Tolerance Against Ischemic Neuronal Injury Can Be Induced by Volatile Anesthetics and Is Inducible NO Synthase Dependent

Krisztian J. Kapinya, MD*; Diana Löwl*; Carsten Fütterer; Martin Maurer, MD; Klaus F. Waschke, MD; Nikolaj K. Isaev, PhD; Ulrich Dirnagl, MD

Background and Purpose—We tested whether volatile anesthetics induce neuroprotection that is maintained for a prolonged time.

Methods—Rats were pretreated for 3 hours with 1 minimal anesthetic concentration of isoflurane or halothane in normal air (anesthetic preconditioning [AP]). The animals were subjected to permanent middle cerebral artery occlusion (MCAO) at 0, 12, 24, or 48 hours after AP. Halothane-pretreated animals were subjected to MCAO 24 hours after AP. Histological evaluation of infarct volumes was performed 4 days after MCAO. Cerebral glucose utilization was measured 24 hours after AP with isoflurane. Primary cortical neuronal cultures were exposed to 1.4% isoflurane for 3 hours. Oxygen-glucose deprivation (OGD) was performed 24 hours after AP. Injury was assessed 24 hours later by measuring the release of lactate dehydrogenase into the medium 24 hours after OGD.

Results—Isoflurane anesthesia at 0, 12, and 24 hours before MCAO or halothane anesthesia 24 hours before MCAO significantly reduced infarct volumes (125+/-60 mm³, P=0.024; 118+/-51 mm³, P=0.008; 120+/-49 mm³, P=0.009; and 121+/-48 mm³, P=0.018, respectively) compared with control volumes (180+/-51 mm³). Three hours of isoflurane anesthesia in rats did not have any effect on local or mean cerebral glucose utilization measured 24 hours later. Western blot analysis from cortical extracts of AP-treated animals revealed an increase of the inducible NO synthase (iNOS) protein beginning 6 hours after AP. The iNOS inhibitor aminoguanidine (200 mg/kg IP) eliminated the infarct-sparing effect of AP. In cultured cortical neurons, isoflurane exposure 24 hours before OGD decreased the OGD-induced release of lactate dehydrogenase by 49% (P=0.002).

Conclusions—Pretreatment with volatile anesthetics induces prolonged neuroprotection in vitro and in vivo, a process in which iNOS seems to be critically involved. (Stroke. 2002;33:1889-1898.)

Key Words: anesthesia ■ cerebral ischemia ■ neuroprotection ■ rats

There is robust evidence that volatile anesthetics applied during cerebral ischemia confer neuroprotection.1-5 In a model of incomplete hemispheric ischemia, Baughman et al1 demonstrated neuroprotection in rats anesthetized with isoflurane or halothane. Warner et al2 demonstrated the neuroprotective effect of halothane or sevoflurane during focal cerebral ischemia in rats. After cardiac arrest in dogs, isoflurane reduces damage of the hippocampal neurons.3 Miura et al4 provided evidence that protection against neuronal injury by isoflurane during near-complete ischemia is superior to protective effects of fentanyl or ketamine anesthesia. In a recent publication, Bhardwaj et al5 presented data on neuroprotection with halothane anesthesia after focal cerebral ischemia induced immediately after the termination of anesthesia. All these studies used volatile anesthetics shortly before and during the ischemic period.

However, it is also known that volatile anesthetics can induce the expression of several genes belonging to the class of immediate-early genes (IEGs), and this effect is not restricted to nervous tissue only.6,7 IEGs are transcription factors induced as a stress response and are implicated in ischemic tolerance induction in the brain.8,9 Thus, induction of IEGs is expected to lead to long-lasting changes in the expression of various gene products.

This leads us to the hypothesis that the well-described neuroprotection during general anesthesia might not be restricted to the duration of anesthesia but may be sustained long after anesthesia has been terminated. In the present

Received November 1, 2001; final revision received March 14, 2002; accepted April 2, 2002.
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Stroke is available at http://www.strokeaha.org DOI: 10.1161/01.STR.0000020092.41820.58
study, we test through in vivo and in vitro experiments whether anesthesia with volatile anesthetics can “precondition” brain tissue and induce tolerance against hypoxia/ischemia. We also address whether anesthesia with isoflurane may have long-lasting effects on cerebral metabolism, which might explain the induction of tolerance. Because isoflurane induces the inducible isofom of NO synthase (iNOS) in macrophages, and because iNOS plays a role in the induction of ischemic preconditioning in the heart and in neuronal cell culture, we speculated that iNOS might also be involved in anesthesia-induced preconditioning (AP).

Materials and Methods

Pretreatment With Volatile Anesthetics (Anesthetic Preconditioning)

Adult male Wistar rats weighing 250 to 300 g were used in the present study (BGVV, Berlin, Germany). All surgical procedures were performed in accordance with the Guidelines for the Use of Animals in Neuroscience Research (Society for Neuroscience) and were approved by the local authorities. Anesthesia was carried out with precalibrated vapors (Dräger Medizintechnik): isoflurane (Forene, Abbott) or halothane (Asid Bonz) was used. Most of the experiments were conducted with isoflurane because it is a clinically widely used volatile anesthetic. To demonstrate the class effect of halogenated volatile anesthetics, delayed neuroprotection was also demonstrated in 2 groups of rats with the use of halothane. Because of its potential cardiodepressant and liver toxic effects, halothane is no longer used clinically, but it is still widely applied in animal experiments. Anesthesia was induced with 4% isoflurane or halothane and maintained for 3 hours with 1.4% isoflurane or 1.2% halothane in air according to the respective minimal anesthetic concentration 1 values for the rat. The anesthesiologists administered the spontaneously breathing animals through a tightly fitting nose cone with a constant flow of 500 mL/min. Body temperature was held constant at 37°C with a temperature-controlled heating pad. After recovery from anesthesia, the animals were transferred back to their cages, where their rectal temperature was kept under the same temperature-controlled environmental condition as described before, until the experiments were performed on them. The preconditioning group was anesthetized by inhalation of 1.4% isoflurane (Forene, Abbott) in air for 3 hours. Body temperature was maintained within a normothermic range (37°C to 38°C) with a temperature-controlled heating pad. The right middle cerebral artery was exposed after a subtemporal craniotomy. The common carotid arteries were then occluded with the use of nontraumatic clips. With the aid of an operating microscope, the middle cerebral artery was coagulated by unipolar cautery and cut below the rhinal fissure. The average duration of anesthesia and instrumentation before MCAO was 60 ± 15 minutes in all experimental groups. At the end of the 60-minute ischemic period, both common carotid artery clips were removed for partial reperfusion, and the wounds were sutured. After recovery from anesthesia, the animals were transferred to their cages, where their rectal temperatures were controlled for another 2 hours. Free access to food and water was allowed until the animals were euthanized.

Determination of Infarct Size

The animals were euthanized 4 days after MCAO. Their brains were removed and snap-frozen in chilled 2-methylbutane (−50°C to −60°C) for cryostat sectioning. Sections (40 μm) at 800-μm intervals were stained with vanadium acidic fuchsin according to the protocol of Victorov et al. The total volume of injury was determined by integration of the area of injury determined on each section with SigmaScan Pro 4.0 image analysis software (Jandel Scientific) after scanning of the histological slides. Four days after MCAO, brain edema was no longer present; therefore, no edema correction was necessary. The infarct volume determination was performed by an investigator blinded to the treatment groups.

Measurement of LCGU

The rats were randomly assigned to 2 experimental groups: the preconditioning group and the control group. The control group was kept under the same temperature-controlled environmental condition as described before, until the experiments were performed on them. The preconditioning group was anesthetized by inhalation of 1.4% isoflurane (Forene, Abbott) in air for 3 hours. Body temperature was held constant at 37°C with a temperature-controlled heating pad after anesthesia for the phase of awakening, the animals were kept under the same environmental conditions as the control group. After 24 hours, the control and pretreated rats were placed in a small box and anesthetized for surgery with 1.4% isoflurane via a nose cone. Body core temperature was held constant at 37°C with a temperature-controlled heating pad. Polyethylene catheters were inserted into the right femoral artery and vein for continuous blood pressure monitoring and for fluid administration, respectively. After surgery, the rats were placed in rat restrainers (Braintree Scientific), infused with saline (4 mL/kg body wt per hour), and allowed to recover for a minimum of 2 hours before measurement of local cerebral glucose utilization (LCGU).

hypotension with a degree and duration comparable to that of AP. Mean arterial blood pressure (MABP) and blood gas values were recorded up to 5 hours after dihydralazine application.

For pharmacological blockade of iNOS activity, a separate set of experiments was carried out in which 200 mg/kg aminoguanidine (AG) (Sigma) dissolved in saline was administered intraperitoneally twice, at the beginning of AP with isoflurane and 12 hours later. This treatment protocol was shown by several studies to inhibit iNOS activity in the rat brain. Control animals received only saline.

Induction of Focal Cerebral Ischemia (Stroke Model)

The experiments were performed in a randomized fashion by an investigator blinded to the treatment groups. Induction of focal cerebral ischemia was performed according to the method of Brint et al., with minor modifications. Briefly, the animals were anesthetized with isoflurane (induction 4%, maintenance 1.4%) in a mixture of O2/N2O (1:2) through a nose cone. The tail artery was cannulated for monitoring MABP and for obtaining blood samples for arterial blood gas and glucose measurements. Arterial blood gases were determined at the beginning and at the end of the ischemia with a blood gas analyzer (Compact 3, AVL Medizintechnik). Body core temperature was maintained within a normothermic range (37°C to 38°C) with a temperature-controlled heating pad. The right middle cerebral artery was exposed after a subtemporal craniotomy. Both common carotid arteries were then occluded with the use of nontraumatic clips. With the aid of an operating microscope, the middle cerebral artery was coagulated by unipolar cautery and cut below the rhinal fissure. The average duration of anesthesia and instrumentation before MCAO was 60 ± 15 minutes in all experimental groups. At the end of the 60-minute ischemic period, both common carotid artery clips were removed for partial reperfusion, and the wounds were sutured. After recovery from anesthesia, the animals were transferred to their cages, where their rectal temperatures were controlled for another 2 hours. Free access to food and water was allowed until the animals were euthanized.
OGD in Primary Neuronal Cell Cultures

To mimic cerebral ischemia in vitro, oxygen-glucose deprivation (OGD) was performed with AP-treated primary neuronal cultures. Cortical cultures were prepared from cerebral cortices of fetal rats (embryonic days 16 through 18; BGVV, Berlin, Germany) as described previously. Briefly, the tissue was digested with trypsin/EDTA (0.05%/0.02% [wt/vol] in PBS, 15 minutes at 37°) followed by mechanical dissociation. Cortical cells were seeded with a density of 3000 cells/mm² in poly-L-lysine (Falcon) and grown in neurobasal medium with serum-free B-27 supplement (Life Technologies/BRL), 100 µM penicillin, 100 µg/mL streptomycin, and 2 mmol/L L-glutamine. Glutamate (25 µmol/L) was added during the initial 3 days in vitro (DIV). Cultures were kept at 36.5°C and 5% CO₂ in a humidified incubator and fed beginning at 4 DIV with cultivating medium (as described above, without glutamate) by replacing half of the medium twice a week.

AP with isoflurane was performed with the neuron-enriched cultures (~85% neuronal cells) after 8 DIV at 36.5°C in a closed chamber (1,040 cm³) in an atmosphere of 1.4% isoflurane, 5% CO₂, 20% O₂, and N₂ (remainder) for a 3-hour period. Cells treated identically but without the addition of isoflurane served as controls. OGD was then performed with the pretreated or control cells 24 hours later. For OGD, medium was set at 36.5°C and the values were summarized to obtain the area-weighted means of all measured sections.

Statistical Analysis

Data are presented as mean ± SD. Infract volumes, Western blotting, and LCGU data of the different treatment groups were compared by using one-way ANOVA. Unpaired t tests with Bonferroni corrections were used for comparisons between groups when significant differences were identified by ANOVA. LDH values from cell culture experiments were compared by the Student nonpaired t test. Physiological variables were analyzed by Kruskal-Wallis ANOVA on ranks and the Dunn correction. The level of statistical significance was set at P < 0.05.

Results

Physiological Variables

Physiological values of the pretreatment period are presented in Table 1. In spontaneously breathing rats, there was a significant increase in the PaCO₂ in the halothane-treated group after 90 minutes and after 180 minutes compared with the nonanesthetized (control) group. Consequently, arterial blood pH was decreased significantly in the halothane-treated group after 90 minutes and 180 minutes compared with the control group. To exclude any possible influence of blood gas alterations, we also performed halothane pretreatment in a group of animals with ventilatory support during the pretreatment period.

Because PaCO₂ and blood pH were normal in the ventilated halothane-treated group and in the isoflurane group and because the induction of tolerance was similar in all 3 groups (see below), we did not consider alkalosis and hypercapnia during halothane + O₂ treatment to be of biological relevance in the present study. However, compared with anesthesia alone in spontaneously breathing animals, oral intubation and mechanical ventilation yield a higher level of stress. There-
fore, the remaining experiments were performed in spontaneously breathing animals exclusively.

MABP was decreased in all the animals treated with halothane or isoflurane (Table 1). To exclude the possibility that systemic hypotension may have caused preconditioning, we induced hypotension in 1 group of animals to a level similar to that in the AP groups by intraperitoneal application of dihydralazine. The mean duration of the hypotensive period (<70 mm Hg) was 220±30 minutes, which was therefore comparable to the duration of AP-caused hypotension. According to the infarct volumes of the hypotensive control group (see below), anesthesia-induced hypotension was also ruled out as a confounding factor in AP.

During MCAO, no significant differences in physiological variables were detected between AP-treated, control, and AG-treated groups (Tables 2, 3, 4, and 5).

### TABLE 1. Selected Physiological Variables of the Pretreatment Period

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>$\text{Paco}_2$, mm Hg</th>
<th>$\text{PaO}_2$, mm Hg</th>
<th>MABP, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=9)</td>
<td></td>
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</tr>
<tr>
<td>90 min</td>
<td>7.43±0.02</td>
<td>36.7±8.0</td>
<td>102.7±6.2</td>
<td>81.8±11.5</td>
</tr>
<tr>
<td>180 min</td>
<td>7.44±0.02</td>
<td>34.9±7.5</td>
<td>102.3±5.7</td>
<td>79.5±13.7</td>
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<tr>
<td>Halothane + O₂ (n=8)</td>
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<td></td>
</tr>
<tr>
<td>90 min</td>
<td>7.29±0.04*</td>
<td>53.2±5.7*</td>
<td>128.3±33.2</td>
<td>61.2±5.2*</td>
</tr>
<tr>
<td>180 min</td>
<td>7.25±0.04*</td>
<td>54.9±6.0*</td>
<td>105.0±20.8</td>
<td>66.9±13.3</td>
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<td>Halothane + ventilatory support (n=9)</td>
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<tr>
<td>90 min</td>
<td>7.45±0.02</td>
<td>39.3±2.1</td>
<td>108.1±6.9</td>
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</tr>
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<td>180 min</td>
<td>7.42±0.03</td>
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<td>108.9±14.0</td>
<td>68.0±10.4</td>
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<td>Isoflurane (n=8)</td>
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<td></td>
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<tr>
<td>90 min</td>
<td>7.40±0.02</td>
<td>38.6±2.5</td>
<td>90.6±5.7</td>
<td>68.2±11.8</td>
</tr>
<tr>
<td>180 min</td>
<td>7.39±0.02</td>
<td>38.2±3.1</td>
<td>88.6±5.9</td>
<td>64.4±9.6*</td>
</tr>
<tr>
<td>Controlled hypotension (n=9)</td>
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<td></td>
</tr>
<tr>
<td>90 min</td>
<td>7.46±0.03</td>
<td>33.1±4.8</td>
<td>110.2±2.8</td>
<td>60±4.0*</td>
</tr>
<tr>
<td>180 min</td>
<td>7.46±0.01</td>
<td>32.8±4.4</td>
<td>110.7±3.0</td>
<td>63±5.8*</td>
</tr>
</tbody>
</table>

Values are mean±SD.
*P<0.05 vs corresponding value of control.

### TABLE 2. Physiological Variables During MCAO in Selected Groups in Which Neuroprotection Was Found

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>$\text{Paco}_2$, mm Hg</th>
<th>$\text{PaO}_2$, mm Hg</th>
<th>MABP, mm Hg</th>
<th>Glucose, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=13)</td>
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<td></td>
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<tr>
<td>Beginning</td>
<td>7.38±0.05</td>
<td>44.7±5.7</td>
<td>138.8±4.8</td>
<td>83.3±11.1</td>
<td>6.8±1.3</td>
</tr>
<tr>
<td>End</td>
<td>7.39±0.03</td>
<td>42.1±5.0</td>
<td>114.1±12.2</td>
<td>69.8±9.5</td>
<td>8.3±3.5</td>
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<tr>
<td>Halothane + O₂ at 24 h (n=11)</td>
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<tr>
<td>Beginning</td>
<td>7.37±0.03</td>
<td>44.3±5.4</td>
<td>156.4±23.2</td>
<td>79.2±10.4</td>
<td>8.0±1.0</td>
</tr>
<tr>
<td>End</td>
<td>7.36±0.04</td>
<td>46.8±3.6</td>
<td>127.1±22.5</td>
<td>61.4±10.4</td>
<td>6.5±0.8</td>
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<td>Isoflurane at 0 h (n=13)</td>
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<tr>
<td>Beginning</td>
<td>7.42±0.06</td>
<td>41.6±6.6</td>
<td>122.3±18.2</td>
<td>93.3±14.0</td>
<td>7.0±2.0</td>
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<tr>
<td>End</td>
<td>7.41±0.03</td>
<td>41.9±5.1</td>
<td>111.7±18.9</td>
<td>79.0±13.9</td>
<td>5.5±1.3</td>
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<td>Isoflurane at 12 h (n=14)</td>
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<td>Beginning</td>
<td>7.38±0.02</td>
<td>46.1±3.7</td>
<td>138.4±10.4</td>
<td>81.7±10.7</td>
<td>6.2±1.6</td>
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<tr>
<td>End</td>
<td>7.37±0.03</td>
<td>47.2±6.2</td>
<td>133.5±15.3</td>
<td>72.5±7.7</td>
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<td>Isoflurane at 24 h (n=15)</td>
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<td>Beginning</td>
<td>7.39±0.02</td>
<td>43.3±4.5</td>
<td>141.8±17.6</td>
<td>80.1±10.4</td>
<td>6.9±0.6</td>
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<tr>
<td>End</td>
<td>7.38±0.03</td>
<td>44.9±3.6</td>
<td>133.5±15.3</td>
<td>61.4±10.0</td>
<td>5.2±0.9</td>
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</tbody>
</table>

Values are mean±SD.
Isoflurane anesthesia at 0 hours (induction of focal ischemia without any interruption of anesthesia), 12 hours, 24 hours, and 48 hours before the induction of MCAO decreased infarct volumes to 125±42 mm$^3$ ($P$=0.024), 118±51 mm$^3$ ($P$=0.008), 120±49 mm$^3$ ($P$=0.009), and 144±50 mm$^3$ ($P$=0.27 [not significant]), respectively. Halothane anesthesia in spontaneously breathing animals 24 hours before MCAO also significantly reduced infarct size (121±48 mm$^3$, $P$=0.018) (Figure 1).

Similarly, in the halothane-treated rats with ventilatory support during pretreatment, the infarct volume was significantly reduced compared with that in briefly anesthetized and intubated rats (201±34 and 145±41 mm$^3$, respectively; $P$=0.012). The infarct volumes of animals with induced mild hypotension 24 hours before MCAO did not differ significantly from the control values (226±32 mm$^3$) (Figure 2).

**Twenty-Four Hours After Isoflurane Anesthesia, Cerebral Glucose Metabolism Is Unaltered**

To test whether the remarkable protection afforded by AP is caused by a long-lasting effect of the anesthetic on brain metabolism, we measured CGU 24 hours after AP with isoflurane. Table 6 shows CGU values for selected brain regions of control and AP animals. In Table 6, emphasis is put on cortical structures that have been damaged by MCAO and in which tolerance was induced. Neither in these nor in any other of the investigated 40 brain regions (not shown) could a significant difference between the control group and the isoflurane-pretreated group be measured.

**Isoflurane Preconditioning Induces Tolerance Against OGD in Neuronal Cultures**

To test whether volatile anesthetic-induced tolerance is a primarily neuronal phenomenon or whether it depends on the effects of the anesthetics on other factors such as blood gas values, cerebral blood flow, vascular tissue, or blood cells, we performed experiments in primary cortical neurons in culture. Pretreatment with isoflurane 24 hours before OGD reduced the LDH release by 49% compared with untreated controls ($P$=0.002). LDH release measured 48 hours after OGD is presented in Figure 3 as a percentage of the control values. The data were acquired from 3 independent experiments with at least 8 wells per condition.

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### TABLE 3. Physiological Variables During MCAO in Intubated Control and Mechanically Ventilated Halothane-Pretreated Groups

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>Paco$_2$ mm Hg</th>
<th>Paco$_2$ mm Hg</th>
<th>MABP, mm Hg</th>
<th>Glucose, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong> ($n=6$)</td>
<td></td>
<td></td>
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<td>Beginning</td>
<td>7.43±0.05</td>
<td>42.8±6.4</td>
<td>122.1±12.6</td>
<td>97.4±6.2</td>
<td>6.1±2.0</td>
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<tr>
<td>End</td>
<td>7.42±0.06</td>
<td>40.6±10.9</td>
<td>105.5±7.8</td>
<td>90.9±8.0</td>
<td>5.6±1.8</td>
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<td><strong>Halothane ventilation at 24 h</strong> ($n=9$)</td>
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<td>Beginning</td>
<td>7.44±0.05</td>
<td>41.6±6.4</td>
<td>121.1±14.6</td>
<td>94.4±6.2</td>
<td>7.2±2.6</td>
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<td>End</td>
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<td>115.5±10.0</td>
<td>88.1±7.7</td>
<td>6.2±1.9</td>
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<td><strong>Controlled hypotension</strong> ($n=9$)</td>
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<tr>
<td>Beginning</td>
<td>7.43±0.04</td>
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<td>109.8±7.3</td>
<td>82.1±3.6</td>
<td>6.4±1.6</td>
</tr>
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</table>

Values are mean±SD.

*Control animals were intubated and extubated immediately for a short period (~5 min) of halothane anesthesia.

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### TABLE 4. Physiological Variables During MCAO in AG-Treated Groups

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>Paco$_2$ mm Hg</th>
<th>Paco$_2$ mm Hg</th>
<th>MABP, mm Hg</th>
<th>Glucose, mmol/L</th>
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<td>7.41±0.07</td>
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<td>118.7±15.0</td>
<td>90.4±15.0</td>
<td>7.4±2.4</td>
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<tr>
<td>End</td>
<td>7.43±0.03</td>
<td>42.3±5.3</td>
<td>116.4±13.0</td>
<td>83.5±8.3</td>
<td>6.0±1.1</td>
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<tr>
<td><strong>Control + AG</strong> ($n=11$)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>122.3±18.7</td>
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<td>39.6±4.4</td>
<td>113.7±13.1</td>
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Values are mean±SD.
iNOS Is Induced After AP With Isoflurane
Western blot analyses from cerebral cortical homogenates from control animals were performed at 0 hours, 6 hours, 12 hours, 18 hours, 24 hours, 36 hours, and 48 hours after AP with isoflurane (n=1100 for each time point). Densitometric analysis of the Western blot images revealed an almost undetectable iNOS expression in the control cortices and an induction of iNOS protein beginning 6 hours after AP and reaching significant differences at 18 and 24 hours compared with the control. A representative blot and the results of the densitometric analysis are depicted in Figure 4.

Inhibition of iNOS Activity Eliminates the Protection After AP With Isoflurane
The effect of iNOS inhibition during and after the pretreatment period (12 hours) was tested in a third set of in vivo experiments. Administration of 200 mg/kg AG did not affect the infarct volumes in the control animals (206±35 mm³ for NaCl-treated controls versus 213±42 for AG-treated controls). However, in the isoflurane-pretreated group, AG treatment eliminated the infarct-sparing effect of the AP (160±31 mm³ for NaCl-treated AP group versus 206±29 mm³ for the AG-treated AP group, P=0.025) (Figure 5).

Discussion
The goal of the present study was to determine whether the well-described acute neuroprotective effect of volatile anesthetics is maintained even after the termination of anesthesia. We focused on focal cerebral ischemia, because it is one of the most relevant acute disorders of the central nervous system (CNS) and can be modeled faithfully in the rat in vivo. Our results indicate that 3 hours of isoflurane anesthesia affords immediate neuroprotection. The neuroprotective effect of anesthesia is maintained even 12 and 24 hours after the anesthetic has been discontinued. The means of the infarct volumes were decreased by 31%, 35%, and 34% of the control values by a 3-hour period of isoflurane anesthesia at 0 hours, 12 hours, and 24 hours, respectively, before the induction of focal cerebral ischemia. Pretreatment with halothane 24 hours before MCAO also decreased the mean infarct volume by 35% in spontaneously breathing animals.

Methodological Considerations
A few methodological issues deserve attention before the neurobiological implications of the present findings are discussed. Halothane-treated spontaneously breathing animals displayed unphysiological blood gases and mild hypotension during AP. When blood gases were controlled by ventilatory support, halothane pretreatment nevertheless decreased the infarct volume by 28%. In additional control experiments, we showed that hypotension induced 24 hours before MCAO for a certain time period and to levels comparable to the AP group levels had no effect on infarct volumes 24 hours later. Thus, we ruled out the possibility that the systemic physiological effects of volatile anesthetics on blood pressure or blood gases were responsible for the induction of protection against focal cerebral ischemia.

The 3 sets of in vivo experimental groups displayed slight differences in mean infarct volumes (compare Figures 1, 2, and 5). This can be explained by the fact that surgery was conducted by 2 different investigators: the time course of AP was performed by D.L. (data on Figure 1), and the experiments involving ventilatory support, induced hypotension, and AG treatment were performed by K.J.K. (data on Figures

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Effects of anesthetic pretreatment with isoflurane and halothane on infarct volumes 4 days after permanent MCAO. *Significant differences from control.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Infarct volumes in the intubated control, the lowered-blood-pressure control (control+hypotension), and the mechanically ventilated halothane-pretreated groups. *Significant differences from control.
2 and 5). Each series was compared with specific control groups, and no comparisons were made across these sets. Therefore, the observed variability is not relevant for the interpretation of the data; the fact that 2 experimenters found almost identical relative-effect sizes points to the robustness and reproducibility of our data.

Recently, Kawaguchi et al26 in a filament model of focal ischemia in rats found that acute neuroprotection by isoflurane only delays but does not prevent damage: after 2 days of survival, a significant reduction of infarct volumes was observed in the isoflurane group compared with the control group, which was no longer apparent when infarct volumes were quantified after 14 days. We have investigated infarct volumes 4 days after MCAO. Thus, we cannot rule out the possibility that weeks after the induction of ischemia, AP-induced neuroprotection is no longer detectable. Although infarct maturation after 2 days in rat models of stroke has been described in the literature,27 there is no evidence for growth of the ischemic lesion beyond day 4 after MCAO. In addition, even if AP-induced neuroprotection may only delay the damage for days, this would still represent an interesting phenomenon pointing to robust and sustained CNS effects of anesthesia for many days.

### Effects of Volatile Anesthetics on Cerebral Metabolic Rate

Isoflurane is known to reduce the cerebral metabolic rate 28; consequently, local and mean CGU levels are decreased by isoflurane in a dose-dependent fashion. 29,30 Because a reduction of metabolic rate is neuroprotective, 31,32 we wondered whether a prolonged reduction of cerebral metabolism might contribute to the delayed neuroprotection observed here. Therefore, we determined local and mean CGU 24 hours after isoflurane pretreatment, at a time point when robust neuroprotection was present. However, in none of the brain regions evaluated did we find a difference in CGU between control and AP-treated rats. This finding is also consistent with studies involving the acute neuroprotective effects of anesthetics: the degree of acute neuroprotection afforded by anesthetics is not correlated with their effectiveness in reducing the rate of cerebral metabolism.33 Interestingly, we have recently described that metabolic inhibition with the blocker of complex II of the respiratory chain, 3-nitroproprionic acid

### Figure 3

Effect of the pretreatment with isoflurane for a period of 3 hours on LDH release 24 hours after OGD in primary cortical cell cultures. \( ^* P = 0.002 \) vs control (Student unpaired 2-tailed t test).

### Figure 4

A, Western blot analysis of iNOS protein from cerebral cortex of rats at various time points after AP with isoflurane (contr. indicates control). B, Densitometric analysis of the Western blots. Intensity of the bands is expressed in arbitrary units (AU) after subtraction of the background signal of each band signal. \( ^* \)Significant differences from control (co.).

### Figure 5

Effect of AG treatment on the infarct volume in control (Contr.) animals and in animals after AP with isoflurane (Isofl.). AG was administered at the beginning of the AP and 12 hours later. Rats were subjected to MCAO 24 hours after AP. \( ^* \)Significant difference from Contr.
(3-NPA), induces dramatic neuroprotection with a maximum of 72 hours after 3-NPA administration. As early as a few hours after 3-NPA administration (at time points even before delayed neuroprotection was demonstrated), brain ATP levels had completely recovered. Therefore, it is possible that there indeed exists a link between anesthesia-induced reduction of metabolic rate and delayed neuroprotection. However, the reduction of the metabolic rate, if involved at all, is not the effector but rather a trigger of neuroprotection.

Effectiveness of AP in Neuronal Cell Culture
Delayed neuroprotection may be the result of a number of very different effects on various systems or cell types in the brain. Besides reductions in the metabolic rate (see above), sustained or delayed increases of cerebral blood flow in the regions affected by cerebral ischemia or interaction with nonneuronal cells, such as vascular or blood cells, may be neuroprotective. To exclude such effects, we performed experiments in cultures of cortical neurons. Indeed, the results obtained in cell culture are fully consistent with our in vivo data. The release of the intracellular enzyme LDH, a robust marker of lethal cell damage to neurons in vitro, was reduced by 49% by pretreatment with isoflurane 24 hours before OGD. Thus, we conclude that, at least partially, AP-induced tolerance by isoflurane is independent of effects on blood gas values and cerebral blood flow and of interactions with vascular or blood cells.

Acute Versus Delayed Neuroprotection
Numerous explanations for the acute neuroprotective effects of volatile anesthetics have been discussed in the literature. Blockade of ischemia-induced glutamate release, induction of γ-aminobutyric acid type A receptor-associated K+ influx, and inhibition of N-methyl-D-aspartate gated ion channels might all be involved in neuroprotection by anesthetics. However, all the available studies focused on the direct effects of anesthesia and did not investigate the effects many hours after anesthesia. Because volatile anesthetics, and especially isoflurane, are poorly metabolized and rapidly washed out of the organism, it is very likely that the delayed effects (>12 hours after administration) reported in the present study do not involve the presence of the agent. This conclusion is further strengthened by the fact that isoflurane also induces delayed neuroprotection in neuronal cultures. In cell culture, the washout of compounds via the gas space and multiple medium changes is greatly facilitated by the very large ratio of extracellular to intracellular space.

It is interesting to note that AP differs from other previously reported models of ischemic preconditioning. In other models of induced tolerance to ischemia, there is a time window in which there is no protection against ischemia: 12 to 14 hours between the early phase of induced tolerance (which occurs within minutes and subsides within hours) and the delayed phase, which is manifested after 24 hours (and is maintained for days). We believe that the continuum of protection of AP is mediated by various mechanisms that overlap in time to produce the sustained level of protection. This is also suggested by the iNOS expression data in the present study, inasmuch as iNOS expression increases only 6 hours after AP, whereas protection was already present immediately after AP. Therefore, the early phase of protection cannot be attributed to iNOS but is more likely due to the acute effect of anesthesia, as discussed below.

Volatile Anesthetics Induce Long-Lasting Changes in Gene Expression
Surprisingly little attention has been paid so far to the effects of volatile anesthetics on gene expression. The few studies available focused on the members of the IEG family of transcription factors, especially on c-fos. Induction of c-fos and jun-B in the rat brain after pentobarbital and halothane anesthesia has been demonstrated by Marota et al. In an immunohistochemical study, Takayama et al demonstrated the induction of c-fos expression as its protein product by various anesthetic agents. The observed changes in gene expression imply that some phenotypical alterations may remain even after cessation of the anesthetic effect. Identification of target genes that are controlled by IEGs might reveal the molecular mechanisms underlying the late effect of volatile anesthetic treatment. Positive findings in the literature linking iNOS or NO to prolonged protection against ischemia tempted us to select iNOS as a potential candidate for transmitting AP. We found a significant induction of iNOS protein expression in the cerebral cortex, and inhibition of iNOS abolished the effect of AP. The possibility that NO is critically involved in protective signaling in the brain is supported by several other reports. NO was found to be involved in the function of the p21WAF1/extracellular signal-regulated kinase cascade, which, in turn, was found to be necessary for ischemic tolerance induction in neuronal cell culture. NO suppresses nuclear factor-κB–mediated gene expression. Furthermore, nitroxylin anion (NO−) has been shown to interact with the NR2A subunit of the N-methyl-D-aspartate receptor, with a subsequent reduction of Ca2+ influx. NO− can be formed from NO by interfering with redox metal–containing proteins in physiological systems. Thus, involvement of iNOS or NO in AP is likely, but further investigation is needed to elucidate the complete pathway.

Clinical Implications
The induction of tolerance (preconditioning) against cerebral ischemia in rodents is possible by numerous stimuli: hypoxia (neonatal rats), global and focal cerebral ischemia itself, treatment with metabolic inhibitors, pretreatment with lipopolysaccharide, and (among others) volatile anesthetics, as described in the present study. However, the question remains: can tolerance also be induced in the human brain? We have recently described evidence for the existence of induced tolerance in the human brain by short ischemic intervals (transient ischemic attacks). Thus, it is tempting to speculate that anesthetics may be used to induce neuroprotection in cases in which damage to the CNS is anticipated (eg, neurosurgery and cardiac surgery and delayed vasospasm after subarachnoid hemorrhage). AP may be a clinically existent, yet unrecognized, entity in cases of repeated general anesthesia with an interval of 12 to 24 hours. In any case, our observations point to up-to-now undescribed prolonged CNS effects of volatile anesthetics, which may account not only for...
protective effects but also for unwanted delayed effects of general anesthesia, such as the frequent phenomenon of perioperative CNS dysfunction.

In summary, in the present study we describe for the first time evidence of a prolonged neuroprotective effect of isoflurane or halothane anesthesia. Although the exact mechanisms underlying this newly described type of ischemic tolerance are unknown, they do not seem to be primarily related to cerebral blood flow or metabolism changes. According to our data, the involvement of iNOS in AP is very likely, but elucidation of the underlying signaling cascades certainly requires further study. AP may have as-yet-unrecognized clinical as well as experimental implications when anesthesia with volatile anesthetics is repetitively applied.

Acknowledgments
This study was supported by the Hermann and Lilly Schilling Foundation and the Deutsche Forschungsgemeinschaft.

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Stroke. 2002;33:1889-1898
doi: 10.1161/01.STR.0000020092.41820.58

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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