Anesthesiology 2004; 101:75–81

Combination of Isoflurane and Caspase Inhibition Reduces Cerebral Injury in Rats Subjected to Focal Cerebral Ischemia

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Background: **Recent data indicate that the neuroprotective efficacy of isoflurane is not sustained. Delayed neuronal death, mediated in part by apoptosis, contributes to the gradual increase in the size of the infarction. These data suggest that isoflurane may not be able to inhibit delayed neuronal death. The prevention of apoptosis by a caspase inhibitor might provide neuroprotection in addition to that provided by isoflurane. The current study was conducted to determine whether isoflurane-mediated neuroprotection can be made more durable with the administration of z-VAD-fmk, a nonspecific caspase inhibitor.**

Methods: **Fasted Wister rats were allocated to awake–zVAD, awake–vehicle, isoflurane–zVAD, or isoflurane–vehicle groups (n = 16/group). Animals were subjected to focal ischemia for 60 min by filament occlusion of the middle cerebral artery. In the awake groups, isoflurane was discontinued after occlusion of the middle cerebral artery. In the isoflurane groups, isoflurane anesthesia was maintained at 1.5 minimum alveolar concentration during occlusion of the middle cerebral artery. Before and after ischemia, daily injections of z-VAD-fmk or vehicle were administered into the lateral cerebral ventricle for 14 days. Neurologic assessment was performed 14 days after ischemia. The volume of cerebral infarction and the number of intact neurons in the periinfarct cortex were determined by image analysis of hematoxylin and eosin–stained coronal brain sections.**

Results: **Infarction volume was less in the isoflurane–zVAD group (23 ± 11 mm^3, mean ± SD) than in isoflurane–vehicle, awake–vehicle, and awake–zVAD groups (82 ± 31, 86 ± 31, and 59 ± 25 mm^3, respectively; $P < 0.05$). In comparison with the awake–vehicle and isoflurane–vehicle groups, the administration of z-VAD-fmk significantly decreased infarction volume ($P < 0.05$). The infarction volume between the awake–vehicle and isoflurane–vehicle groups was not different. The number of intact neurons within the periinfarct cortex was significantly less in the awake–vehicle group than in the other three groups ($P < 0.05$). The isoflurane–zVAD group demonstrated better neurologic function than the awake–vehicle group ($P < 0.05$).**

Conclusion: **These findings are consistent with the premise that ongoing delayed neuronal death, in part mediated by apoptosis, contributes to the progression of cerebral infarction during the recovery period, and its inhibition can provide sustained neuroprotection.**

This article is featured in "This Month in Anesthesiology." Please see this issue of ANESTHESIOLOGY, page 5A.

EXPERIMENTAL studies have shown that volatile anesthetics can reduce neuronal injury in the setting of focal or global cerebral ischemia.[1–7] In most studies, the recovery period was relatively short (1–7 days). Recent data have shown that postischemic neuronal death is a dynamic process in which neurons continue to die over a long period of time after the initiating ischemic injury.[8,9] Although isoflurane can also reduce ischemic neuronal injury after focal ischemia in comparison with the awake state after short postischemic recovery intervals (2 days later), data from our laboratory have shown that this neuroprotective efficacy is not sustained (2 weeks later).[10] These data suggest that isoflurane delays the development of cerebral infarction but does not prevent it.

A number of mechanisms contribute to postischemic neuronal injury. During the ischemic and early reperfusion periods, glutamate-mediated excitotoxicity and ischemic neuronal depolarizations lead to rapid neuronal death. Isoflurane can suppress excitotoxicity and ischemic depolarizations.[11–19] These effects of isoflurane contribute to its neuroprotective efficacy. In the later stages of postischemic recovery, the development of inflammation within the brain and of neuronal apoptosis lead to delayed neuronal death and the gradual expansion of the cerebral infarct. An important mechanism by which neuronal apoptosis occurs is *via* the activation of caspases. Caspase activation results in the proteolytic cleavage of a number of vital cellular components and subsequent neuronal apoptosis. The activation of caspases seems to play an important role in mediating neuronal cell death by apoptosis after focal cerebral ischemia,[20] and the administration of caspase inhibitors has been reported to reduce the volume of infarction after focal cerebral ischemia.[21–24]

The available data suggest that, in the setting of focal ischemia *in vivo*, isoflurane does not prevent neuronal apoptosis. Work from our laboratory has shown that in rodents subjected to focal cerebral ischemia, apoptosis continues to occur for several days after ischemia and that the administration of isoflurane during the ischemic interval does not mitigate this apoptosis.[25] The lack of an effect on apoptosis may explain the failure of isoflurane to prevent infarct expansion after focal ischemia. How-

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Received from the Veterans Affairs Medical Center and the University of California, San Diego, California. Submitted for publication August 27, 2003. Accepted for publication December 20, 2003. Supported by grant No. GMS52098 from the National Institutes of Health, Bethesda, Maryland (to Dr. Patel).

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ever, it is possible that caspase inhibition might prevent this infarct expansion and that the combination of isoflurane (suppression of excitotoxic injury) and caspase inhibition (reduction in neuronal apoptosis) might provide sustained neuroprotection. The current study was conducted to determine the effect of the combination of isoflurane and caspase inhibition, produced by the nonspecific broad caspase inhibitor z-VAD-fmk, on neuronal injury in a rodent model of focal ischemia.

Materials and Methods

The study was approved by the local institutional Animal Care and Use Committee (Veterans Affairs Medical Center, San Diego, California). All experimental procedures were performed in accordance with the guidelines established in the Public Health Service *Guide for the Care and Use of Laboratory Animals*.**

Male Wistar rats (Simonson Laboratories, San Diego, CA) weighing 270–330 g were fasted overnight. Access to water was provided. The rats were anesthetized with an inspired concentration of 5% isoflurane (Ohmeda, Liberty Corner, NJ). After tracheal intubation, the animals' lungs were mechanically ventilated with a gas mixture of 30% oxygen and 70% nitrogen. The end-tidal concentration of isoflurane was reduced to 2.5%. A needle thermistor (Mon-a-Therm; Mallinckrodt, St. Louis, MO) was inserted between the temporalis muscle and the skull, and the pericranial temperature was servocontrolled to 37.0 ± 0.2°C by surface heating or cooling. A cannula was inserted in the tail artery using PE-50 tubing. The mean arterial pressure was monitored continuously. With the use of randomization tables, the animals were allocated to awake–zVAD, awake–vehicle, isoflurane–zVAD, or isoflurane–vehicle groups (n = 16/group).

The animals were mounted on a stereotactic frame (Kopf Instruments, Tujunga, CA), and their heads were secured. A midline scalp incision was made, and a 1.5-mm burr hole was drilled 0.8 mm posterior and 1.5 mm right lateral from the bregma. A 23-gauge guide cannula was inserted by micromanipulator into the cerebral ventricle to a depth 4.0 mm from the surface of the cranium. Dental cement was used to fix the guide cannula to the cranium. A 30-gauge stylet was inserted into the cannula to maintain patency. The animal was removed from the frame headrest and was transferred to the surgical table for the right middle cerebral artery occlusion (MCAO). Thirty minutes before initiation of the right MCAO, 2 h after the termination of MCAO, and every 24 h for 14 days, z-VAD-fmk (0.5 μg in 5 μl over 5 min) or vehicle were administered intracerebroventricularly using a 30-gauge blunt needle *via* the guide cannula. This dose was determined based on previous reports[26,27] that demonstrated the neuroprotective efficacy of zVAD. The drug vehicle was 0.4% dimethyl sulfoxide in artificial cerebrospinal fluid (composition: 132 mM NaCl, 2.95 mM KCl, 1.71 mM $CaCl_2$, 0.65 mM $MgCl_2$, 24.6 mM $NaHCO_3$, and 3.69 mM D-glucose). Surgical preparation during isoflurane anesthesia was complete in approximately 45–60 min.

Focal cerebral ischemia was induced according to the technique of Zea-Longa *et al.*[28] The right common carotid artery was exposed *via* a midline pretracheal incision. The vagus and sympathetic nerves were carefully separated from the artery. The external carotid artery was ligated 2 mm distal to the bifurcation of the common carotid artery. The internal carotid artery was dissected distally, and the pterygopalatine artery was ligated. The common carotid artery then was ligated 5–10 mm proximal to its bifurcation. Baseline values for arterial oxygen (Pa_{O_2}) and carbon dioxide (Pa_{CO_2}) tensions and pH, plasma glucose concentration, hematocrit, mean arterial pressure, and heart rate were measured and recorded. *Via* a small arteriotomy, a 0.25-mm-diameter nylon monofilament, previously coated with silicone, was inserted into the proximal common carotid artery and was advanced into the internal carotid artery to a distance of 18–20 mm from the carotid artery bifurcation until slight resistance was felt.

After induction of focal ischemia, isoflurane administration in awake groups was discontinued. On resumption of spontaneous ventilation, mechanical ventilation was discontinued, and the endotracheal tube was removed. The animals were transferred to a heated and humidified incubator, through which oxygen was flushed continuously. The animals were anesthetized briefly with isoflurane 6 min before the end of the 60-min ischemic interval. The pretracheal incision was reopened, and the monofilament was removed from the common carotid artery at the end of the 60-min ischemic interval. The tail artery catheter was removed, and the wound was sutured. The animals were then allowed to awaken.

In the isoflurane groups, the end-tidal concentration of isoflurane was reduced to 1.8% (approximately 1.5 times the minimum alveolar concentration [MAC])[29] after MCAO. At the end of 60-min ischemic interval, the monofilament was removed. The tail artery catheter was removed, and the wound was sutured. All wounds were infiltrated with 0.25% bupivacaine (total dose, 0.5 mg). Isoflurane administration was then discontinued. On resumption of spontaneous ventilation, mechanical ventilation was discontinued, and the endotracheal tube was removed. The animals were transferred to the incubator as described above. During the recovery period, the pericranial temperature was recorded at 1-h intervals for 3 h. Thereafter, the temperature probe was removed. The rectal temperature was monitored every 24 h for 14 days.

** PHS Guide for the Care and Use of Laboratory Animals. Available at: http://www.nap.edu/readingroom/books/labrats/. Accessed May 15, 2004.

Two groups of animals, n = 4/group, underwent cannulation of the lateral cerebral ventricle as described above. These groups received either z-VAD-fmk or vehicle by intracerebral ventricular injection. The brains from these animals were removed 3 days after injection and were used as nonischemic controls to determine whether z-VAD-fmk (or vehicle) administration resulted in neuronal injury.

All animals that were subjected to MCAO underwent a neurologic evaluation 2 h after focal ischemia. Those animals that did not manifest clinical evidence of neurologic injury were then excluded from the study.

Neurologic evaluation was performed 14 days after ischemia. Each rat was assigned a score according to an eight-point behavioral rating scale[30]: 0 = no neurologic deficit; 1 = failure to extend left forepaw fully; 2 = decreased grip of the left forelimb; 3 = spontaneous movement in all direction, contralateral circling only if pulled by the tail; 4 = circling or walking to the left (or right); 5 = walking only if stimulated; 6 = unresponsiveness to stimulation, with a depressed level of consciousness; and 7 = dead. Neurologic testing was performed by a single observer who was blinded to group assignment. The animal's body weight was measured before the experiment and 14 days after ischemia.

Assessment of Ischemic Cerebral Damage

The animals were anesthetized with chloral hydrate after neurologic examination. They were killed by transcardiac perfusion with 200 ml heparinized saline followed by 200 ml phosphate-buffered formaldehyde, 4%. The animals were decapitated, and their brains were removed carefully, immersed in fixative, and refrigerated at a temperature of approximately 4°C for 24–48 h. The brains then were prepared for histologic analysis. After dehydration in graded concentrations of ethanol and butanol, the brains were embedded in paraffin. Six-micron-thick coronal sections were obtained at 0.75-mm intervals and stained with hematoxylin and eosin. During tissue processing, the implanted guide cannula placement was evaluated. The animals in which the guide cannula was not in the lateral ventricle were excluded from this study. The animals that had subarachnoid hemorrhage were also excluded.

Infarction was assessed using light microscopy, and within each section, the area of infarction was traced. Infarction area was defined as pan-necrosis with loss of neuropil. The area of infarction was determined by image analysis using National Institutes of Health Image 1.62 software and an Apple Power Macintosh G4 computer (Apple Computer, Cupertino, CA). The total volume of injury was determined by integration of the area of injury in each section (between 9 and 12 sections of the brain, spanning the entire region of ischemic injury, were analyzed) according to the technique of Swanson *et al.*[31]

For quantitation of neuronal damage, a modification of the method of Lei[32] was used. Coronal sections at the level of the anterior commissure and 750 μm rostral and caudal[33] from that level were evaluated. In each section, the number of intact neurons per 0.25 mm^2 tissue in the periinfarct cortex (cortical tissue immediately adjacent to the infarction boundary) was counted in three contiguous fields. The neuron counts were then averaged. The histologic analysis was performed by an observer who was not aware of the experimental group assignment.

Statistical Analysis

The study population size was determined by a power analysis of the data from a previous investigation from our laboratory.[10] With the assumption of a type I error protection of 0.05 and a power of 0.80, 13 rats in each of the four groups were required. Unanticipated death of animals was expected and a sample size of 16 animals/group was deemed necessary for appropriate study power.

Physiologic variables were analyzed by repeated-measures analysis of variance. Cerebral infarction volumes and neuron counts were analyzed by factorial analysis of variance (Statview 4.5; Abacus Concepts, Berkeley, CA). Unpaired *t* tests with Bonferroni corrections were used for *post hoc* intergroup comparisons. Neurologic scores were analyzed by the Kruskal-Wallis test followed by the Mann–Whitney U test with Bonferroni correction. A *P* value of less than 0.05 was considered to be statistically significant. All data except neurologic scores are presented as mean ± SD. Neurologic scores are reported as 10th, 25th, median, 75th, and 90th percentile ranges.

Results

The physiologic variables are presented in table 1. There were no significant differences in preischemic weight, mean arterial pressure, heart rate, pH, arterial carbon dioxide tension, arterial oxygen tension, glucose concentration, and hematocrit among the four experimental groups. There were no significant differences in the pericranial and rectal temperatures among the groups. The weight loss in the isoflurane–zVAD group was less than in the awake–vehicle group ($P < 0.01$).

Of a total of 64 animals, 4 (1 in the awake–vehicle group, 1 in the isoflurane–zVAD group, and 2 in the isoflurane–vehicle group) were excluded from this study because of misplacement of a guide cannula, development of subarachnoid hemorrhage, or technical experimental problems. Of this total of 60 remaining animals, 5 (including 1 in the awake–zVAD group, 2 in the awake–vehicle group, 1 in the isoflurane–zVAD group, and 1 in the isoflurane–vehicle group) died before histologic analysis. These animals were considered to have experienced neurologic deaths.

Table 1. Physiologic Variables in the Four Experimental Groups

	Awake–Vehicle	Isoflurane–Vehicle	Awake–zVAD	Isoflurane–zVAD
Number	15	14	16	15
Weight, g	301 ± 18	296 ± 22	298 ± 20	292 ± 18
MAP, mmHg				
Before MCAO	91 ± 12	91 ± 9	95 ± 11	93 ± 10
Reperfusion	87 ± 6	89 ± 7	91 ± 5	93 ± 6
HR, beats/min				
Before MCAO	368 ± 32	385 ± 33	373 ± 26	383 ± 28
Reperfusion	397 ± 15	388 ± 23	392 ± 15	397 ± 17
*p*H				
Before MCAO	7.43 ± 0.01	7.43 ± 0.02	7.44 ± 0.02	7.43 ± 0.02
Reperfusion	7.34 ± 0.04	7.36 ± 0.04	7.35 ± 0.03	7.35 ± 0.03
$Paco_2$, mmHg				
Before MCAO	38 ± 2	38 ± 3	37 ± 1	38 ± 3
Reperfusion	40 ± 4	39 ± 4	39 ± 3	40 ± 4
Pao_2, mmHg				
Before MCAO	126 ± 12	128 ± 16	130 ± 13	133 ± 9
Reperfusion	132 ± 16	130 ± 20	127 ± 16	130 ± 13
Hematocrit, %				
Before MCAO	44 ± 2	45 ± 2	44 ± 3	44 ± 2
Reperfusion	41 ± 4	40 ± 4	41 ± 3	40 ± 3
Glucose, mg/dl				
Before MCAO	119 ± 15	116 ± 12	120 ± 11	112 ± 12
Reperfusion	127 ± 20	122 ± 14	124 ± 14	120 ± 15

Data are presented as mean ± SD.

HR = heart rate; MAP = mean arterial pressure; MCAO = middle cerebral artery occlusion; reperfusion = 5 min after reperfusion; $Paco_2$ = arterial carbon dioxide tension; Pao_2 = arterial oxygen tension.

The results of behavioral testing are shown in figure 1. Fourteen days after ischemia, the isoflurane–zVAD group demonstrated a better neurologic outcome than the awake–vehicle group ($P < 0.05$).

Cerebral infarction volumes are presented in figure 2. Total infarction volume (cortex and subcortex infarction) was less in the isoflurane–zVAD group (23 ± 11 mm^3, mean ± SD) than in isoflurane–vehicle, awake–vehicle, and awake–zVAD groups (82 ± 31, 86 ± 31, and 49 ± 25 mm^3, respectively; $P < 0.05$). z-VAD-fmk also reduced cerebral injury in the awake–VAD group in comparison with both vehicle groups ($P < 0.05$). The cortex infarction volume was significantly less in both zVAD groups in comparison with both vehicle groups ($P < 0.05$). The subcortex infarction volume was significantly less in the isoflurane–zVAD group in comparison with the other three groups.

The number of histologically preserved neurons within the periinfarct cortex in the four groups is presented in figure 3. The awake–vehicle group had significantly fewer intact neurons in comparison with the other three experimental groups ($P < 0.05$).

The administration of either vehicle or z-VAD-fmk to

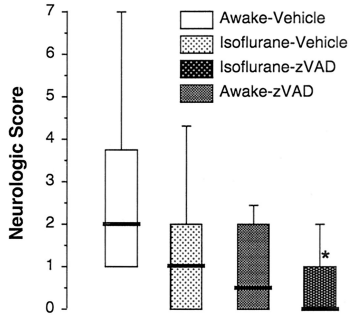

Fig. 1. Neurologic scores, on a scale from 0 (no injury) to 7 (neurologic death), were obtained 14 days after ischemia. Data are presented as median (*horizontal bar*) and 25–75% range (*box*) and 90% range (*vertical bar*). * $P < 0.05$ *versus* awake–vehicle group; # $P < 0.05$ *versus* isoflurane–vehicle group.

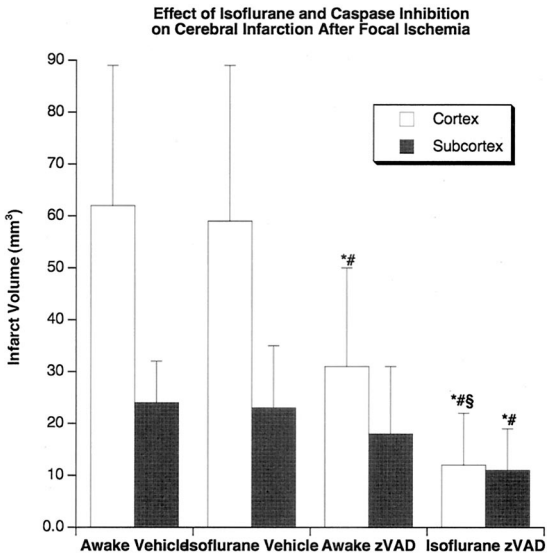

Fig. 2. Cortex and subcortex infarction volumes in the four experimental groups were measured 2 weeks after focal ischemia. Data are presented as mean ± SD. * $P < 0.05$ *versus* awake-vehicle group; # $P < 0.05$ *versus* isoflurane–vehicle group; § $P < 0.05$ *versus* awake–zVAD group.

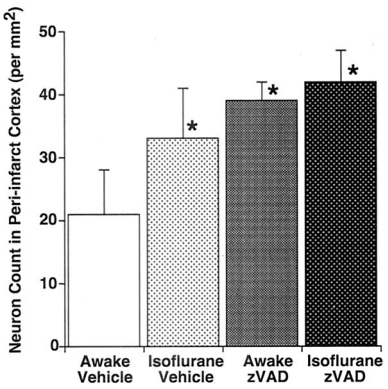

Fig. 3. Histologically intact neurons in 1-mm^2 area of the peri-infarct cortex were counted in three contiguous regions, and the results were averaged. Data are presented as mean ± SD. * $P < 0.01$ *versus* awake–vehicle group.

sham-operated rats that did not undergo MCAO did not result in any histologic or clinical neurologic injury.

Discussion

The results of the current study indicate that a combination of isoflurane and caspase inhibition can reduce cerebral injury produced by focal ischemia. The neuroprotective efficacy of this combination is apparent even after a 2-week recovery period. Although the administration of z-VAD-fmk alone also reduced cerebral infarction, its efficacy was less than that of the combination of isoflurane and z-VAD-fmk. The histologic reduction in cerebral injury was consistent with the improvement in neurologic outcome that was observed in the animals that received isoflurane and z-VAD-fmk. Isoflurane, when administered alone, did not reduce cerebral infarction 2 weeks after ischemia. The isoflurane-vehicle–treated animals demonstrated better neurologic function 14 days after ischemia. The results are consistent with the premise that ongoing delayed neuronal death contributes to postischemic cerebral infarction in rodents subjected to focal ischemia and that the reduction of this delayed death, in part mediated by apoptosis, with caspase inhibition can provide sustained neuroprotection.

Although the short-term neuroprotective efficacy of isoflurane has been clearly established, our results indicate that isoflurane neuroprotection is not persistent. This phenomenon, in which early neuroprotection is not sustained after longer recovery intervals, has also been reported for other neuroprotective agents such as a-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid and *N*-methyl-D-aspartate receptor antagonists.[34] Du *et al.*[8] have shown that cerebral infarction after focal cerebral ischemia undergoes a gradual expansion; the authors suggested that this expansion was due in part to delayed neuronal death *via* apoptosis. Li *et al.*[35] have shown that apoptosis can be observed as late as 4 weeks after ischemia. Moreover, apoptotic cells are localized primarily within the inner boundary zones of the evolving infarct.[35] These data suggest that apoptosis may contribute to the expansion of the ischemic lesion. In a recent study from our laboratory, in which we evaluated the effect of isoflurane on postischemic apoptosis, we demonstrated that isoflurane delayed the development of apoptosis but did not prevent it. In fact, apoptosis was apparent even 7 days after focal ischemia.[25] Collectively, these data indicate that isoflurane does not ultimately prevent apoptotic neuronal death.

A number of mechanisms contribute to the development of postischemic neuronal apoptosis. Mitochondrial injury, the release of cytochrome c, and the subsequent activation of caspases 9 and 3 have received the most attention.[36] More recent data also indicate that tumor necrosis factor, a major inflammatory cytokine, leads to direct activation of caspase 8. Activated caspase 8 then cleaves downstream caspases and results in apoptosis.[37,38] In the setting of focal ischemia, caspase-8 activation has been implicated in the development of apoptosis.[39–41] z-VAD-fmk is a potent but nonspecific broad spectrum caspase inhibitor. Therefore, it is logical to expect that broad inhibition of caspases might reduce postischemic neuronal injury. In fact, the neuroprotective efficacy of z-VAD-fmk has previously been demonstrated in rodent models of focal cerebral ischemia.[21,23] The results of current study are consistent with those reports. These results are consistent with the premise that activation of caspases occurs during and after focal ischemia and this activation results in neuronal apoptosis. The corollary is that inhibition of caspases can reduce apoptosis and can provide neuroprotection that is apparent after a 2-week recovery interval.

Of interest is our observation that the number of preserved neurons in the periinfarct cortex was greater in the animals that received either isoflurane or z-VAD-fmk. The mechanism by which isoflurane, when administered alone, preserved neurons in the periinfarct cortex after ischemia but did not decrease infarct size is not clear. One possible mechanism is the inhibition of ischemic depolarizations that occur during focal ischemia. These ischemic depolarizations have been shown to increase neuronal calcium influx during ischemia, thereby increasing brain injury probably mediated by apoptosis.[40,42] Recent data reported by Back *et al.*[43] indicate that ischemic depolarizations during focal ischemia do not increase the infarct volume but contribute significantly to the development of scattered neuronal injury within the cortex adjacent to the infarct. Previous work in our laboratory has shown that isoflurane can reduce the frequency of ischemic depolarizations during focal ischemia.[43] Together, these studies suggest that the increase in the number of intact neurons in the penumbra that was observed in the current study might be mediated in part by a reduction in the frequency of ischemic depolarizations during isoflurane anesthesia.

A recent report by Sullivan[44] showed that isoflurane can prevent delayed cell death in an organotypic slice culture model of oxygen-glucose deprivation–mediated neuronal injury. The neuroprotective effect of isoflurane on delayed cell death, evaluated 14 days after injury, was compared to that of the *N*-methyl-D-aspartate antagonist MK-801. In contrast to the results of the current study, sustained isoflurane neuroprotection was observed even 14 days after injury. This reduction in injury was similar to that achieved with MK-801 treatment. It should be noted, however, that the *in vitro* hippocampal slice preparation cannot replicate cerebral blood flow changes and postischemic inflammation that are characteristic of the focal ischemia model that was used in the current investigation. Recent reports have highlighted the importance of postischemic inflammation, perhaps mediated *via* Fas signaling,[45,46] to the development of delayed neuronal death. Therefore, the discrepancy between our results[10] and the results from Sullivan[44] are probably a function of the differences in the experimental models that were used.

In summary, a combination of isoflurane and z-VAD-fmk, a nonspecific caspase inhibitor, decreased focal ischemia induced cerebral infarction when the injury was evaluated after a recovery period of 14 days. This combination demonstrated greater efficacy than the administration of z-VAD-fmk alone. By contrast, isoflurane, when administered alone, did not reduce ischemic cerebral injury. The results indicate that caspase-mediated neuronal apoptosis contributes to the development of injury in the setting of focal ischemia and that the inhibition of caspases can result in neuroprotection that is sustained. Our findings are also consistent with the notion that combination therapy with agents that target different aspects of the pathophysiology of cerebral ischemia (*e.g.*, excitotoxicity and apoptosis) is more likely to be effective in reducing ischemic cerebral injury than is the administration of individual agents alone.

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