Neuroprotection by Selective Nitric Oxide Synthase Inhibition at 24 Hours After Perinatal Hypoxia-Ischemia

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Background and Purpose—Perinatal hypoxia-ischemia is a major cause of neonatal morbidity and mortality. Until now no established neuroprotective intervention after perinatal hypoxia-ischemia has been available. The delay in cell death after perinatal hypoxia-ischemia creates possibilities for therapeutic intervention after the initial insult. Excessive nitric oxide and reactive oxygen species generated on hypoxia-ischemia and reperfusion play a key role in the neurotoxic cascade. The present study examines the neuroprotective properties of neuronal and inducible but not endothelial nitric oxide synthase inhibition by 2-iminobiotin in a piglet model of perinatal hypoxia-ischemia.

Methods—Twenty-three newborn piglets were subjected to 60 minutes of hypoxia-ischemia, followed by 24 hours of reperfusion and reoxygenation. Five additional piglets served as sham-operated controls. On reperfusion, piglets were randomly treated with either vehicle (n = 12) or 2-iminobiotin (n = 11). At 24 hours after hypoxia-ischemia, the cerebral energy state, presence of vasogenic edema, amount of apparently normal neuronal cells, caspase-3 activity, amount of terminal deoxynucleotidyl transferase–mediated dUTP-biotin in situ nick end labeling (TUNEL)–positive cells, and degree of tyrosine nitration were assessed.

Results—A 90% improvement in cerebral energy state, 90% reduction in vasogenic edema, and 60% to 80% reduction in apoptosis-related neuronal cell death were demonstrated in 2-iminobiotin–treated piglets at 24 hours after hypoxia-ischemia. A significant reduction in tyrosine nitration in the cerebral cortex was observed in 2-iminobiotin–treated piglets, indicating decreased formation of reactive nitrogen species.

Conclusions—Simultaneous and selective inhibition of neuronal and inducible nitric oxide synthase by 2-iminobiotin is a promising strategy for neuroprotection after perinatal hypoxia-ischemia. (Stroke. 2002;33:2304-2310.)

Key Words: caspases ■ cerebral ischemia, global ■ magnetic resonance imaging ■ neuroprotection ■ reperfusion injury ■ spectroscopy, nuclear magnetic resonance

During perinatal hypoxia-ischemia, lack of oxygen and nutrients to the brain results in initiation of a complex cascade, which intensifies on reperfusion and reoxygenation and leads to secondary energy failure and neuronal cell death. A major pathway leading toward neuronal injury involves elevation of extracellular glutamate and activation of glutamate receptors, with a subsequent increase in intracellular calcium and generation of reactive oxygen species (ROS) and nitric oxide (NO).1 NO plays important physiological roles as well as pathophysiological roles in a wide range of diseases. Three different isoforms of nitric oxide synthase (NOS) have been identified thus far: the constitutive endothelial and neuronal NOS and the inducible NOS. All NOS isoforms are upregulated after hypoxia-ischemia but in different time profiles.2 One of the mechanisms by which excessive NO can be involved in hypoxia-ischemia is by its rapid reaction with the superoxide radical to form peroxynitrite. Peroxynitrite and other related reactive nitrogen species (RNS) produce nitration of lipids, DNA, and proteins, thereby irreversibly modifying brain cell constituents.3 Studies in knockout mice and intervention strategies with selective NOS antagonists claimed that excessive NO production due to neuronal and inducible NOS activation is neurotoxic, whereas endothelial NO production acts neuroprotectively by preventing the postreperfusion decrease in cerebral blood flow.2,4 To our knowledge the effects of administration of a combined neuronal and inducible but not endothelial NOS inhibitor have not been tested in a model of...
perinatal hypoxia-ischemia. Furthermore, in the clinical situation the compound should preferably be administered intravenously for rapid passage to the brain compartment. 2-Iminobiotin fulfills the aforementioned conditions.5 In this study we tested the hypothesis that neuronal and inducible NOS inhibition is important to prevent secondary energy failure and subsequent neuronal death at 24 hours in a piglet model of perinatal hypoxia-ischemia.6 7 NO, ROS, and peroxynitrite also have toxic effects on mitochondria,8 which in turn are involved in the activation of apoptotic processes partly through caspase-dependent mechanisms.9 Caspases are cysteine proteases that are activated as part of a proteolytic cascade leading to disruption of cellular homeostasis and death. Recently, it was shown that caspase-3 is activated after hypoxia-ischemia in the immature brain and that blockade of the caspases attenuates injury.10 12 Therefore, a secondary aim was to evaluate to what extent blockade of neuronal NOS and inducible NOS activity affected terminal deoxynucleotidyl transferase–mediated dUTP-biotin in situ nick end labeling (TUNEL) and caspase-3 activity after the insult.

**Materials and Methods**

**Animal Model**

We used 23 newborn Dutch store piglets (mean gestational age, 115 ± 1 days; weight, 1.7 ± 0.3 kg) with a postnatal age range from 1 to 3 days. After anesthesia with 4% isoflurane in a N2O/O2 mixture (79%/21%), the piglets were intubated. For blood pressure measurement, blood gas analysis, and drug infusions, arterial and venous catheters were inserted. During this procedure, anesthesia was maintained with 1.5% isoflurane in the same N2O/O2 mixture. Lidocaine 1% was injected before each skin incision. Vascular cuffs (OC2a, In Vivo Metric) were placed around both common carotid arteries. Amoxicillin (50 mg/kg twice daily), gentamicin (5 mg/kg per day), and atropine (0.01 mg/kg) were administered intravenously. During the experiment rectal temperature was kept constant by a heat lamp and/or water blanket. After regaining hemodynamic stability, we transported the piglets to a 4.7-T magnet for MRI and MR spectroscopy (MRS) measurements. Anesthesia was maintained with 1.5% isoflurane in the N2O/O2 mixture, and animals were paralyzed with pancuronium bromide (0.25 mg/kg IV). Hypoxia-ischemia was induced by clamping both common carotid arteries and reducing the fraction of inspired oxygen for 1 hour, evaluating the extent of MR-measured cerebral edema after a hypoxic-ischemic insult in mice correlates with the long-term histological outcome.13 To investigate whether vasogenic edema was present, multislice T2-weighted MRI was performed at baseline and at 24 hours after hypoxia-ischemia with a repetition time of 3 seconds; echo times of 25, 40, and 70 ms; a slice thickness of 2 mm; 16 slices; and 2 transitions. Quantitative T2 maps were generated from the T2-weighted MR images, and time course changes of T2 relaxation times from baseline to 24 hours after hypoxia-ischemia (ΔT2) were assessed in brain areas known to be sensitive to hypoxia-ischemia, eg, cortex, striatum, thalamus, and white matter.

**Assay of Caspase-3–Like Activity**

At 24 hours after hypoxia-ischemia, animals were killed with an overdose of pentobarbital, and the brain was rapidly perfused with normal saline to remove the excess of blood. Right hemispheres were dissected into striatum and parietal and temporal cortex and were snap-frozen in liquid nitrogen and stored at −80°C until further analysis. Tissue pieces were homogenized by sonication in 10 volumes of ice-cold 50 mmol/L Tris-HCl (pH 7.3) containing 5 mmol/L EDTA, aliquoted, and stored at −80°C. The protein concentrations were determined according to Whitaker and Graham14 and adapted for microplates with the use of a Spectramax Plus plate reader (Molecular Devices). Samples of homogenate (25 μL) were mixed with 75 μL of extraction buffer as described earlier.10 Cleavage of DEVD-AMC was measured at 37°C with a Spectramax Gemini microplate fluorimeter (Molecular Devices) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm and expressed as picomoles AMC released per milligram protein per minute.

**Histology**

Left brain halves were only taken out after intracardial perfusion with 4% phosphate-buffered formaldehyde. The hemispheres were accurately placed into a self-made leaden mall and were cut at fixed intervals into 3 parts for easier processing for light microscopy. Coronal sections (7 μm) of these brain parts were cut, mounted on silane-coated glass slides, and stained with cresyl violet. The striatum was counted at 17.50 mm in the anterior plane, as shown in a stereotaxic atlas of the pig brain.17 The parietal cortex and hippocampus were both counted at 3.00 mm in the posterior plane.17 Quantification of neuronal viability was performed in the central 3 lobes of the parietal cortex and in the caput of the caudate nucleus of the striatum with the use of a grid of 100 compartments at ×200 magnification. All normal-appearing neurons in the CA1, CA2, CA3, and CA4 regions of the hippocampus and the dentate gyrus were counted. The averaged value was used as the amount of normal-appearing cells in the hippocampus. Normal-appearing neurons were morphologically identified by the presence of typical nuclei with clear nucleoplasm and a distinct nucleolus, surrounded by purple-stained cytoplasm. Neurons were defined damaged when no distinction could be made between nucleus and cytoplasm (pyknotic or necrotic).

**Immunohistochemistry**

The presence of 3-nitrotyrosine–modified substrates in cortex, striatum, and hippocampus was assessed by immunohistochemistry with the use of polyclonal antibodies against 3-nitrotyrosine (Upstate), as modified substrates in cortex, striatum, and hippocampus and scored on a 3-point scale. In this scale a score of 1 indicates that <25% of nuclei are stained; 2, between 25% and 75% of nuclei are stained; and 3, >75% of nuclei are stained.
Statistical Analysis

Data were presented as mean ± SEM and were analyzed by ANOVA for repeated measures, in which time after hypoxia-ischemia served as “within” factor and therapy as “between” factor. When a significant difference was detected, analysis was performed nonparametrically by means of the Mann-Whitney U test for 2 independent samples and the Wilcoxon signed rank test for 2 dependent samples. MRS and MRI data of prematurely dead piglets were excluded at the analysis stage.

Table 1. Physiological Data

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>End of 1-h HI</th>
<th>24 h After Start of HI</th>
</tr>
</thead>
<tbody>
<tr>
<td>MABP, mm Hg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>55 ± 4</td>
<td>75 ± 8*</td>
<td>58 ± 5</td>
</tr>
<tr>
<td>2-Iminobiotin</td>
<td>53 ± 3</td>
<td>70 ± 6*</td>
<td>59 ± 5</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>160 ± 7</td>
<td>210 ± 14*</td>
<td>194 ± 15*</td>
</tr>
<tr>
<td>2-Iminobiotin</td>
<td>157 ± 5</td>
<td>235 ± 8*</td>
<td>172 ± 6</td>
</tr>
<tr>
<td>PacO₂, mm Hg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>44 ± 2</td>
<td>46 ± 3</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>2-Iminobiotin</td>
<td>47 ± 2</td>
<td>44 ± 3</td>
<td>44 ± 7</td>
</tr>
<tr>
<td>PacO₂, mm Hg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>100 ± 4</td>
<td>33 ± 1*</td>
<td>123 ± 11</td>
</tr>
<tr>
<td>2-Iminobiotin</td>
<td>100 ± 11</td>
<td>30 ± 2*</td>
<td>119 ± 9</td>
</tr>
</tbody>
</table>

HI indicates hypoxia-ischemia.

*P<0.05 compared with baseline.

Results

Five of the 23 piglets died before 24 hours after hypoxia-ischemia: 3 in the vehicle-treated group at 5, 10, and 19 hours after hypoxia-ischemia as a result of hypoxic-ischemic complications (necrotizing enterocolitis, cardiomyopathy, and status epilepticus and bronchial obstruction) and 2 in the 2-iminobiotin–treated group at 11 and 14 hours after hypoxia-ischemia. The areas under the PCr/Pi curve, as measured between baseline and the end of 60 minutes of hypoxia-ischemia, were significantly increased at the end of 1 hour of hypoxia-ischemia, whereas the arterial P O₂ was significantly decreased in both groups. At 24 hours after hypoxia-ischemia the heart rate remained increased in the vehicle-treated piglets but not in the 2-iminobiotin–treated piglets.

Magnetic Resonance Spectroscopy

The areas under the PCr/Pi curve, as measured between baseline and the end of 60 minutes of hypoxia-ischemia, were not significantly different between treatment groups, indicating that the piglets were subjected to the same degree of hypoxic-ischemic brain injury. 31P-MRS spectra of a newborn piglet at baseline and at 24 hours after hypoxia-ischemia for

Table 2. 31P-MRS Metabolite Ratios

<table>
<thead>
<tr>
<th></th>
<th>PCr/Pi</th>
<th>ATP/EPP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>2-Iminobiotin</td>
</tr>
<tr>
<td>Baseline</td>
<td>2.37 ± 0.22</td>
<td>2.15 ± 0.14</td>
</tr>
<tr>
<td>End of 60-min HI</td>
<td>0.40 ± 0.10†</td>
<td>0.26 ± 0.03†</td>
</tr>
<tr>
<td>1 h of reperfusion</td>
<td>1.65 ± 0.26</td>
<td>2.14 ± 0.14</td>
</tr>
<tr>
<td>2 h of reperfusion</td>
<td>1.87 ± 0.31</td>
<td>2.23 ± 0.18</td>
</tr>
<tr>
<td>24 h of reperfusion</td>
<td>0.80 ± 0.24†</td>
<td>2.00 ± 0.25†</td>
</tr>
</tbody>
</table>

PCr/Pi ratios and ATP/EPP ratios (in arbitrary units) of vehicle-treated piglets (n=12) and 2-iminobiotin–treated piglets (n=11) during baseline until 2 h of reperfusion and at 24 h after hypoxia-ischemia (HI) are shown.

*P<0.05, †P<0.01 compared with baseline; ‡P<0.005 compared with vehicle-treated piglets.
vehicle- and 2-iminobiotin–treated piglets are shown in Figure 1a, 1b, and 1c. Results for 31 P-MRS metabolites are presented in Table 2. At 24 hours after hypoxia-ischemia, 2-iminobiotin–treated piglets had preserved PCr/Pi and ATP/EPP ratios compared with vehicle-treated piglets (P<0.005). The PCr/Pi and ATP/EPP ratios were significantly reduced for vehicle-treated piglets at 24 hours after hypoxia-ischemia compared with baseline (P≤0.005), demonstrating the presence of secondary energy failure. PCr/Pi and ATP/EPP ratios of the 2-iminobiotin–treated piglets at 24 hours after hypoxia-ischemia were as high as baseline.

Magnetic Resonance Imaging
Vehicle-treated piglets showed a significant increase in T2 relaxation times over time (Figure 1d and 1e; Table 3). 2-iminobiotin prevented the formation of vasogenic edema in cortex (Figure 1f). Significant changes in ΔT2 values were present between vehicle- and 2-iminobiotin–treated piglets in cortex and striatum (P<0.05). In thalamus and white matter, no increase in T2 relaxation times was observed after hypoxia-ischemia for both groups.

Caspase-3 Activity, Histology, and TUNEL Labeling
The activity of caspase-3, the executor of apoptotic cell death, was reduced by 93% in cortex and 71% in striatum after 2-iminobiotin treatment (Figure 2).

Representative examples of microscopic slices from the parietal cortex of a vehicle- and 2-iminobiotin–treated piglet are shown in Figure 3a and 3b, after cresyl violet staining to demonstrate the amount of apparently normal neurons and after TUNEL labeling to identify the amount of cell death. A significant difference in number of apparently normal neurons was demonstrated between vehicle- and 2-iminobiotin–treated piglets in parietal cortex (65% reduction), striatum (82% reduction), and hippocampus (59% reduction) (Table 4). 2-iminobiotin–treated piglets showed a significant reduction in TUNEL labeling in parietal cortex, striatum, and hippocampal areas.

An inverse relation was demonstrated between the number of apparently normal neurons and the TUNEL score in cortex (r=0.67, P≤0.005), striatum (r=0.69, P<0.005), and in hippocampus (r=0.83, P<0.0005). No statistical differences in histology and TUNEL labeling were detected between 2-iminobiotin–treated and sham-operated piglets (Table 4).

Tyrosine Nitration
2-iminobiotin–treated piglets showed less tyrosine nitration compared with vehicle-treated piglets in parietal cortex (Figure 4; Table 4). The reduced amount of nitrated tyrosine in neurons in the cortex of 2-iminobiotin–treated piglets suggests inhibition of neuronal NOS and inducible NOS activity. An inverse correlation exists between the number of apparently normal cells and the tyrosine nitration score in cortex (r=0.84, P<0.0005). The number of TUNEL-positive cells relates to the degree of tyrosine nitration in cortex (r=0.64, P<0.005) and hippocampal area (r=0.54, P<0.05).

### Table 3. Water T2 Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>24 h After HI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>2-iminobiotin</td>
</tr>
<tr>
<td>Parietal cortex</td>
<td>80±3</td>
<td>79±1</td>
</tr>
<tr>
<td>Striatum</td>
<td>72±2</td>
<td>73±1</td>
</tr>
<tr>
<td>White matter</td>
<td>59±1</td>
<td>59±1</td>
</tr>
<tr>
<td>Thalamus</td>
<td>67±1</td>
<td>66±1</td>
</tr>
</tbody>
</table>

Water T2 characteristics (in ms) measured with T2-weighted MRI at baseline and at 24 h after hypoxia-ischemia (HI) for vehicle-treated piglets (n=12) and 2-iminobiotin–treated piglets (n=11) are shown. ΔT2: *P<0.05 compared with vehicle-treated piglets.

### Table 4. Histology, TUNEL Labeling, and Tyrosine Nitration Immunohistochemistry

<table>
<thead>
<tr>
<th></th>
<th>Parietal Cortex</th>
<th>Striatum</th>
<th>Hippocampus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>2-iminobiotin</td>
<td>Sham</td>
</tr>
<tr>
<td>Cresyl violet</td>
<td>155±37</td>
<td>281±36*</td>
<td>349±71</td>
</tr>
<tr>
<td>TUNEL</td>
<td>2.2±0.2</td>
<td>1.5±0.2*</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td>Tyrosine nitration</td>
<td>2.4±0.2</td>
<td>1.7±0.2*</td>
<td>2.0±0.3</td>
</tr>
</tbody>
</table>

Histology and immunohistochemistry were performed at 24 h after hypoxia-ischemia. Piglets were treated with vehicle (n=12) or 2-iminobiotin (n=11) or were sham-operated (n=5). Sections were stained with cresyl violet, and the amount of apparently normal cells was counted in a grid consisting of 100 compartments. Hippocampus indicates the mean value from CA1 to CA4, plus dentate gyrus. TUNEL labeling and tyrosine nitration were scored on a 3-point scale. Although these data are ordinal data, the mean±SEM is reported for reasons of clarity. *P<0.05 compared with vehicle-treated piglets.
Discussion

2-Iminobiotin administration directly on reperfusion and reoxygenation led to an improved cerebral energy state, prevented the formation of vasogenic edema, and corresponded histopathologically with preservation of viable cells and a striking reduction in apoptosis-related parameters such as caspase-3 activity and TUNEL labeling. This is the first study to report beneficial effects of a combined neuronal and inducible but not endothelial NOS inhibitor that can be intravenously administered after hypoxia-ischemia.

This study was conducted in a newborn piglet model of perinatal hypoxia-ischemia in term neonates. 2-Iminobiotin was administered directly on reperfusion, mimicking the clinical setting of an asphyxiated neonate. Previously, we demonstrated that 2-iminobiotin preserved the electrocortical brain activity at 24 hours after hypoxia-ischemia.19 The preservation of the number of apparently normal cells in cortex, striatum, and hippocampus validated the beneficial effects of 2-iminobiotin treatment after hypoxia-ischemia.

In this study we observed the piglets for a 24-hour period after hypoxia-ischemia. Secondary energy failure in newborn piglets occurs from 6 until 72 hours after hypoxia-ischemia. The major decrease in PCr/Pi ratio takes place between 6 and 24 hours after hypoxia-ischemia, although some further decrease is seen between 24 and 48 hours.7 To confirm that brain cell injury is not only postponed but also reduced, long-term studies are warranted. However, the decreased neuronal cell loss at 24 hours after hypoxia-ischemia, the reduction in TUNEL-positive cells, and the absence of caspase-3 activity in the 2-iminobiotin–treated piglets suggest that additional brain injury is less likely to occur at later stages. Nevertheless, one must be cautious because the benefits of neuroprotective interventions may disappear as the period of recovery is extended.20,21 The present study demonstrates that time is gained to intervene in the process of neuronal regeneration, for example, by the administration of neurotrophic factors.

TUNEL labeling is not specific for apoptotic cell death because it detects double-stranded DNA nicks bearing the 3′ OH groups, which can also be present after random cleaving of the DNA, for instance, through ROS-mediated injury.22 Previous data demonstrate that activation of caspase-3 is a key event in hypoxia-ischemia injury leading to programmed cell death, especially in the immature brain.23 Hypoxia-ischemia induces a massive increase of caspase-3 and cleavage of its substrates.10,11 Inhibition of caspase activation after hypoxia-ischemia either directly12 or by administration of the brain-derived neurotrophic factor attenuates injury.24 The present data suggest that activation of inducible NOS/neuronal NOS may actually be part of the triggering process leading to activation of caspases. One might speculate that NO injures mitochondria and promotes the release of proapo-

Figure 3. Histology and TUNEL labeling in parietal cortex. Representative example of parietal cortex in vehicle-treated (a) and 2-iminobiotin–treated (b) piglet at 24 hours after hypoxia-ischemia. Sections were stained with cresyl violet to identify the amount of apparently normal neurons. TUNEL labeling (visible as brown-stained nuclei) was performed to estimate the number of neurons with DNA damage. The 2-iminobiotin–treated piglets showed more apparently normal neurons and less brain cell damage.

Figure 4. Tyrosine nitration in the cerebral cortex. Representative example of tyrosine nitration (visible as brown-stained cytoplasm) in the cerebral cortex of a vehicle-treated (a) and 2-iminobiotin–treated (b) piglet at 24 hours after hypoxia-ischemia. The 2-iminobiotin–treated piglet showed less tyrosine nitration than the vehicle-treated piglet.
ptotic proteins (cytochrome C, procaspases) into the cytosol with subsequent apoptosome formation and upstream activation of caspase-3,4 or that caspase-3 is activated via the extrinsic pathway due to cell surface receptors like tumor necrosis factor-α or Fas.22 Other studies reported that low doses of NO may have antiapoptotic characteristics by blocking caspase activity via S-nitrotyrosylation.25 Since these studies were performed in isolated endothelial cells, it cannot be excluded that the endothelial isoform of NOS was involved in the reduction of cell death.26 Furthermore, it was shown earlier in fetal sheep and newborn piglets that the NO production after hypoxia-ischemia is both massive and long-lasting.27,28 This offers additional support for a proapoptotic effect of NO under these conditions.

Nonselective treatment with NOS inhibitors has provided conflicting results.29,30 It has been shown that pretreatment with 7-nitroindazole, a selective neuronal NOS inhibitor, suppressed both peaks of NO metabolites (during hypoxia-ischemia as well as on reperfusion) and provided neuroprotection in a 7-day-old rat model of hypoxia-ischemia.4,31 This is in agreement with the results in a middle cerebral artery occlusion (MCAO) model in mice and rats, in which neuronal NOS inhibition before the onset of hypoxia-ischemia reduced the infarct volume.20,32 These observations can be explained from the fact that during the hypoxic-ischemic event NO reversibly inhibits mitochondrial respiration by binding to the oxygen binding site of cytochrome oxidase in competition with oxygen, thereby aggravating the cytotoxic injury.33 Treatment with a selective neuronal NOS inhibitor directly on occlusion in a permanent MCAO rat model or directly on reperfusion in a transient MCAO rat model provided neuroprotection, but not after delayed administration.34,35 O’Neill et al.36 also demonstrated that treatment with a selective neuronal NOS and endothelial NOS inhibitor (ARL-17477) directly on reperfusion provided neuroprotection after short-term global ischemia in the gerbil but not after delayed administration. This low-dose selective neuronal NOS and endothelial NOS inhibitor was not neuroprotective after 2 hours of transient intraluminal MCAO in the adult rat,36 while earlier studies reported a neuroprotective effect after 7 days in the same model.37 Treatment with the selective neuronal NOS inhibitor 7-nitroindazole a longer time after occlusion or reperfusion was not neuroprotective in the MCAO model in rats20,34,36 and showed only a trend toward neuroprotection in a mouse hypoxia-ischemia model when used in high dosages.38 This indicates that neuronal NOS production is predominantly deleterious during or immediately after the hypoxic-ischemic insult and to a much lesser extent in a later phase after reperfusion.

In a 7-day-old rat model, it was shown that aminoguanidine, a selective inducible NOS inhibitor, administered before the onset of hypoxia-ischemia did not reduce the first peak of NO production during hypoxia-ischemia and only partly suppressed the second peak after reperfusion.4 However, inducible NOS inhibition after reperfusion in a rat MCAO model19,40 showed a 30% to 40% reduction in infarct volume from 72 hours after MCAO. Furthermore, it was shown in a 7-day-old rat hypoxia-ischemia model that combined pre- and post–hypoxia-ischemia inducible NOS inhibition reduced infarct volumes by approximately 90%.41 One can conclude from these studies that inducible NOS inhibition is particularly neuroprotective in the reperfusion phase, although an additional neuroprotective effect from inducible NOS inhibition during the insult cannot be excluded.

In a study in piglets it was shown that transient global cerebral ischemia is accompanied by enhanced endothelial NOS immunoreactivity in the blood vessels of the hippocampus.42 It has been suggested that the NO produced by this mechanism may have neuroprotective effects due to a possible improvement in cerebral blood flow.2 This observation is in agreement with the fact that transgenic mice that lack endothelial NOS experience increased infarct volumes after MCAO and show a decrease in infarct volumes after treatment with a nonselective NOS inhibitor.53 The positive outcome in some studies with the use of nonselective NOS inhibitors might be caused by the rather mild endothelial NOS inhibitory properties of the compound used.59

In earlier studies it was shown that 2-iminobiotin is a reversible inhibitor of both the neuronal and the inducible isoforms of NOS but not the endothelial NOS isoforms.5 In 3 additional piglets we tested the effect of 2-iminobiotin treatment on physiological parameters. No increases in MABP, heart rate, and breathing frequency were observed (data not shown), providing evidence that 2-iminobiotin does not block the endothelial isoform of NOS in such a way that it affects the MABP. The cerebral energy state, as measured with 31P-MRS, and the electrocortical electroencephalogram also remained stable after 2-iminobiotin administration (data not shown).

In our study 2-iminobiotin, administered on reperfusion, significantly decreased the amount of cell death at 24 hours after hypoxia-ischemia. This was supported by the observation that the degree of tyrosine nitration in the brain used in this study as a biomarker for the presence of RNS was reduced in the cortex after 2-iminobiotin administration. Future experimental studies are needed to assess the dose-response characteristics of 2-iminobiotin. In addition, the effect of 2-iminobiotin treatment administered before the insult should be investigated. If proven effective, 2-iminobiotin could be administered in high-risk pregnancies, when fetal hypoxia-ischemia is imminent or cannot be avoided.

In conclusion, the present study demonstrated that selective inhibition of neuronal NOS and inducible NOS after global hypoxia-ischemia in newborn piglets is neuroprotective at 24 hours after the insult. Thus, selective neuronal and inducible NOS inhibitors administered directly on reperfusion may afford promising avenues in the treatment of this socially, emotionally, and financially devastating neonatal disorder.

Acknowledgments
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References


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