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Isoflurane Provides Long-term Protection against Focal Cerebral Ischemia in the Rat

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Background: **Long-term neuroprotection by isoflurane has been questioned. The authors examined factors in experimental models potentially critical to definition of enduring isoflurane neuroprotection.**

Methods: **Rats were prepared for temporary middle cerebral artery occlusion (MCAO). Pericranial normothermia was maintained. Neurologic deficits (range, 0–48; 0 = no deficit) and cerebral infarct volumes were measured. In experiment 1, rats underwent 50 or 80 min MCAO while awake or anesthetized with 1.8% isoflurane. Blood pressure was controlled with phenylephrine. Outcome was evaluated 2 weeks later. In experiment 2, rats underwent 50 min MCAO while awake or anesthetized with isoflurane, with outcome evaluated 8 weeks later. In experiment 3, rats underwent 50 min MCAO while awake or anesthetized with isoflurane and 2 weeks recovery. Effects of phenylephrine and the mitochondrial adenosine triphosphate–sensitive K^+ channel antagonist 5-hydroxydecanoate were studied. In experiment 4, isoflurane-anesthetized rats underwent 50 min MCAO with permanent or temporary common carotid artery occlusion, with outcome evaluated 2 weeks later.**

Results: **In experiment 1, isoflurane reduced neurologic deficit (median ± interquartile range; awake *vs.* isoflurane: 11 ± 12 *vs.* 8 ± 6 for 80 min and 13 ± 4 *vs.* 3 ± 9 for 50 min; $P = 0.0006$) and infarct size (160 ± 97 *vs.* 84 ± 62 mm^3 for 80 min and 169 ± 78 *vs.* 68 ± 61 mm^3 for 50 min; $P < 0.0001$). In experiment 2, isoflurane protection persisted at 8 weeks after ischemia. In experiment 3, there was no effect of phenylephrine or 5-hydroxydecanoate. In experiment 4, permanent common carotid ligation increased infarct size threefold *versus* temporary occlusion.**

Conclusions: **Isoflurane repeatedly improved long-term neurologic and histologic outcome from focal ischemia independent of ischemia duration, perfusion pressure, or pretreatment with 5-hydroxydecanoate.**

VOLATILE anesthetics are used in countless surgical procedures, some of which present substantial risk of ischemic–hypoxic injuries to the central nervous system. Because volatile anesthetics have profound pharmacologic effects on neural tissue, it can be postulated that these same drugs present potential interactions with pathologic responses to energy deprivation.

There is a long history of investigations designed to assess the impact of anesthetic agents on ischemic outcome.[1–7] Results have been discrepant and are likely attributable to both physiologic effects of anesthetics, which may or may not have been controlled, and to differences in experimental models used to test this question. Recently, the preponderance of studies has identified a favorable effect of volatile anesthetics on ischemic brain.[5–7] However, study of volatile anesthetics and other purported neuroprotective strategies have indicated that neuroprotection may at best be transient.[8–13] That is, although neuroprotective strategies slow the rate of postischemic cell death, over time, the final outcome becomes similar to tissue that underwent an ischemic injury and recovery in the absence of therapeutic intervention.

Definition of the permanence of volatile anesthetic neuroprotection is important for several reasons. First, if such intervention does not provide sustained protection, it likely has little clinical value. Second, understanding the long-term effect of therapeutic intervention is essential in defining the mechanism by which such protection is afforded. Understanding the mechanism of action can serve to define the limits of pharmacologic neuroprotection and also to provide insight into development of compounds that might selectively target critical pathways stimulated by ischemia offering greater potency with fewer side effects.

In the following studies, we first hypothesized that volatile anesthetics provide neuroprotection, but that the persistence of improved outcome is dependent on the duration of the ischemic injury.[14,15] Upon finding persistent protection by isoflurane against temporary focal ischemia, we probed potential methodologic factors that might explain the discrepancy between our findings and those of previous investigations. Finally, after observing long-term protection from isoflurane in this model, we tested the hypothesis that this protection could be accounted for by interactions between isoflurane and mitochondrial K^+ adenosine triphosphate (ATP) channels, which have previously been shown to play an important role in isoflurane preconditioning.[16]

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Materials and Methods

The following studies were approved by the Duke University Animal Care and Use Committee, Durham, North Carolina. Male Wistar rats (10 weeks of age; Harlan Sprague-Dawley, Indianapolis, IN) were housed in a temperature-controlled environment with an artificial light–dark cycle (12 h). They were fasted from food but allowed free access to water for 12 h before ischemia.

MCAO Procedure

Rats were anesthetized in a chamber with 5% isoflurane in 100% oxygen. The trachea was intubated, and the lungs were mechanically ventilated (40% oxygen–balance nitrogen). The inspired isoflurane concentration was reduced to 1.5–2%, and animals were prepared for middle cerebral artery occlusion (MCAO) using modifications of the techniques described by Memezawa *et al.*[17] and Longa *et al.*[18] Surgery was performed with aseptic technique, and all surgical fields were infiltrated with 0.25% bupivacaine.

The tail artery was cannulated and used to monitor mean arterial blood pressure (MAP) and sample blood. The tail vein was cannulated for drug infusion. A calibrated flexible thermistor was percutaneously implanted beneath the right temporal muscle adjacent to the skull and sutured in place. A silicon harness (Covance Infusion Harness, CIH95; Instech Laboratories, Inc., Plymouth Meeting, PA) was positioned on the rat's torso. The thermistor wire was connected to the harness, which was then passed through a flexible coil attached to both the harness and a swivel commutator (model SL2C 2 channel; Plastic Ones, Roanoke, VA). Pericranial temperature was continuously recorded and servo-controlled (YSI model 73ATA; YSI Inc., Yellow Springs, OH) by surface heating and cooling (heating lamp or fan, respectively) to maintain pericranial temperature at 37.5° ± 0.2°C from surgery onset until indicated by experimental protocol.

A midline ventral cervical skin incision was made, and the right common carotid artery was identified. The external carotid artery was isolated, ligated, and divided. The internal carotid artery was dissected distally until the origin of the pterygopalatine artery was visualized. After surgical preparation, a 30-min interval was allowed for physiologic stabilization. Heparin (50 U) was given intravenously. To achieve MCAO, a 0.25-mm-diameter nylon filament coated with silicon (0.38 mm diameter) was inserted into the external carotid artery stump and advanced 19–20 mm from the carotid artery bifurcation into the internal carotid artery until a slight resistance was felt. After the intended MCAO duration, the filament and intravascular catheters were removed. The wounds were closed, isoflurane was discontinued, the rats were allowed to awaken, and the trachea was extubated. Arterial carbon dioxide and oxygen partial pressures and arterial pH were measured 15 min before and 25 min after MCAO onset. Hematocrit and blood glucose concentration were determined 15 min before MCAO onset. MAP was continuously monitored until 15 min after reperfusion.

Measurement of Neurologic Outcome

We used a previously described scoring system that evaluates general status, simple motor deficit, complex motor deficit, and sensory deficit.[19] The score given to each animal at the completion of the testing was the sum of the four individual scores, with 0 being the minimum (best) score and 48 the maximum possible (worst) score. The same experienced observer, who was blinded to group assignment, assigned all scores.

Measurement of Cerebral Infarct Size

After neurologic evaluation, animals were weighed, deeply anesthetized with isoflurane, and decapitated. The brains were removed, snap frozen at −20°C in 2-methylbutane, and stored at −80°C for later analysis. Infarct volume was measured by using the method of Swanson *et al.*[20] Serial quadruplicate 20-μm-thick coronal sections were taken by using a cryotome at 720-μm intervals over the rostral–caudal extent of the infarct. The sections were dried and stained with hematoxylin and eosin. A representative section from each 720-μm interval was digitized with a video camera controlled by an image analyzer (MCID Elite; Interfocus Imaging, Linton, England). The image of each section was stored as a 1,280 × 960-pixel matrix and displayed on a video monitor. With the observer blinded to experimental conditions, the following regions of interest were cursor-outlined: noninfarcted ipsilateral cerebral cortex, noninfarcted ipsilateral subcortex, contralateral cerebral cortex, and contralateral subcortex. The area within each region of interest (mm^2) was determined by automated counting of the calibrated pixels contained within the region of interest. Ipsilateral noninfarcted cortex and subcortex areas were subtracted from the corresponding contralateral region of interest values. Infarct volumes (mm^3) were computed as running sums of subtracted infarct area multiplied by the known interval (*e.g.*, 720 μm) between sections over the rostral–caudal extent of the infarct calculated as an orthogonal projection.

Experiment 1: Effects of MCAO Duration on Isoflurane Protection

This experiment examined the dependence of sustained isoflurane neuroprotection on MCAO duration. Rats were assigned randomly to undergo either 50 or 80 min MCAO while anesthetized with isoflurane or while "awake" (n = 15 rats per condition).

For the "awake" groups, isoflurane was abruptly discontinued at MCAO onset. The wound was closed dur-

ing anesthesia emergence, and the trachea was extubated. The rat, along with the tethered thermistor assembly, was placed in a 6-l transparent plastic animal enclosure, with the fraction of inspired oxygen being 40%. This system allowed continuous observation, thermoregulation, and monitoring of MAP. Seven minutes before the end of MCAO, isoflurane was introduced into the chamber. The anesthetized rat was removed, the filament was withdrawn to allow reperfusion after either 50 or 80 min MCAO, and intravascular catheters were removed. Anesthesia was discontinued, and the rat was placed in a 20-l transparent plastic animal enclosure. Pericranial temperature was continuously recorded and servo-regulated at 37.5° ± 0.2°C for the next 20 h using automated surface heating and cooling. Animals breathed supplemental oxygen in room air for the first 1 h after reperfusion and then room air for 19 h with free access to food and water. After 20 h, the animals were removed from the enclosure and briefly anesthetized with isoflurane to remove the harness and thermistor. The animals were returned to their cages for further recovery.

In the isoflurane groups, end-tidal isoflurane concentration was adjusted to 1.8% (1.5 minimum alveolar concentration)[21] 30 min before MCAO onset and maintained throughout MCAO. Pilot studies had been performed that indicated that rats anesthetized with 1.8% isoflurane typically have a MAP approximately 20–25 mmHg less than those subjected to MCAO while "awake." Therefore, phenylephrine (0–15 $\mu g \cdot kg^{-1} \cdot min^{-1}$) was infused *via* the tail vein catheter throughout MCAO to provide MAP values similar to those observed in the "awake" group, which received a similar volume of vehicle (0.9% NaCl). After either 50 or 80 min MCAO, the filament was withdrawn, and rats were subsequently treated as described for the "awake" groups.

All rats in this experiment were allowed to survive for 2 weeks after MCAO, after which neurologic function and cerebral infarct size were measured.

Experiment 2: Effects of Isoflurane on 8-Week MCAO Outcome

Experiment 1 was precisely replicated with two exceptions. Only two groups were studied. These rats were subjected to 50 min MCAO after random assignment to either 1.8% isoflurane (n = 15) or "awake" (n = 15) groups. All rats were allowed to survive for 8 weeks after MCAO, after which neurologic function and cerebral infarct size were measured.

Experiment 3: Effects of Intraischemic Phenylephrine Infusion and a Mitochondrial ATP-sensitive K^+ Channel Antagonist on MCAO Outcome

The 50-min MCAO protocol described in experiment 1 was replicated with random assignment to the following treatment groups (n = 14–15 rats per group): (1) "awake," (2) 1.8% isoflurane with phenylephrine infusion, (3) 1.8% isoflurane without phenylephrine infusion, (4) "awake" plus 30 mg/kg intraperitoneal 5-hydroxydecanoic acid (Sigma-Aldrich, Inc., St. Louis, MO) 30 min before MCAO induction, or 5) 1.8% isoflurane with phenylephrine infusion plus 30 mg/kg intraperitoneal 5-hydroxydecanoic acid. Blood pressure was monitored from the tail artery catheter throughout the ischemic injury and recorded at 5-min intervals. Rats were allowed to survive for 2 weeks after MCAO, after which neurologic function and cerebral infarct size were measured.

Experiment 4: Effects of Common Carotid Occlusion on MCAO Outcome

This experiment followed the same physiologic monitoring and control protocol described for in experiment 1, with two exceptions: (1) pericranial temperature was controlled during ischemia but only for the first 30 min after reperfusion onset, and (2) phenylephrine was not used to support MAP. Rats were assigned randomly to two groups (n = 15 for each). In both groups, rats were anesthetized with 1.8% isoflurane during 50 min MCAO. In one group, the right common carotid artery was occluded only during filament insertion and removal. In the other group, the right common carotid artery was permanently ligated just before commencing MCAO. Rats were allowed to survive for 2 weeks after MCAO, after which neurologic function and cerebral infarct size were measured.

Statistical Analysis

Two-way analysis of variance was used to compare groups in experiment 1 using anesthetic condition ("awake" *vs.* isoflurane) and MCAO duration (50 *vs.* 80 min) as independent factors for cerebral infarct volume. Physiologic data were compared qualitatively to preserve statistical power. For experiments 2 and 4, cerebral infarct volumes were compared with the Student *t* test. For experiment 3, cerebral infarct volumes were compared with one-way analysis of variance. Planned *post hoc* comparisons for neurologic score and infarct sizes were (1) "awake" *versus* isoflurane, (2) isoflurane *versus* isoflurane with phenylephrine, (3) "awake" *versus* "awake" plus hydroxydecanoate, and (4) isoflurane with phenylephrine without hydroxydecanoate *versus* isoflurane with phenylephrine with hydroxydecanoate. MAP was compared among groups with repeated-measures analysis of variance. Neurologic scores were compared in all experiments using nonparametric tests (Kruskal–Wallis H statistic and Mann–Whitney U statistic where appropriate). Statistical significance was accepted when $P \leq 0.05$ in all cases except experiment 3 *post hoc* analysis when a Bonferroni correction was applied ($P < 0.0125$) for cerebral infarct volumes. Parametric values

Table 1. Physiologic Values for Experiment 1

	80 min Awake	80 min Isoflurane	50 min Awake	50 min Isoflurane
Preischemia				
Body weight, g	305 ± 13	304 ± 11	302 ± 10	303 ± 9
MAP, mmHg	81 ± 19	83 ± 17	87 ± 17	92 ± 11
Hematocrit, %	41 ± 2	41 ± 2	41 ± 2	40 ± 1
Glucose, g/dl	81 ± 11	84 ± 12	78 ± 12	79 ± 10
Arterial pH	7.43 ± 0.02	7.43 ± 0.02	7.43 ± 0.03	7.42 ± 0.03
$Paco_2$, mmHg	36 ± 3	35 ± 4	35 ± 5	37 ± 5
Pao_2, mmHg	137 ± 21	132 ± 19	129 ± 17	134 ± 24
Intraischemia				
MAP, mmHg				
15 min	118 ± 11	114 ± 7	119 ± 8	116 ± 6
25 min	113 ± 11	113 ± 6	117 ± 7	116 ± 9
35 min	113 ± 11	113 ± 8	115 ± 6	115 ± 8
65 min	116 ± 9	111 ± 5	—	—
Arterial pH, mmHg	7.46 ± 0.02	7.43 ± 0.02	7.47 ± 0.03	7.42 ± 0.03
$Paco_2$, mmHg	33 ± 4	35 ± 4	32 ± 3	36 ± 3
Pao_2, mmHg	170 ± 28	168 ± 22	160 ± 22	158 ± 29
Pericranial temperature, °C	37.5 ± 0.1	37.5 ± 0.1	37.5 ± 0.1	37.5 ± 0.1
Postischemia				
Pericranial temperature, °C				
0 h	37.3 ± 0.2	37.4 ± 0.1	37.4 ± 0.1	37.4 ± 0.1
4 h	37.5 ± 0.1	37.5 ± 0.1	37.5 ± 0.1	37.5 ± 0.1
8 h	37.5 ± 0.1	37.4 ± 0.1	37.5 ± 0.1	37.5 ± 0.1
12 h	37.5 ± 0.1	37.4 ± 0.1	37.5 ± 0.1	37.4 ± 0.1
16 h	37.5 ± 0.1	37.4 ± 0.1	37.5 ± 0.1	37.5 ± 0.1
20 h	37.4 ± 0.1	37.4 ± 0.1	37.4 ± 0.1	37.4 ± 0.1

Values are mean ± SD.

MAP = mean arterial pressure; $Paco_2$ = arterial blood carbon dioxide partial pressure; Pao_2 = arterial blood oxygen partial pressure.

are presented as mean ± SD. Nonparametric values are presented as median ± interquartile range.

Results

Experiment 1

Physiologic values are presented in table 1. There were no physiologically relevant differences among groups for all monitored variables. As intended, pericranial temperature was maintained within 37.5° ± 0.2°C during MCAO and the entire 20-h recovery interval.

Isoflurane anesthesia improved 2-week neurologic function ($P = 0.0006$), but there was no effect of MCAO duration on this variable ($P = 0.64$; fig. 1). Isoflurane decreased total infarct volume at 14 days after ischemia ($P < 0.0001$; fig. 1). This protection was present in both cortical ($P = 0.0001$) and subcortical structures ($P = 0.0006$; fig. 2). However, there was no effect of ischemia duration on cortical ($P = 0.97$), subcortical ($P = 0.63$), or total infarct size ($P = 0.81$; fig. 1), and there was no interaction between factors ($P = 0.48$).

Experiment 2

Physiologic values were similar to those reported in experiment 1 and thus are not reported. After 8 weeks' recovery, rats anesthetized with isoflurane during MCAO exhibited reduced neurologic deficit ($P = 0.05$) and smaller total infarct sizes ($P = 0.03$) than those allowed

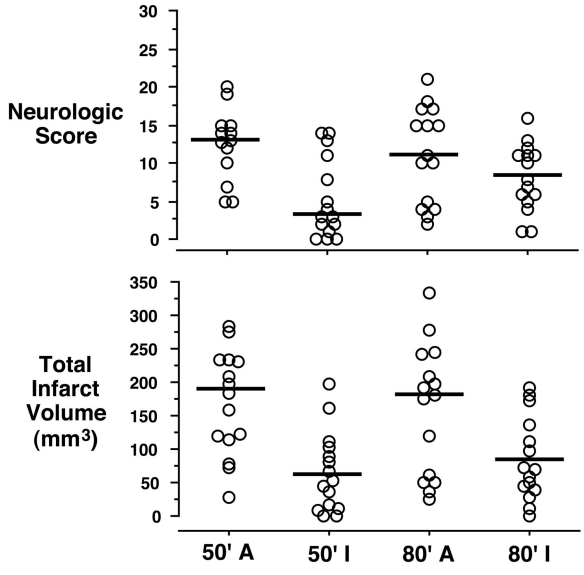

Fig. 1. Effects of anesthetic state and middle cerebral artery occlusion duration on 14-day neurologic score (0–48 point scale; 0 = no deficit) and total infarct volume. *Open circles* indicate values for individual animals. *Horizontal bars* indicate group median values for neurologic scores and mean values for infarct volumes. Isoflurane reduced neurologic deficit ($P = 0.0006$) and total infarct size ($P < 0.0001$). There was no effect of middle cerebral artery occlusion duration on either outcome variable. 50′ A and 50′ I indicate 50 min of middle cerebral artery occlusion with rats maintained "awake" or anesthetized with 1.8% isoflurane, respectively. 80′ A and 80′ I indicate 80 min of middle cerebral artery occlusion with rats maintained "awake" or anesthetized with 1.8% isoflurane, respectively.

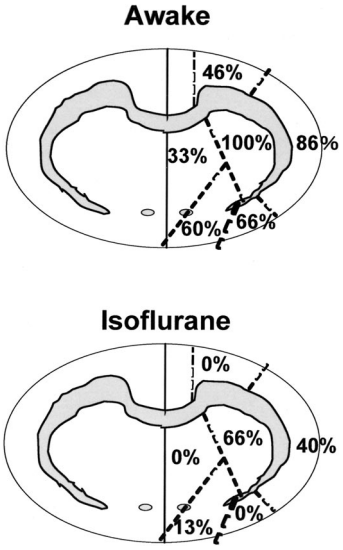

Fig. 2. Coronal sections (bregma + 0.70 mm) were analyzed for presence of infarcted tissue in sectors of the neocortex and subcortex. All rats subjected to 50 min of middle cerebral artery occlusion in experiment 1 underwent this analysis. Values presented indicate the fraction of the total group (n = 15 per condition) in which infarct was evident in the respective sectors. A marked reduction in the incidence of infarct was present throughout the hemisphere ipsilateral to middle cerebral artery occlusion.

to awaken during the ischemic injury (fig. 3). Similar effects were present in both cortex ($P = 0.03$) and subcortex ($P = 0.02$). Figure 4 depicts the relation between total infarct volume and neurologic score.

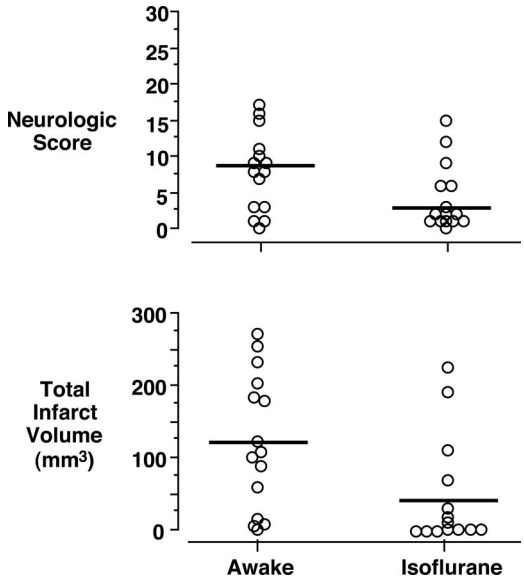

Fig. 3. Rats (n = 15 per group) were subjected to 50 min of middle cerebral artery occlusion while anesthetized with isoflurane or "awake" and allowed to recover for 8 weeks. Both neurologic deficit ($P = 0.05$) and total infarct size ($P = 0.03$) were reduced in the isoflurane group. *Open circles* indicate values for individual animals. *Horizontal bars* indicate group median values for neurologic scores and mean values for infarct volumes.

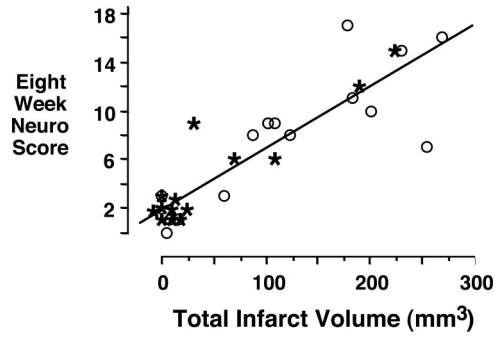

Fig. 4. Scatter plot of individual rat values for total cerebral infarct volume and neurologic score measured 8 weeks after recovery from 50 min of middle cerebral artery occlusion. *Open circles* indicate rats allowed to emerge from anesthesia during middle cerebral artery occlusion. *Stars* indicate animals that remained anesthetized with 1.8% isoflurane throughout middle cerebral artery occlusion. A *best-fit line* is provided indicating the relation between cerebral infarct size and neurologic score (neurologic score = −10.097 + 15.418 × total infarct volume; $R^2 = 0.78$).

Experiment 3

Physiologic values were similar to those reported in experiment 1 with the exception of MAP in isoflurane-anesthetized rats left untreated with phenylephrine (fig. 5). Isoflurane alone (2 ± 4) improved neurologic score relative to the "awake" group (9 ± 7; $P = 0.002$). There was no difference between isoflurane-anesthetized rats with (2 ± 7) or without phenylephrine (2 ± 4; $P = 0.82$). There also was no effect of 5-hydroxydecanoic acid in either the awake group (10 ± 13; $P = 0.89$) or the isoflurane group (3 ± 9; $P = 0.82$). There was a main effect for treatment group on total infarct volume ($P =$

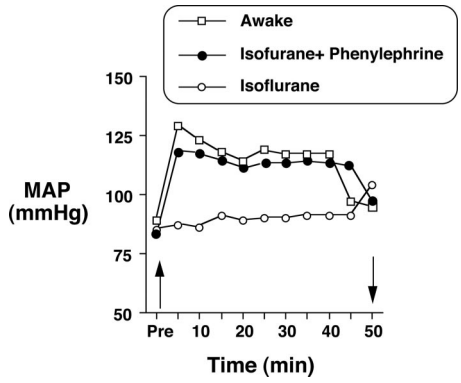

Fig. 5. Mean arterial blood pressure values (MAP) derived from three groups in experiment 2. Rats were subjected to 50 min of middle cerebral artery occlusion. In the "awake" group, isoflurane was discontinued at onset of ischemia and reintroduced just before onset of reperfusion. In the isoflurane group, 1.8% isoflurane was continued throughout middle cerebral artery occlusion. In a second group of isoflurane-anesthetized rats, phenylephrine was infused intravenously throughout the ischemic injury to maintain MAP values similar to those of the "awake" group. There was no difference between the "awake" and isoflurane with phenylephrine groups ($P = 0.13$). However, MAP values in the isoflurane group were lower in the isoflurane group *versus* either the "awake" group or the isoflurane with phenylephrine group ($P < 0.0001$). *Up-going* and *down-going arrows* indicate onset and offset of middle cerebral artery occlusion, respectively. Values are group means.

0.0009). As in experiment 1, isoflurane with phenylephrine (44 ± 56 mm^3) reduced total infarct volume compared with the "awake" group (113 ± 67 mm^3; P = 0.01). There was no difference between isoflurane-anesthetized rats with (44 ± 56 mm^3) or without phenylephrine (52 ± 50 mm^3; P = 0.67). The mitochondrial ATP–sensitive K^+ channel antagonist 5-hydroxydecanoic acid had no effect on neurologic score in either the "awake" group (10 ± 13; P = 0.89) or the isoflurane plus phenylephrine group (3 ± 9; P = 0.82). Similarly, 5-hydroxydecanoic acid had no effect on total infarct volume in either the "awake" group (125 ± 79 mm^3; P = 0.66) or the isoflurane plus phenylephrine group (56 ± 50 mm^3; P = 0.55).

Experiment 4

Physiologic values were similar to those reported in experiment 1 and thus are not reported. Permanent common carotid artery ligation enlarged total infarct size relative to rats in which it was only temporarily occluded during filament insertion and removal (76 ± 62 *vs.* 26 ± 32 mm^3 respectively; P = 0.01) but had no effect on neurologic score (3 ± 4 *vs.* 2 ± 4; P = 0.15).

Discussion

Answering the question as to whether volatile anesthetics are neuroprotective has faced two challenges. First, physiologic effects of volatile anesthetics (*e.g.*, effects on blood glucose, hemodynamics, and thermoregulation) might serve to confound comparison against control groups. Second, because induction of an experimental ischemic injury necessarily incurs use of surgical anesthesia, it has been difficult to propose an appropriate control state against which volatile anesthetic efficacy can be measured. Consequently, early studies investigating volatile anesthetic efficacy provided discrepant results.[2,3,22–24]

It has become evident that minor changes in brain temperature during ischemia can have profound effects on ischemic outcome. Severe hippocampal CA1 damage resulting from profound forebrain ischemia is nearly abolished by reducing brain temperature only 2°–3°C.[25] In contrast, focal ischemic cerebral infarct size can be nearly tripled by simply increasing brain temperature 1.2°C.[26] Further, mild postischemic hyperthermia having onset even 24 h after reperfusion from an ischemic injury can appreciably worsen final damage.[27] The clinical relevance of these findings has been indicated by the observation that acute brain injury frequently invokes thermal disregulation,[28] which has been correlated with worsened stroke outcome.[29] Consequently, meaningful assessment of therapeutic interventions, intended to protect the brain during an ischemic injury, must be isolated from potential impacts on brain temperature over extended intervals. This reality led to a series of studies performed in different laboratories that provided consistent results regarding volatile anesthetic neuroprotective efficacy. When brain temperature was controlled during ischemia, halothane, isoflurane, desflurane, and sevoflurane provided substantial neuroprotection.[5,7,30,31]

However, it has become evident that another factor must be considered when examining outcome from cerebral ischemia. Du *et al.*[32] subjected isoflurane-anesthetized rats to either 30 or 90 min MCAO. Cerebral infarct size was assessed at either 24 h or 2 weeks after ischemia. Whereas 30 min MCAO resulted in substantially smaller infarcts at 24 h after ischemia, infarct sizes were the same, regardless of MCAO duration, if outcome was measured 2 weeks after ischemia. This indicates that a slow (and likely apoptotic) cell death can persist for several weeks after MCAO. Therefore, acute evaluation of infarct size does not reliably predict final outcome. It also has become evident that acute evaluation does not predict therapeutic efficacy. This was most saliently demonstrated regarding effects of therapeutic postischemic hypothermia. Work using only brief periods of induced mild hypothermia found marked protection with acute observation intervals, but not when outcome was assessed weeks later.[8] In contrast, prolonged postischemic induced hypothermia was found to provide sustained protection leading to the two successful human trials of induced hypothermia used after restoration of spontaneous circulation subsequent to out-of-hospital ventricular fibrillation cardiac arrest.[33–35]

Given these considerations, the issue of volatile anesthetic neuroprotection has been revisited using long-term recovery models of brain ischemia. Kawaguchi *et al.*[10] replicated the experimental concept of Du *et al.*[32] Rats were anesthetized with isoflurane or awakened during 70 min MCAO. Outcome was assessed either 2 days or 14 days after MCAO. As was observed by Du *et al.*[32], the postischemic observation interval was critical in defining efficacy. At 2 days, isoflurane appeared highly protective, whereas at 14 days, no difference between isoflurane and awake groups was observed. Similarly, Elsersy *et al.*[11] subjected rats to a severe forebrain ischemic injury when anesthetized with isoflurane or nitrous oxide–fentanyl. At 5 days after ischemia, isoflurane rats had substantially more living cells in the selectively vulnerable hippocampal CA1, whereas at 3 weeks and 3 months after ischemia, there was no difference between groups. These results are consistent with another report in which both a glutamate and an N-type calcium channel antagonist only served to delay eventual CA1 demise.[9] These findings suggest that volatile anesthetics cause only transient protection. It would follow that when long-term outcome is assessed, volatile anesthetics, as a sole neuroprotectants, have little value.

Evidence to the contrary has emerged. Sullivan *et al.*[36] studied rat organotypic hippocampal slices that were

either treated or untreated with isoflurane during an episode of oxygen–glucose deprivation. Isoflurane-treated slices had substantially less damage than untreated slices independent of observation interval (2–14 days after oxygen–glucose deprivation). In an *in vivo* experiment, Pape *et al.*[31] subjected rats to hemispheric ischemia while anesthetized with 2% sevoflurane or fentanyl–nitrous oxide. In the sevoflurane group, histologic damage was markedly reduced, even when measured 28 days after ischemia.

A possible explanation for the discrepant results is that volatile anesthetic efficacy is dependent on both the type of injury and injury severity. It was thus speculated that there may be a limit to volatile anesthetic neuroprotection, with sustained protection available only against mild injuries.[14,15]

We endeavored to test this hypothesis in experiment 1. Rats were subjected to 50 *versus* 80 min ischemia while anesthetized with isoflurane or while "awake." A duration of 80 min was accepted as intermediate between that of Du *et al.*[32] and Kawaguchi *et al.,*[10] reflecting two protocols that demonstrated delayed postischemic demise. Pilot studies also found that MCAO intervals less than 50 min provided an inconsistent incidence of cerebral infarction. More important, we believed that the necessities to both emerge from and reinduce anesthesia during MCAO in the "awake" groups smeared the concept of a true awake condition. Approximately 10–15 min of the MCAO interval in fact occurs in the face of substantial isoflurane concentrations, as defined by absence of the righting reflex. Thus, we accepted that 50 min was the minimum MCAO duration that would allow a substantive difference between groups with respect to anesthetic state, and this therefore became the minimum MCAO duration examined.

Experiment 1 provided two findings. First, regardless of MCAO duration, isoflurane-anesthetized rats had markedly better 2-week outcomes than did the "awake" group. This is inconsistent with the findings of Kawaguchi *et al.*[10] Second, there was no effect of ischemia duration on MCAO size in the "awake" group. This may indicate a limitation of the ischemia model, *i.e.*, with 50 min MCAO we may have exceeded the ischemia duration threshold necessary to cause eventual infarction in the same volume of tissue infarcted by 80 min MCAO. Alternatively, because we examined outcome at 2 weeks after MCAO, we may have failed to observe the slower infarct maturation associated with a mild injury as suggested by Du *et al.*[32] Regardless of mechanism, we believe that we have identified the limits of our model in testing isoflurane efficacy, and under those circumstances, it provided substantial and sustained protection.

Ongoing work in our laboratory has allowed us to develop the husbandry skills necessary to extend the postischemic survival interval to 8 weeks. We next considered the possibility that 14 days was too short an outcome interval to allow dissipation of the isoflurane protection observed at 14 days. However, even after 8 weeks' recovery in experiment 2, isoflurane-treated rats retained evidence of major improvement in outcome. Therefore, the protective effect is robust and sustained over an interval that might hold human relevance.

That left us with the issue of resolving the discrepancy between our data and those of Kawaguchi *et al.*[10] It is important to note that their work has been replicated twice and must be considered real.[12,13] Comparison of our model and that of Kawaguchi *et al.*[10] found several methodologic differences, two of which we explored. Kawaguchi *et al.*[10] did not control MAP in their isoflurane-anesthetized group. They reported, and our pilot studies supported the fact, that intraischemic MAP is approximately 25 mmHg less in anesthetized *versus* "awake" rats. Perhaps the fact that we controlled MAP in our isoflurane group with phenylephrine accounted for the different findings. To explore this, experiment 3 was performed. Despite substantial MAP differences between isoflurane-anesthetized rats treated or untreated with phenylephrine, both groups had similar 14-day outcomes, and these outcomes were both substantively better than those awakened during MCAO. Therefore, we could not associate differences in findings to MAP or use of phenylephrine.

The filament MCAO model requires surgical dissection of the carotid artery and bisection of the external carotid artery to provide access to the internal carotid lumen. This procedure is facilitated by occlusion of the ipsilateral common carotid artery. A difference between our procedure and that of Kawaguchi *et al.*[10] was the duration of common carotid occlusion. Kawaguchi *et al.*[10] reported that the artery was permanently ligated. In contrast, our practice is to occlude the artery only during filament insertion and removal. We speculated that permanent common carotid occlusion would present ischemic stress persisting beyond MCAO and the isoflurane exposure interval. One would not expect protection from isoflurane against this ongoing ischemic stress. If so, rats subjected to MCAO and permanent carotid occlusion should develop larger infarcts than those for which the common carotid is perfused during the 14-day recovery interval. The data from experiment 4 support this. Total infarct size at 14 days was approximately threefold larger in those rats with permanent common carotid ligation. However, this was not reflected in neurologic scores, perhaps indicating reduced sensitivity of our neurologic score in detecting deficits associated with the relatively small infarcts present in both groups. Further research examining blood flow patterns during subacute recovery in groups with and without permanent common carotid artery occlusion would be of interest. There is a substantial body of evidence that chronic hypoperfusion results in ongoing cell loss and neurologic deficit when examined over extended outcome intervals.[37] If persistent ischemia were observed with permanent carotid artery

occlusion, it would help explain the discrepancy between the two experimental models.

Finally, we explored the potential role of mitochondrial K^+-ATP channels in the mechanism of isoflurane neuroprotection. This notion was derived from literature reporting that the mitochondrial K^+-ATP channel plays an important role in both ischemic and isoflurane preconditioning in rat brain.[16,38] Mitochondrial K^+-ATP channel blockers inhibit both forms of preconditioning. We speculated that this interaction with isoflurane pharmacology might also be important as a neuroprotective mechanism. Using dosing strategies for 5-hydroxydecanoic acid, a selective mitochondrial K^+-ATP channel blocker, previously reported to inhibit preconditioning,[38,39] we found no effect on isoflurane protection. These data therefore do not support the mitochondrial K^+-ATP channel as a critical site of isoflurane's neuroprotective mechanism of action.

In conclusion, a series of studies assessed long-term efficacy of isoflurane when present during a focal ischemic injury in the rat. We repeatedly found substantial and sustained protection by isoflurane in the model used. This was independent of ischemia duration or perfusion pressure. However, the magnitude of infarct size was dependent on the MCAO model used. It remains a matter of further investigation to determine the clinical relevance of these findings and the mechanisms by which volatile anesthetics permanently reduce experimental focal ischemic brain injury.

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