a 50%/50% mixture of N2O and O2 via facemask.

#### Perinatal Hypoxic-Ischemic Injury

The 9-day-old C57BL/6 male mouse pups were exposed to hypoxic-ischemic injury as previously described. One hour after ligature of the left common carotid artery, pups were subjected to hypoxia for 50 minutes with or without addition of NO to the gas mixture (50 ppm). After 72 hours brains were removed, 5-µm paraffin coronal sections were prepared for quantification of brain injury by MAP 2 staining. Brain injury was then evaluated. A pathological score (0–22) was determined for different brain regions.

#### CBF Measurement in Mice

Regional CBF was measured through the intact skull by laser Doppler flowmetry as previously described. In mice subjected to MCA occlusion, regional CBF was determined in the ischemic core region (regional CBF <20% of baseline), the penumbra (regional CBF 25%–40% of baseline), or the surrounding normal cortex (regional CBF >80% of baseline).

#### Intravital Microscopy

Intravital fluorescence microscopy of brain microvessels was performed as previously described. A cranial window was prepared over the MCA territory and pial microvessels were visualized by intravital microscopy of FITC-dextran. The diameters of at least two arterioles venules were analyzed. Intact vasoreactivity was confirmed at the end of each experiment by CO2-induced vasodilation.
Inhibition of Soluble Guanylyl Cyclase

Topically, 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was used at a final concentration of 10 μmol/L after careful mechanical permeabilization of the dura mater using a custom-made micropipet.

NO Imaging

In vivo NO imaging was performed by adaptation of our previously reported in situ technique,21 ie, the cortex was superfused with the NO-sensitive dye 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) for 30 minutes after microperforation of the dura mater. DAF-FM fluorescence was imaged at 480 nm.

Measurement of S-Nitroso-Hemoglobin and Nitrite

Concentrations of NO adducts were measured in snap-frozen arterial blood of mice with or without NO inhalation by a previously described chemiluminescence technique.18

Positron-Emission Tomography

Mice received 14 MBq of 18F-fluorodeoxyglucose intravenously 10 minutes after MCA occlusion. Positron emission tomography (PET) scanning was performed 20 to 40 minutes thereafter. Sheep were subjected to serial 15O-H2O PET imaging to determine CBF by using a high-resolution clinical scanner (ECAT EXACT HR+; Siemens/CTI, Knoxville, TN).

Determination of Bleeding Time

Tail bleeding time after 30 minutes of iNO was determined in mice. A 1-cm segment of the tail was amputated; subsequently, the tail was placed horizontally in saline kept at 37°C. Bleeding time was visually determined and timed.

Determination of Infarct Size by Histomorphometry

Lesion volume was assessed in 12 sequential cryosections. Infarct volumes were normalized to the contralateral hemisphere.

Permanent MCA Occlusion in Sheep

Adult female merino sheep (bred at Leipzig University Veterinary Faculty Breeding Facility, Leipzig, Germany) were subjected to permanent distal middle cerebral artery occlusion as described previously.15 Baseline PET imaging was performed afterward. Two hours after induction of ischemia, one group of animals received 50 ppm of iNO for 60 minutes, whereas controls were ventilated normally. Animals were then subjected to serial PET scans (t=30 minutes, t=55 minutes, t=90 minutes).

Functional Outcome

Neurological function was assessed by the Neurological Severity Score as previously described.19 Mice that did not survive until the end of the observation period received the worst score achieved by an animal of their respective group on the respective day. Body weight was recorded as a parameter of general well-being.

Statistical Analysis

All data are given as means±SEM. Repeated measurements in one animal were analyzed by the Friedman test. Independent groups were compared by the Mann-Whitney rank-sum test. Statistical significance was assumed at P<0.05. All calculations were performed with a standard statistical software package (Sigma Stat 3.0; Jandel Scientific).

Results

Cerebrovascular Effects of NO Inhalation in Normally Perfused Brain

In the first series of experiments, we investigated whether iNO exerts cerebrovascular effects in healthy brain. In anesthetized mice, we monitored regional CBF by laser Doppler fluximetry and microvascular diameters of pial arterioles and venules by intravital fluorescence microscopy during inhalation of 50 ppm NO. At this concentration, no effects on the systemic circulation or gas exchange were observed (for physiological parameters for all experiments see Online Table I) and iNO did not alter CBF (Figure 1A). Absent effects on cerebral vascular resistance were reflected by unchanged diameters of cerebral arterioles, the major resistance vessels in the brain (Figure 1B). In contrast to arterioles, pial venules dilated by 10%±1% during NO inhalation and gradually returned to baseline diameters after discontinuation of iNO (Figure 1C, D), thereby demonstrating that iNO does not affect cerebral resistance vessels and, thus, cerebral perfusion. The vasodilatory response in mouse pial venules was dose-dependent within the clinically relevant dose range of iNO in humans (5–50 ppm; Figure 1E).

To investigate whether iNO reaches the brain vasculature and induces cerebral venodilation directly, we blocked cerebral NO signaling by topical application of the soluble guanylyl cyclase blocker ODQ onto the cerebral cortex. ODQ completely inhibited the iNO-induced dilation of pial venules, thereby demonstrating that the response was attributable to local NO effects in the brain (Figure 2A). To further prove that NO reaches the cerebral microvasculature on NO inhalation, we visualized NO in vivo by molecular fluorescence imaging with the NO-sensitive DAF-FM. DAF does not directly react with NO but detects nitrosonium ions (NO+), therefore assaying NO oxidation products such as N2O3 and also S-nitrosothiols. In pial venules of healthy mice, DAF-FM fluorescence in the microvascular wall remained stable at baseline but increased during NO inhalation concomitantly with vasodilatation (Figure 2B). Data clearly show that under physiological conditions iNO reaches the brain and dilates pial venules directly and not through an indirect pulmonary effect. This implies that bioactive NO most probably needs to be transported from the lung to the brain. To investigate the mechanisms of this putative transport, we measured two proposed NO carriers, nitrite and S-nitrosohemoglobin, in arterial blood of mice inhaling NO. The concentration of both molecules increased significantly on NO inhalation (Figure 2C, D), thereby suggesting that bioactive NO is transported from the lung to the brain via the blood stream.

Cerebrovascular Effects of NO Inhalation in Cerebral Ischemia

To investigate why iNO is only released in cerebral venules and dilates only this component of the cerebral vasculature while arterioles remain unaffected, we hypothesized that the selective release of NO in venules was mediated by the different conditions present in arterioles and venules, ie, PO2, PCO2, or pH. The most straightforward experimental strategy to test this hypothesis was to investigate the vasodilatory effect of iNO under conditions in which arteriolar and venular PO2, PCO2, and pH gradients become similar, ie, during hypoperfusion. We induced cerebral hypoperfusion by bilateral carotid artery banding. In control mice, reduction of CBF to 30% of baseline decreased diameters of pial arterioles and venules by 10% because of the reduction in volume flow (Figure 3A, B; control). As expected, NO inhalation in-
creased the diameter of pial venules (Figure 3A; iNO). Most interestingly, however, iNO also dilated pial arterioles by 22% \pm 3.2% (Figure 3B; iNO). To investigate if this arteriolar vasodilatation was caused by NO, we visualized endothelial NO by intravital imaging with DAF-FM (Figure 3C). Normally perfused pial arterioles did not show any relevant increase in fluorescence on NO inhalation, yet the fluorescence signal increased significantly when iNO was administered during cerebral hypoperfusion (Figure 3C, D). These data suggest that iNO is transported from the lung to the brain via the blood stream and is released in cerebral venules by an oxygenation-dependent mechanism. Because during hypoperfusion and ischemia the oxygenation gradient between venules and arterioles assimilates, NO is also released in cerebral resistance vessels under these conditions. Accordingly, NO applied by this specific route may serve as a selective arteriolar vasodilator in hypoperfused tissue.

Because most stroke patients have atherosclerosis leading to endothelial dysfunction and impaired cerebrovascular NO production, we tested whether iNO causes arteriolar vasodilatation also under this clinically highly relevant condition. Using a mouse model of atherosclerosis, we demonstrated that iNO dilates atherosclerotic arterioles to the same degree as healthy vessels, suggesting that the therapeutic potential of iNO is not affected by atherosclerosis (Online Figure II).

Next, we examined whether iNO dilates penumbral venules and arterioles in a well-established mouse model of ischemic stroke (MCA occlusion). NO inhalation induced a marked vasodilatation of pial venules (Figure 4A) and arterioles (Figure 4B) in the ischemic penumbra. Laser Doppler
fluximetry demonstrated that these vasoactive effects resulted in a selective increase of CBF in the ischemic penumbra, whereas perfusion of the surrounding normal cortex and the central necrotic infarct core remained unchanged (Figure 4C). Measurement of absolute CBF by [14C]iodoantipyrine autoradiography verified this finding (Online Figure IIIA, B).

The observed approximate 40% increase in penumbral blood flow was associated with a proportionally improved metabolic function in the penumbra, as demonstrated by micro-PET analysis with 18F-fluorodeoxy-glucose (Figure 4D, E). In addition, PET analysis revealed that the area of brain tissue deficient of oxidative metabolism was significantly smaller in iNO-treated as compared to control mice (Figure 4E), indicating that iNO restores oxidative metabolism in the ischemic penumbra.

Effect of NO Inhalation on Neuronal Cell Damage and Outcome in Ischemic Stroke

To evaluate whether the iNO-induced increase in penumbral blood flow and glucose metabolism attenuates ischemic brain damage, we subjected mice to 45 minutes of MCA occlusion followed by 24 hours of reperfusion and quantified the volume of infarcted tissue on cresyl violet-stained coronal brain sections. No histological evidence for microhemorrhage or macrohemorrhage was observed in control or in NO-treated animals. When NO inhalation (50 ppm) was started 10 minutes and 1 hour after induction of transient cerebral ischemia or 10 minutes after permanent MCA occlusion and maintained for 60 minutes, ischemic tissue injury was markedly attenuated and infarct volume was reduced by >40% as compared to control mice (Figure 5A, B). Because iNO is supposed to improve penumbral blood flow only when applied during ischemia, we performed a control experiment starting iNO treatment 75 minutes after reperfusion, ie, 2 hours after MCA occlusion. Under this condition, as expected, no protective effect was observed (Figure 5B; iNO 2 hours).

All experiments performed so far suggest that iNO increases CBF exclusively in the ischemic penumbra, ie, in hypoperfused brain tissue at risk for infarction. If this assumption is correct, then iNO should be able to maintain penumbral viability as long as hypoperfusion persists. To test this hypothesis, we performed permanent MCA occlusion and ventilated mice with 50 ppm NO beginning from 10 minutes after onset of ischemia for a total period of 12 hours (longer occlusion periods were not possible because of ischemia-
induced mortality rate >80% in the control group). Even over this extended ischemic period NO inhalation reduced total infarct volume by almost 40% (Figure 5C). Thus, NO inhalation appears to prevent ischemic brain tissue damage as long as ischemia persists.

To investigate whether these acute beneficial effects of iNO also translate into long-term neurological improvement, functional outcome was assessed over a period of 7 days after MCA occlusion. Mice treated with iNO (50 ppm) for 24 hours after onset of ischemia performed significantly better in a neurological test battery analyzing motor function, agility, and coordination (*P<0.05, †P<0.01, ‡P<0.001; n=7 each). C, In vivo imaging of NO in the vascular wall of hypoperfused mouse brain. Representative intravital fluorescence images of a cerebral arteriole loaded with the NO-sensitive dye DAF-FM. Images were obtained under control conditions, ie, at normal cerebral blood flow (top and center) and during low flow conditions (30%–40% of baseline CBF; bottom). DAF-FM fluorescence in the arteriolar vessel wall only increased under low-flow conditions. D, Quantification of NO delivery to the vascular wall of normal and hypoperfused mouse brain before and after iNO (INO). At normal CBF, NO inhalation only delivers NO to cerebral venules. During hypoperfusion, however, arteriolar NO delivery is also dramatically enhanced (*P<0.01 vs baseline; §P<0.01 vs iNO; n=4).

Effect of NO Inhalation in an Ovine Model of Ischemic Stroke

So far, we demonstrated that iNO acts as a selective vasodilator in hypoxic brain tissue, thus improving perfusion of the ischemic penumbra and reducing infarct size in murine models of ischemic stroke. To investigate if iNO is equally effective across species, we tested iNO in a clinically relevant large animal stroke model in sheep. Adult sheep were subjected to permanent occlusion of the MCA. After a baseline PET scan 2 hours after MCA occlusion, NO inhalation was initiated (2 hours after MCA occlusion); CBF was then quantified repeatedly during and after iNO by [15O]H2O PET. To investigate whether the observed iNO effect was confined to the ischemic penumbra, the penumbra was identified by absolute CBF measurements (8–22 mL/100 g×min; Figure 7A, pink). The iNO selectively increased CBF in the ischemic penumbra as compared to the condition before iNO application and as compared to a control animal not receiving NO (encircled; Figure 7A, ~10 minutes, 55 minutes). Quantification of penumbral CBF before and after iNO showed that the penumbral volume decreases significantly by approximately 50% but remains unchanged in control animals (P<0.05 vs baseline and vs control; Figure 7C). These findings substantiate the view that inhalation of NO causes selective vasodilatation of vessels in lowly perfused brain tissue. Met-hemoglobin levels were <1.3% of total hemoglobin in all animals.

Effect of NO Inhalation on Perinatal Hypoxic-Ischemic Brain Damage

To test our hypothesis that iNO is effective in hypoxic regions only and to validate our findings using iNO in experimental stroke in a different model and in a different laboratory, iNO was performed in a well-standardized model of murine perinatal hypoxia-ischemia (unilateral common carotid artery occlusion plus hypoxia). The iNO was also protective in this model. Tissue loss and pathological scores were significantly attenuated by 50 ppm iNO 3 days after the insult (P<0.03; Figure 6A, B).
Potential Side Effects of iNO
A reduction of systemic blood pressure attributable to release of NO in peripheral resistance vessels and an increase in bleeding complications are the major concerns regarding possible systemic side effects of iNO as a stroke therapeutic. In mice, iNO did not exert any effects on blood pressure (Online Table I) or primary hemostasis. Tail bleeding time showed no difference between iNO-treated and control animals (data not shown).

Discussion
Our experiments demonstrate that iNO is delivered to pial microvessels in a bioactive form, dilates cerebral resistance vessels selectively in hypoperfused tissue, and, thus, improves collateral blood flow to areas in need. Therefore, iNO improves metabolic function and prevents the death of the ischemic penumbra under conditions of transient or permanent reduction of CBF. Therefore, iNO may represent a novel treatment for ischemic conditions in which collateral blood flow is critical for tissue survival, eg, after myocardial or, as shown here, in cerebral ischemia.

Previously, the vascular action of iNO was considered to be confined to the lung. However, recent data suggest that it extends beyond the pulmonary circulation. The iNO has been shown to exert anti-inflammatory effects in mesenteric microvessels,20 to increase forearm, renal, and myocardial blood flow,21–23 and to increase the concentration of cyclic guanosine monophosphate in the aortic wall.24 These phenomena were attributed to the formation of bioactive NO carriers in the lung that circulate in blood and release NO in extrapulmonary blood vessels. The nature of the NO carriers formed during NO inhalation is still undergoing debate and may involve nitrite,25,26 S-nitroso hemoglobin,27–30 or other S-nitrosothiols (eg, of cysteine or glutathione),27,30 which, among others, may be formed when NO reacts with oxygen to form N2O3.31,32 Consistent with this concept, previous studies using iNO or inhaled ethyl nitrite have shown protection in experimental paradigms in which vasoconstriction plays an important pathophysiological role, eg, experimental subarachnoid hemorrhage.33,34

The observed increase in both S-nitroso-hemoglobin and nitrite in the current study and in other studies using NO

![Figure 4. Nitric oxide (NO) inhalation increases penumbral perfusion in experimental stroke. Diameter of cerebral venules (A) and arterioles (B) in the ischemic penumbra of mice subjected to focal cerebral ischemia by middle cerebral artery occlusion (MCAo). Inhalation of 50 ppm NO increased diameters of both arterioles and venules as compared to baseline values before inhaled NO (iNO; *P<0.05, †P<0.01; n=5 each). C, Regional cerebral blood flow (rCBF) in normal brain, the ischemic core, and the ischemic penumbra during inhalation of 50 ppm NO. The iNO increased blood flow selectively in the ischemic penumbra (I2P<0.01 vs normal brain; n=5–10 each). D, 18F-fluorodeoxyglucose micropositron emission tomography (PET) and computed tomography (CT) analysis of glucose metabolism in ischemic brain of control mice (MCAo) and in animals ventilated with 50 ppm NO (MCAo + iNO). The area of reduced glucose uptake is smaller, indicating restoration of cerebral glucose metabolism in NO-treated animals. E, Quantification of 18F-fluorodeoxyglucose activity reveals higher glucose (normalized to the activity within contralateral hemisphere; regions of interest marked in the inset in green) in the penumbra of NO-treated animals as compared to controls indicating restoration of glucose metabolism after iNO (*P<0.05 vs control animals; n=5 each).]
inhalation suggests that either of these intermediates alone or together may contribute to the reported neuroprotective effect of iNO. However, given the complex nature of the methodology used to measure NO and NO adducts, further studies are needed to elucidate which specific compound mediates the observed cerebrovascular effect of iNO, specifically under physiological conditions/normoxia in which nitrite concentrations in the range of the plasma levels observed in the present study did not result in detectable increases in total forearm blood flow or volume.

Both molecules measured in the current study have been proposed to release NO in response to local oxygen partial pressure (PO2) gradients, either by an allosteric regulation or by an oxygenation-dependent enzymatic function of hemoglobin as a nitrite reductase. Also, a pathway in which nitrite is the source for S-nitroso-hemoglobin has been proposed. The specific role of hemoglobin versus nitrite as NO carriers has been discussed in various studies and the relevance of one over the other is still undergoing dispute. Yet, there is common agreement that circulating blood can transport NO bioactivity (as also evidenced by the current study) and release NO during the conformational transition of hemoglobin from the oxygenated R-state to the deoxygenated T-state.

Under physiological conditions, hemoglobin deoxygenates predominantly in capillaries and venules. Hence, as demonstrated by the current results, release of NO occurs specifically at these sites and causes exclusive dilatation of venules, whereas arterioles remain unaffected. Accordingly, iNO should not have any effect on systemic blood pressure and tissue perfusion. This is supported by the current results in mice showing no effect of iNO on normal blood pressure and on CBF in normally perfused brain and by studies in humans showing that iNO at clinically relevant concentrations (≤50 ppm) may have a minor but not significant effect on systemic blood pressure.

Under conditions of reduced tissue perfusion, however, iNO not only dilates venules but also has a marked vasodilation effect (Figure 5). Nitric oxide (NO) inhalation reduces infarct size after transient and permanent cerebral ischemia and improved functional outcome while exerting no influence on bleeding time. A, Representative photomicrographs of cresyl violet-stained brain sections obtained after 45 minutes of cerebral ischemia and 24 hours of reperfusion. Start of inhaled NO (iNO; right; 50 ppm for 60 minutes) 10 minutes after middle cerebral artery (MCA) occlusion reduces ischemic injury as compared to widespread cortical infarction in control animals (left). B, Quantification of infarct volume in untreated animals (control) and in animals receiving iNO 10 minutes (iNO 10’) or 2 hours (iNO 2 hours) after MCA occlusion. Early administration of iNO effectively reduced infarct size (†P<0.01 vs control; n=7 each). C, Infarct volume determined after 12 hours of permanent focal cerebral ischemia. NO inhalation significantly reduced cerebral infarction (†P<0.01 vs control; n=7 each). Mice treated with NO inhalation for 24 hours after transient focal ischemia show improved neurological outcome and recovery 7 days after brain injury. D, The iNO-treated animals perform significantly better in neurological testing (Neurological Severity Score [NSS]; n=10 each; †P<0.01) and E, recover significantly better from post-stroke weight loss (n=10 each; *P<0.05).
The intriguing fact is that during hypoxia, ischemia, or anemia, a phenomenon, known as Robin Hood effect, which resulted in enhanced glucose metabolism and long-term survival of penumbral brain tissue otherwise doomed to die by ischemia. It is important to consider that although the observed increase in CBF in the ischemic penumbra may seem modest in absolute values, there is ample evidence in the literature that tissue perfusion is not linearly related to neurological outcome; enhancement of perfusion to modest levels above the penumbral threshold as observed by laser Doppler flowmetry therefore can significantly improve tissue viability and survival (as evidenced by FGD-PET in mice) and, thus, neurological outcome.

In addition to the selective increase of CBF, the neuroprotective effect of iNO also may be mediated by reduced inflammatory response. However, we could not confirm this mechanism for cerebral ischemia because leukocyte infiltration was not altered by iNO in the current experiment (Online Figure IV).

Various experimental studies have previously demonstrated the beneficial effect of NO derivates for the treatment of ischemic stroke. Infusion of the NO precursor L-arginine or NO donors were shown to increase CBF after experimental stroke; however, so far, none of these promising approaches could be translated into the clinic because of various reasons. Some approaches impaired the endogenous NO production, whereas others lacked a clinically feasible mode of administration or, most importantly, caused significant reduction of systemic blood pressure, eg, L-arginine infusion. As compared to previous attempts that tried to utilize NO-based strategies for the treatment of tissue ischemia, the main advantages of iNO are its specific effect on hypoperfused tissue without any effect on systemic blood pressure, its immediate action, simple and well-investigated route of administration, its efficacy across species, and its excellent safety profile that is based on the use of iNO in several thousand patients with pulmonary disorders. Because of these properties iNO may be readily implemented into primary care and serve as an ideal stroke therapy until interventional or spontaneous reperfusion occurs. Because currently <5% of stroke patients receive thrombolysis by rtPA, iNO is likely to have a significant clinical impact.

Despite these positive properties that suggest iNO as an almost ideal stroke therapeutic, several points need to be carefully addressed before a clinical study can be initiated. One of the most important points is certainly the potential effect of iNO on bleeding time. It has been proposed that high concentrations of iNO may affect hemostasis and thus may result in bleeding complications. We addressed this important issue in the current study but did not detect any indication that at the applied dose of 50 ppm, iNO may prolong bleeding time. This finding is supported by anecdotal data about the safe use of iNO during lung and brain surgery, and by several clinical studies that did not report increased bleeding complications after iNO in patients. Nevertheless, this issue needs to be addressed carefully before a clinical study can be initiated.

Taken together, our experiments suggest that iNO exerts the properties of an ideal therapeutic agent for cerebral ischemia. The iNO has no hemodynamic effects on normally
perfused tissue but increases blood flow selectively in ischemic tissue. Because iNO is already approved for use in humans, its clinical evaluation for ischemic conditions in which collateral blood flow is important, eg, ischemic stroke or myocardial infarction, may be possible in the near future.

Sources of Funding
This work was supported by a grant from Friedrich Baur Foundation (N.P.) and the GEMI Fund (N.P., W.M.K.). No funding bodies played any role in the study design, data collection and analysis, preparation, writing, or decision to publish this manuscript. All animal experiments were conducted according to protocols approved by the responsible government authorities in Germany and Sweden.

Disclosures
None.

References


Novelty and Significance

What Is Known?

- Stroke is a major cause of death and disability in industrialized countries.
- Current therapy is thrombolysis with recombinant tissue plasminogen activator (rtPA), but <5% of patients are eligible for this treatment because of side effects or restrictions.
- Nitric oxide (NO) is a strong vasodilator, but systemic application of NO or NO precursors has failed to be neuroprotective in clinical trials because of side effects.

What New Information Does This Article Contribute?

- Inhaled NO (iNO) results in the formation of NO carriers in blood that release NO only in brain areas with low oxygen content, ie, in cerebral veins in normally perfused brain and in collateral arterioles in the ischemic brain.
- By this mechanism, iNO significantly increased collateral blood flow to ischemic but viable brain tissue and reduced brain damage and neurological dysfunction after experimental stroke.
- The iNO behaves as an ideal vasodilator. It has no effect on blood flow in normally perfused tissue, eg, no systemic hypotension; it has no steal effect, but it increases blood flow in ischemic tissue. Thus, iNO represents a novel therapeutic concept for the treatment of ischemic disorders in which collateral blood flow is present.

Stroke is a major cause of death and disability. Prompt re-establishment of perfusion is the only available therapy that salvages cerebral tissue around the infarct core and thus improves neurological outcome. This is achieved by thrombolysis with rtPA, but the applicability of this therapy is limited because of restrictions on rtPA treatment. Inhalation of NO has been used since the early 1990s to treat pulmonary vasoconstriction without influencing other vascular beds. In the current study, we demonstrate for the first time a cerebrovascular effect of iNO by using in vivo microscopy. Under physiological conditions, iNO led to a significant dilatation of cerebral arterioles by nitric oxide-dependent mechanisms and increases blood flow dependent cerebral blood flow augmentation by L-arginine after chronic statin treatment. J Cereb Blood Flow Metab. 2000;20:709–717.


Inhalation of Nitric Oxide Prevents Ischemic Brain Damage in Experimental Stroke by Selective Dilatation of Collateral Arterioles


Circ Res. 2012;110:727-738; originally published online December 29, 2011;
doi: 10.1161/CIRCRESAHA.111.253419

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/110/5/727

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2011/12/29/CIRCRESAHA.111.253419.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Detailed Methods

Animals. Male, 6-10 week-old C57BL/6 mice (25-30 g) were purchased from Charles River Laboratories. They were kept in Macrolon type I cages at 5 animals/cage and allowed access to water and food ad libitum. Animals were kept on a 12h/12h day/night cycle.

Anesthesia

Intravital microscopy. Anesthesia for all intravital microscopy experiments was induced by intraperitoneal injection of medetomidine (0.5 mg/kg bodyweight), midazolam (5 mg/kg bodyweight), and fentanyl (0.05 mg/kg bodyweight). Before injection, animals were briefly anesthetized with 2.5% isoflurane in 70% N₂O and 30% O₂ for 30 seconds in order to allow for atraumatic intraperitoneal injection. After induction of anesthesia, animals were intubated with a 22G silicone coated cannula, and mechanically ventilated with a mouse respirator (MiniVent 805, Hugo Sachs, Marchstetten, Germany). Ventilation was controlled via measurement of end-tidal CO₂ using a microcapnograph (Columbus Instruments, Columbus, OH). Body temperature was maintained at 37°C using a heating pad with a feedback mechanism via a rectal temperature sensor (FHC, Bowdoinham, ME, USA). Eyes were protected with lacrilube ointment. Mean arterial pressure was continuously measured via a silicone catheter placed in the left femoral vein.

See supplementary materials for description of ApoE⁻/⁻ experiments.

Transient and permanent focal cerebral ischemia - chronic experiments (24h/12h/7d). Experimental middle cerebral artery occlusion was induced under 1.2% isoflurane in 70% N₂O and 30% O₂ administered by a face mask in spontaneously breathing adult mice. Body temperature was maintained at 37°C using a heating pad with a feedback mechanism via a rectal temperature sensor (FHC, Bowdoinham, ME, USA). Eyes were protected with lacrilube ointment. In the wake up phase animals were supplied with 100% oxygen until spontaneous motor function was recovered; then animals were kept in an incubator kept at 32°C for 1 hour.

Bilateral carotid banding. Male C57bl/6 mice were anesthetized, intubated, and prepared for intravital microscopy as described above. A silk filament (5-0) was placed around both carotid arteries after surgical preparation. The end of each filament loop was held by micro screws. Cerebral perfusion was measured by Laser Doppler Fluxmetry over both MCA territories. By adjusting the length of the filament loop via the microscrew cerebral blood flow was lowered to 30% of baseline for the experiments.

Cerebral blood flow measurement in mice. rCBF was measured through the intact skull by laser Doppler Fluxmetry. In mice subjected to MCA occlusion, rCBF was determined in the ischemic core region (rCBF <20% of baseline), the penumbra (rCBF 25-40% of baseline), or the surrounding normal cortex (rCBF >80% of baseline). For this, glass fiber probes were glued to the intact skull. Once fixed, probes were not moved until the end of the observation period. Valued obtained during a 10 minute baseline measurement were calculated to 100%, all subsequent values obtained are expressed as percentage of baseline. See Online Figure I for schematic drawing of mouse skull.

Perinatal hypoxia. Pups were anesthetized with isoflurane (1.0-1.5%) in a 50%/50% mixture of N₂O and O₂ via facemask in spontaneously breathing pups.

Permanent focal ischemia in sheep. Adult female merino sheep were obtained from the Leipzig University department of Veterinary Medicine. Anesthesia was induced as outlined previously by i.v. injection of 2% xylazin (0.1 mg/kg BW), ketamine (4 mg/kg BW), and diazepam (0.2 mg/kg BW). After orotracheal intubation anesthesia was continued with isoflurane (1.5 – 2% in oxygen). Blood gas analysis was performed using a standard clinical analyzer (ABL System 625, Radiometer, Copenhagen, Denmark) with samples obtained from the tarsal/metatarsal artery. Blood pressure and ECG were continuously measured.
After induction of anesthesia and orotracheal intubation the skin between the ear and eye was incised on the left side of the head to expose the branches of the superficial temporal artery and the accompanying vein. After occlusion of these vessels by high frequency bipolar forceps (LigaSure, Valleylab, Boulder, CO, USA), the origin of the temporalis muscle was incised at the temporalis line and the muscle was carefully elevated from the parietal bone. After exposure of the skull bone surface, a hole was drilled into the parietal plate using a set of neurosurgical burrs and was then extended using Kerrison rongeurs. On local incision of the dura mater, the MCA was permanently occluded. Then, the temporalis muscle was fixed on the temporalis line and the skin was sutured using 2-0 absorbable filaments (Ethicon Ltd, Norderstedt, Germany). After termination of surgery, animals were disconnected from artificial ventilation and immediately taken to a specialized awakening box. Intratracheal and intraesophageal tubes were removed when the swallowing reflex was restored.

Inhalation of nitric oxide (iNO). Nitric oxide (268 mg/m³ N₂) (Linde AG, Munich, Germany) was mixed with oxygen and room air to obtain final concentrations of 5 to 50 ppm NO in 30% O₂. NO₂ was below 1.2 mmHg at all times. Controls received 30% oxygen. For long term inhalation, mice were kept in temperature controlled gas proof cages with an in- and outlet valve under continuous monitoring of oxygen, NO and N₂O concentrations (O₂: Oxydig, Draeger, Luebeck, Germany; NOx: ITX, industrial scientific corporation, Oakdale, PA. N₂O concentration was below 1.2 ppm at all times.

Cerebral hypoperfusion. Both common carotid arteries were occluded until cerebral blood flow was reduced to 30-40% of baseline as assessed by laser Doppler fluxmetry.

Transient and permanent focal cerebral ischemia. Mice were subjected to transient middle cerebral artery occlusion by an intraluminal filament as previously described. In short, after placement of Laser Doppler Probe over the right MCA territory, mice were placed on their back on a heating pad. A longitudinal incision was performed on the lateral neck, the common carotid artery and the carotid bifurcation was exposed. After ligation of the internal carotid artery the vessel was incised using micro scissors and a silicone coated polymer filament (9-0) was inserted into the vessel. It was then advanced within the vessel until it occluded the middle cerebral artery. MCA occlusion was verified by a drop in Laser Doppler flow of at least 80% of baseline. After occlusion had been verified the filament was fixed by an external suture. The skin wound was then closed using atraumatic 5-0 sutures. Animals were then allowed to wake up. For transient ischemia, the filament was removed after 45 min (after induction of a second anesthesia) and mice were sacrificed after 24 h of reperfusion. For permanent ischemia, the filament was left in place until the end of the experiment 12 h after MCA occlusion.

Perinatal hypoxic-ischemic injury. 9d-old C57/BL6 male mouse pups were exposed to hypoxic-ischemic injury as previously described. In short, 1h after ligature of the left common carotid artery pups were subjected to hypoxia for 50 min with or without addition of NO to the gas mixture (50 ppm). After 72h brains were removed, 5µm paraffin coronal sections were prepared for quantification of brain injury by MAP 2 staining. Brain injury was then evaluated. A pathological score (0-22) was determined for different brain regions.

Cerebral blood flow measurement in mice. rCBF was measured through the intact skull by laser Doppler Fluxmetry (Perimed, Järåsfält, Sweden) as previously described. In mice subjected to MCA occlusion, rCBF was determined in the ischemic core region (rCBF <20% of baseline), the penumbra (rCBF 25-40% of baseline), or the surrounding normal cortex (rCBF >80% of baseline).

Intravital microscopy. Intravital fluorescence microscopy of brain microvessels was performed as previously described. A cranial window was prepared over the territory of the MCA leaving the dura mater intact and microvessels were visualized by intravenous injection of FITC-dextran into the femoral vein. The diameters of at least two arterioles venules were...
analyzed. Vessel size ranged from venules: 30 ± 14 µm, in venules and 26 ± 8 µm in arterioles. Intact vasoreactivity was confirmed at the end of each experiment by CO₂-induced vasodilation.

**Inhibition of soluble guanylyl cyclase.** 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Sigma-Aldrich Chemie GmbH, Munich, Germany) was dissolved in isotonic saline and ethanol (final concentration: 0.5%) and used topically at a final concentration of 10 µM after careful mechanical permeabilization of the dura mater using a specially manufactured micropin.

**NO imaging.** In vivo NO imaging was performed by adaptation of our previously reported in situ technique, i.e. the cortex was superfused with the NO-sensitive dye 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM; 5 µmol/L; Molecular Probes, Eugene, OR) for 30 min after microperforation of the dura mater. DAF-FM fluorescence was imaged at 480 nm.

**Measurement of S-nitroso-hemoglobin and nitrite.** Concentrations of NO adducts were measured in snap frozen arterial blood of mice with or without NO inhalation by a previously described chemiluminescence technique.

**Positron-Emission Tomography (PET).** Mice received 14 MBq of ¹⁸F-fluorodeoxyglucose i.v. 10 min after MCA occlusion. PET scanning was performed 20-40 min thereafter (microPET Focus 120, CTI Molecular Imaging, Knoxville, TN).

**H₂O PET in sheep.** Adult merino ewes were subjected to permanent MCA occlusion (pMCAO) as described previously. The sheep were subjected to serial [¹⁵O]H₂O CBF PET scans by using a high resolution clinical scanner (ECAT EXACT HR+, Siemens/CTI, Knoxville, TN). The PET scanner characteristics were recently published elsewhere. Baseline PET imaging was performed ~2h after pMCAO. Directly after the baseline scan, one group of animals (n=3) received 50 ppm of NOi for 60 minutes while controls (n=3) were ventilated normally. All sheep were subjected to follow-up PET scans 30min and 55min after start of iNO/air as well as 30min after end of iNO/air. The serial PET scanning was followed by MRI (1.5 T clinical whole body scanner; Gyroscan Intera; Philips, Koninklijke, The Netherlands; Sequences: T1 3D, DWI, PWI, MRA, T2 turbo spin echo, T2 flair, T2*) at 5h pMCAO. For each PET scan, ~1 GBq [¹⁵O]H₂O as produced from [¹⁵O]O₂ (PETtrace cyclotron (GE Healthcare, USA)) by catalyst-mediated reaction with H₂ and subsequent dialysis exchange in an automated system (Veenstra, Joure, The Netherlands) was i.v. administered by an automated injection system. Dynamic 3D PET acquisition was carried out for 5min. Prior to radiotracer injection, a 10 min transmission scan using three rotating ⁶⁸Ge rod sources was performed for attenuation measurement. In order to reduce the contribution of out-of-field scattered radiation, the PET scanner was equipped with a “U” shaped lead-plate shielding system (NeuroShield®, Scanwell Systems, Montreal, Canada) placed in the neck region of the sheep. The PET data obtained were corrected for radioactive decay, death time, attenuation and scatter. The resulting image data were reconstructed by means of the iterative OSEM algorithm (10 iterations, 16 sub-sets). The PET acquisition was parallel by continuous arterial blood sampling using an ALLOG AB blood sampler (Allogg Mariefred, Sweden) which was carried out to obtain arterial input function for kinetic modeling. Kinetic modeling of the PET data was carried out using the PMOD software (version 3.0, PMOD Technologies Ltd., Zurich, Switzerland) employing the method of Alpert et al. This resulted in voxel-based absolute CBF values. Brain tissue was defined in these data after co-registration with individual T1 3D MRI data. In a next step, automated threshold detection was carried out in PMOD, whereby the following widely accepted thresholds were applied: 0-8 ml/min/100g for infraction core, 8-22 ml/min/100g for ischemic penumbra, and over 22 ml/min/100g for normal brain. By that, it was possible to operator-independently perform volumetry for these three pMCAO-related brain compartments (see Online Fig. VI).
**Determination of infarct size by histomorphometry.** After 24h animals were anaesthetised and sacrificed by cervical dislocation. Brains were removed and immediately frozen in powdered dry ice. 11 10µm coronal sections every 500µm were prepared on a Cryostat (Cryostar MH 560, Microm, Walldorf, Germany). The sections were Nissl-stained. Each section then was photographed digitally at a magnification of at least 12,5fold; both hemispheres and the infarct area were then measured in each section using a standard image analysing system (analySIS® 3.2 for Olympus DP - soft, soft imaging system, Muenster, Germany). Volume were then calculated according to the following formula: (Area₁/₂) x 0,5 + A₂ x 0,5.....+(A₁₁/₂) x 0,5. To correct for hemispheric swelling indirect infarct volume (D) was determined by subtraction of ipsilateral non-ischemic cortex from the contralateral hemispheric area (see Figure 1A, D=C-B). Volume then was calculated to the following formula: $V_{indir} = 0.5*(D₁/2 + D₂ + D₃ + ..... + D₁₁/2)$. All values given in the manuscript are indirect infarct volumes.

Supplemental Figure 5 shows infarct volume before normalization to contralateral hemisphere for permanent (Online Fig. VB) and transient MCA occlusion (Online Fig. VC).

**Permanent middle cerebral artery occlusion in sheep.** Adult female merino sheep were subjected to permanent middle cerebral artery occlusion as described above. Baseline PET imaging was performed afterwards. 2 hours after induction of ischemia one group of animals received 50 ppm of iNO for 60 minutes while controls were ventilated normally. Animals were then subjected to serial PET scans (t=30min, t=55min, t=90 min).

**Functional outcome.** Neurological function was assessed by the Neurological Severity Score (NSS) as previously described.¹⁶ In short, mice were subjected to 10 tasks evaluating motor function, coordination, spatial orientation, and learning. Each task is assessed with 0-2 points, best score is 0 points, maximum score is 20 NSS points. Mice that did not survive until the end of the observation period received the worst score achieved by an animal of their respective group on the respective day. Body weight was recorded as a parameter of general wellbeing.

**Statistical analysis.** All data are given as means ± SEM. Sample size calculation was performed using a standard statistical software package (Sigma Stat 3.0, Jandel Scientific, Erkrath, Germany); a relevant biological difference was assumed as 30%; standard deviation ranged from 15-20% (dependent on the parameter at hand), alpha error was 0.05, beta error 0.2.

Repeated measurements in one animal were analysed by the Friedman test. Independent groups were compared by the Mann-Whitney Rank Sum test. Statistical significance was assumed at P<0.05. All calculations were performed with a standard statistical software package (Sigma Stat 3.0, see above).
Supplemental Methods and Results

Effect of NO inhalation on atherosclerotic vessels

Most patients suffering from stroke have atherosclerosis and, subsequently, endothelial dysfunction to a certain degree. Therefore, we wanted to evaluate if iNO–induced cerebral vasodilatation also occurs in the context of impaired cerebrovascular NO production, i.e. in atherosclerotic mice suffering from endothelial dysfunction. Based on the assumed mode of action of iNO, we hypothesized that iNO-induced vasodilation should be independent of endothelial NO production.

Method. ApoE deficient mice (strain B6.129P2-Apoetmt1Unc/J, Jackson Laboratories, Bar Harbour, MA) were fed with a high cholesterol diet (Purina mouse chow 5015, Altromin, Lippe, Germany) for 3 months. Under these dietary conditions mice regularly develop medium to severe atherosclerosis. They were then subjected to cerebral hypoperfusion by bilateral carotid banding as described above. Vessel diameter of cerebral arterioles and venules was assessed by intravital microscopy.

Results. The vasodilatatory effect of iNO in atherosclerotic cerebral venules and arterioles was identical to the response observed in healthy blood vessels (Online Fig. II) indicating that NO delivered to the brain by iNO exerts its vasodilatatory effect directly through the vascular musculature and independently of the ability of the endothelium to produce NO.

Measurement of absolute cerebral blood flow by [14C] autoradiography

Focal cerebral ischemia was induced by MCA occlusion as described in the main manuscript. NO inhalation was started 10 minutes after onset of ischemia. [14C]iodoantipyrine-autoradiography was performed as previously described. Briefly, animals received an intraperitoneal injection of 20 µCi [14C]iodoantipyrine 20 min after initiation of iNO, i.e. 30 min after onset of ischemia. Animals were sacrificed by whole body snap freezing 30 seconds later. 12 coronal cryosections were prepared every 500µm and exposed to autoradiography film. Baseline activity was determined in a blood sample taken from the left ventricle.

Results. iNO treated animals showed significantly higher blood flow in the area adjacent to the ischemic core region. Online Fig. IIIA shows representative coronal sections in pseudocolor. The encircled area represents the area of the brain perfused by collaterals deriving from the anterior cerebral artery. iNO specifically increases collateral blood flow while no effect was observed in the control group. See Online Fig. IIIB for quantification.

Effect of NO inhalation on intraparenchymal leukocyte accumulation.

Previous reports suggest that inhaled nitric oxide may exert anti-inflammatory properties. In order to evaluate an anti-inflammatory effect of iNO we evaluated leukocyte infiltration into the brain after MCAo in mice.

Method. Male C57 bl/ 6 mice were subjected to 45 minutes of MCA occlusion as described above. 10 minutes after induction of MCAo until 24h after reperfusion animals were subjected either to NO inhalation or control ventilation. 24 hours after MCAo animals were deeply anesthetized with chloral hydrate and transcardially perfused with 4% paraformaldehyde. After paraffin embedding 12 sequential 4µm coronal sections were prepared every 500 µm. We used rat-anti mouse CD45 as antibody (BD Pharmingen Biosciences, Heidelberg, Germany; 1:50). CD45 cells were visualized using a standard staining kit (Vectastain Elite ABC Peroxidase Kit, Vector Laboratories, Burlingame, CA). CD45 positive cells were evaluated in the left striatum at 10 fold magnification; the ROI was chosen in the striatum, at the border zone of the infarct.

Results. Leukocyte infiltration did not differ between iNO treated and control animals (see Online Fig. IV).
Supplemental Tables

Table I.

Physiological parameters for all mouse experiments (Mean ± SEM)

**iNO under physiological conditions**

**MAP (mmHg)**

<table>
<thead>
<tr>
<th>min</th>
<th>-20</th>
<th>-10</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>69 ± 2</td>
<td>68 ± 4</td>
<td>67 ± 2</td>
<td>66 ± 2</td>
<td>66 ± 3</td>
<td>64 ± 2</td>
<td>62 ± 2</td>
<td>62 ± 2</td>
<td></td>
</tr>
<tr>
<td>iNO</td>
<td>79 ± 8</td>
<td>77 ± 8</td>
<td>74 ± 7</td>
<td>70 ± 5</td>
<td>68 ± 6</td>
<td>68 ± 4</td>
<td>68 ± 3</td>
<td>70 ± 4</td>
<td>67 ± 4</td>
</tr>
</tbody>
</table>

**Blood gas analysis**

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>PO₂ (mmHg)</th>
<th>PCO₂ (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>baseline</td>
<td>End</td>
<td>baseline</td>
</tr>
<tr>
<td>control</td>
<td>7.37±0.05</td>
<td>7.27±0.09</td>
<td>102 ± 22</td>
</tr>
<tr>
<td>iNO</td>
<td>7.34±0.03</td>
<td>7.24±0.03</td>
<td>80 ± 5</td>
</tr>
</tbody>
</table>

**iNO and hypoperfusion**

**MAP (mmHg)**

<table>
<thead>
<tr>
<th>min</th>
<th>-30</th>
<th>-20</th>
<th>-10</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>82 ± 8</td>
<td>69 ± 3</td>
<td>98 ± 21</td>
<td>97±15</td>
<td>91±17</td>
<td>91 ± 37</td>
<td>89 ±15</td>
<td>85 ±10</td>
<td>78 ±11</td>
<td>78 ± 3</td>
</tr>
<tr>
<td>iNO</td>
<td>83 ± 7</td>
<td>81 ± 7</td>
<td>103 ± 9</td>
<td>102 ± 27</td>
<td>105 ± 36</td>
<td>101 ± 39</td>
<td>86 ± 8</td>
<td>82 ± 9</td>
<td>87±10</td>
<td>82 ± 16</td>
</tr>
</tbody>
</table>
## Blood gas analysis

<table>
<thead>
<tr>
<th>pH</th>
<th>PO₂ (mmHg)</th>
<th>PCO₂ (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>baseline</td>
<td>end</td>
<td>baseline</td>
</tr>
<tr>
<td>control</td>
<td>7.30 ± 0.04</td>
<td>7.24 ± 0.08</td>
</tr>
<tr>
<td>iNO</td>
<td>7.30 ± 0.06</td>
<td>7.25 ± 0.07</td>
</tr>
</tbody>
</table>

## iNO and ODQ

**MAP (mmHg)**

<table>
<thead>
<tr>
<th>min</th>
<th>-30</th>
<th>-20</th>
<th>-10</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNO</td>
<td>81</td>
<td>82</td>
<td>78</td>
<td>77</td>
<td>81</td>
<td>76</td>
<td>74</td>
<td>72</td>
<td>67</td>
<td>70</td>
</tr>
</tbody>
</table>

## Blood gas analysis

<table>
<thead>
<tr>
<th>pH</th>
<th>PO₂ (mmHg)</th>
<th>PCO₂ (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>baseline</td>
<td>end</td>
<td>baseline</td>
</tr>
<tr>
<td>iNO</td>
<td>7.24 ± 0.06</td>
<td>7.15 ± 0.1</td>
</tr>
</tbody>
</table>

## iNO and MCAo

**MAP (mmHg)**

<table>
<thead>
<tr>
<th>Min</th>
<th>-30</th>
<th>-20</th>
<th>-10</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNO</td>
<td>107</td>
<td>99</td>
<td>91</td>
<td>85</td>
<td>99</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>91</td>
</tr>
</tbody>
</table>
### Blood gas analysis

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>PO₂ (mmHg)</th>
<th>PCO₂ (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>baseline</td>
<td>end</td>
<td>baseline</td>
</tr>
<tr>
<td>iNO</td>
<td>7.31 ± 0.12</td>
<td>7.12 ± 0.11</td>
<td>140 ± 44</td>
</tr>
</tbody>
</table>

### Table II.

Physiological parameters ± iNO in Sheep

#### Blood gas analysis

<table>
<thead>
<tr>
<th></th>
<th>PO₂ (mmHg)</th>
<th>PCO₂ (mmHg)</th>
<th>metHb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre MCAo</td>
<td>215 ± 82</td>
<td>49 ± 10</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Post MCAo</td>
<td>227 ± 103</td>
<td>53 ± 11</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>iNO</td>
<td>273 ± 96</td>
<td>47 ± 5</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>End of experiment</td>
<td>67 ± 5.2</td>
<td>38 ± 5</td>
<td>0.7 ± 0.2</td>
</tr>
</tbody>
</table>

(spontaneous breathing)
Reference List


**ONLINE FIGURE LEGENDS**

**Online Figure I**
Anatomical location of Laser Doppler probes on the mouse skull.

**Online Figure II**
Diameter of cerebral vessels in ApoE -/- mice subjected to bilateral carotid banding. In hypoperfused brain of atherosclerotic mice NO inhalation induces dilation of cerebral venules and arterioles (*P<0.05 vs. baseline; n=5 each).

**Online Figure III**
(A) Cerebral blood flow measured by [14C]iodoantipyrine autoradiography in ischemic mouse brain with (+iNO) and without iNO (C). For each condition two representative sections from a rostral and a caudal brain region are shown. The dotted area represents the ischemic core, the solid line the penumbra. Animals receiving iNO showed increased penumbral blood flow. (B) Quantification of cerebral blood flow measured by [14C]iodoantipyrine autoradiography in the core and the penumbra of control or iNO ventilated mice subjected to middle cerebral artery occlusion. iNO treated animals have a significantly higher penumbral blood flow as compared to control animals (†P<0.01 vs. control animals, n=5 each).

**Online Figure IV**
(A) Schematic drawing of the area investigated. The ROI was adjusted individually to contain the border zone between ischemic and non ischemic tissue. (B) Immunohistochemical staining for CD 45 revealed positive cells in both groups. However, there was no significant difference in leukocyte infiltration between iNO and control animals (C).

**Online Figure V**
(A) Schematic drawing of areas measured. (B, C) Infarct volume quantified by histomorphometry as measured. i.e. before normalisation to contralateral hemisphere. Nitric oxide inhalation reduces infarct size following transient and permanent cerebral ischemia. (B) Infarct volume determined after 12 h permanent focal cerebral ischemia. NO inhalation significantly reduced cerebral infarction (†P<0.01 vs. control. n=7 each). (C) Quantification of infarct volume in untreated animals (control) and in animals receiving NOi 10 min (iNO 10’) or 2 h (iNO 2 h) after MCA occlusion. Early administration of iNO effectively reduced infarct size (†P<0.01 vs. control. n=7 each).

**Online Figure VI**
Exemplary PET picture with volumetry for the infarct core, the penumbra, and normally perfused brain quantified by calculation of absolute CBF.
Online Fig. I
Anatomical location of Laser Doppler probes on the mouse skull.
Diameter of cerebral vessels in ApoE-/- mice subjected to bilateral carotid banding. In hypoperfused brain of atherosclerotic mice NO inhalation induces dilation of cerebral venules and arterioles (*P<0.05 vs. baseline; n=5 each).
Online Figure III

(A) Cerebral blood flow measured by $^{[14]C}$iodoantipyrine autoradiography in ischemic mouse brain with (+iNO) and without iNO (C). For each condition two representative sections from a rostral and a caudal brain region are shown. The dotted area represents the ischemic core, the solid line the penumbra. Animals receiving iNO showed increased penumbral blood flow.

(B) Quantification of cerebral blood flow measured by $^{[14]C}$iodoantipyrine auto-radio-graphy in the core and the penumbra of control or iNO ventilated mice subjected to middle cerebral artery occlusion. iNO treated animals have a significantly higher penumbral blood flow as compared to control animals ($\dagger P<0.01$ vs. control animals, $n=5$ each).
Online Figure IV

(A) Schematic drawing of the area investigated. The ROI was adjusted individually to contain the border zone between ischemic and non ischemic tissue. (B) Immunohistochemical staining for CD 45 revealed positive cells in both groups. However, there was no significant difference in leukocyte infiltration between iNO and control animals (C).
Online Figure V

(A) Schematic drawing of areas measured. (B, C) Infarct volume quantified by histomorphometry as measured, i.e. before normalisation to contralateral hemisphere. Nitric oxide inhalation reduces infarct size following transient and permanent cerebral ischemia. (B) Infarct volume determined after 12 h permanent focal cerebral ischemia. NO inhalation significantly reduced cerebral infarction (†P<0.01 vs. control. n=7 each). (C) Quantification of infarct volume in untreated animals (control) and in animals receiving NOi 10 min (iNO 10') or 2 h (iNO 2 h) after MCA occlusion. Early administration of iNO effectively reduced infarct size (†P<0.01 vs. control. n=7 each).
Online Figure VI
Exemplary PET picture with volumetry for the infarct core, the penumbra, and normally perfused brain quantified by calculation of absolute CBF.