

Isoflurane Pretreatment Ameliorates Postischemic Neurologic Dysfunction and Preserves Hippocampal Ca^{2+} /Calmodulin-dependent Protein Kinase in a Canine Cardiac Arrest Model

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Background: Inhalational anesthetics are neuroprotective in rat models of global ischemia. To determine whether isoflurane at a clinically relevant concentration is neuroprotective in a canine model of cardiac arrest, we measured neurologic function and hippocampal Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) content 20 h after cardiac arrest.

Methods: We tested the neuroprotective effect of 30 min of 1.5% isoflurane exposure before 8 min of global ischemia induced with ventricular fibrillation. Animals were randomized to four groups: control, isoflurane-control, ischemia, and isoflurane-ischemia. After resuscitation and 20 h of intensive care, each animal's neurologic deficit score was determined by two blinded evaluators. The hippocampal content of CaMKII, determined by immunoblotting, was measured by an individual blinded to the treatment groups. CaMKII activity was measured in samples from the cortex, hippocampus, and striatum of animals in each group.

Results: Isoflurane-ischemic animals had a median neurologic deficit score of 22.6% compared with 43.8% for the ischemic animals ($P < 0.05$). Hippocampal levels of the β -subunit of CaMKII (CaMKII β) were relatively preserved in isoflurane-ischemic animals ($68 \pm 4\%$ of control) compared with ischemic animals ($48 \pm 2\%$ of control; $P < 0.001$), although both groups

were statistically significantly lower than control ($P < 0.001$ ischemia vs. control and $P < 0.05$ isoflurane-ischemia vs. control).

Conclusions: Isoflurane is an effective neuroprotective drug in a canine cardiac arrest model in terms of both functional and biochemical criteria. (Key words: Global cerebral ischemia; neuronal calcium; resuscitation; volatile anesthetic.)

ISOFLURANE is a volatile anesthetic that is often used as a primary anesthetic agent during neurosurgical operations. It has mild cardiovascular side-effects and is potentially a neuroprotective drug under certain circumstances. Because isoflurane has many effects on neuronal Ca^{2+} regulation, we investigated its neuroprotective effects in a canine cardiac arrest model of global cerebral ischemia.

At least three important and interrelated pathophysiologic mechanisms are active following a period of global cerebral ischemia and reperfusion: elevation of intracellular Ca^{2+} , release of excitotoxic amino acids, and generation of oxygen free radicals.¹ Within minutes of the initiation of total cerebral ischemia intraneuronal Ca^{2+} increases from approximately 100 nM to approximately 30 μ M.^{2,3} We have previously shown that 10 min of global ischemia in a canine cardiac arrest model leads to a marked increase in the number of L-type Ca^{2+} channels in both cerebral cortex and striatum.^{4,5} L-type Ca^{2+} channels might be one of the several routes of Ca^{2+} entry into the ischemic cell. Because isoflurane can decrease the number of calcium channels, Ca^{2+} entry through Ca^{2+} channels, and depolarization-evoked peak synaptosomal Ca^{2+} ,^{6,7} we hypothesized that isoflurane would be neuroprotective by virtue of its ability to minimize Ca^{2+} elevation during and after ischemia and reperfusion. Our goal was to test the potential neuroprotective activity of isoflurane pretreatment at a clinically relevant concentration.

Materials and Methods

The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the Hospital for Special Surgery. Twenty-one adult, female, beagle-like dogs were randomized to four groups: control, isoflurane-control, ischemia, and isoflurane-ischemia. Anesthesia was induced with sodium thiopental, 12.5 mg/kg, and the trachea intubated with a 7-mm

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Received from the Excitable Tissues Laboratory and the Department of Anesthesiology, Hospital for Special Surgery, New York, New York; Memorial Hospital, Memorial Sloan Kettering Cancer Center, New York, New York; and the Departments of Anesthesiology, Physiology and Biophysics, and Pharmacology, Weill College of Medicine of Cornell University, New York, New York. Submitted for publication March 6, 2000. Accepted for publication June 20, 2000. Supported by funds from the National Institutes of General Medical Sciences, Bethesda, Maryland (grant No. RO1 GM50686 to Dr. Blanck); the Hospital for Special Surgery Anesthesia Research Fund, New York, New York; and the Charina Foundation, New York, New York.

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(inner diameter) endotracheal tube. The animals were then given 37.5 mg/kg of α -chloralose intravenously and paralyzed with 2 mg of pancuronium. Animals were ventilated at a tidal volume of approximately 18 ml/kg with 25% O₂ and 75% N₂O to an arterial carbon dioxide tension of 25–35 mmHg with a total gas flow of 3 l. Temperature was maintained between 36.2°C and 37.8°C with a heating blanket and heating lamp. Thirty minutes after induction of anesthesia, an additional 37.5 mg/kg of α -chloralose was given, and 250 mg of ceftriaxone was given as antibiotic prophylaxis. Left femoral arterial and venous catheters were placed. The arterial catheter was attached to a pressure transducer for continuous blood pressure monitoring. A Swan-Ganz catheter was passed through the femoral vein to the pulmonary artery and attached to a pressure transducer. The experimental treatments consisted of the following:

Control (n = 3): Baseline anesthetic was administered and invasive monitoring placed, followed by a thoracotomy and pericardiectomy, but no ischemia time was induced. Approximately 60 min later, the chest was closed, and the animals were observed for an additional 18–20 h while receiving a maintenance anesthetic of morphine (0.1 mg · kg⁻¹ · h) and pancuronium (0.1 mg · kg⁻¹ · h⁻¹).

Isoflurane-control (n = 3): This group received the same treatment as control plus 30 min of 1.5% isoflurane after the thoracotomy and pericardiectomy and before maintenance anesthesia for 20 h.

Ischemia (n = 7): This group received the same treatment as control, but after the thoracotomy and pericardiectomy, there was a 30-min stabilization period followed by 8 min of apnea and ventricular fibrillation induced electrically by directly stimulating the left atrium (25 Hz at 25 V). After the 8 min of apnea and fibrillation, ventilation was resumed with 100% O₂ at a rate of 28/min, and manual open-chest cardiac massage was begun at a rate of 60/min and maintained for 3 min. Simultaneously, 20 μ g/kg of epinephrine and 1 mEq/kg of NaHCO₃ was administered. Internal cardiac defibrillation was performed at 35 J. On return of sinus rhythm, an epinephrine infusion was initiated, titrating the systolic arterial blood pressure to 100 mmHg.

Isoflurane-ischemia (n = 8): This group was treated identically to the ischemic group except before fibrillation, the animals were given 1.5% isoflurane for 30 min. Isoflurane was terminated at the time fibrillation was induced.

After stabilization of vital signs, a chest tube was inserted, the chest was closed in four layers, the chest cavity was evacuated, and the chest tube was connected to a Heimlich chest valve. Animals were turned from side to side every 3 h during the 20-h maintenance period to reduce lung atelectasis. Cardiovascular parameters and arterial blood gases were measured throughout the ex-

periment. The morphine-pancuronium drip was turned off 2 h before the awakening time. Before awakening, neuromuscular blockade was reversed with neostigmine and glycopyrrolate, morphine was reversed with 0.6 mg/kg of naloxone, and the animals were extubated. The animals were examined using a 410-point neurologic deficit scoring (NDS) system to evaluate postischemic neurologic injury in the dog (0%, normal; 100% brain dead).^{8,9} The examination involves evaluation of the level of consciousness, respiratory pattern, cranial nerve and motor function, and behavior. The scores, determined by two individuals blinded to the treatment groups, were averaged. After neurologic evaluation, the animals were anesthetized with 200 mg of sodium thiopental, 30 mg of morphine, and 2 mg of pancuronium and the brains and hearts were rapidly excised. Each brain was placed on ice, the two hemispheres were separated, and the hippocampus, cortex, and striatum were identified and separated.

Animal Care

The animals were anesthetized with N₂O, α -chloralose and morphine sulfate during the prearrest period. After arrest, the animals were maintained with a morphine (0.1 mg · kg⁻¹ · h⁻¹) and pancuronium (0.1 mg · kg⁻¹ · h⁻¹) drip and given bolus injections of morphine sulfate if heart rate exceeded 180/min or mean arterial blood pressure (MAP) was greater than 140 mmHg. The administration of pancuronium was stopped 2 h before reversal and awakening. To ensure that the animals were not awake and were paralyzed, several precautions were taken.

The model is essentially identical to that of Rosenthal and Fiskum, who used a similar morphine regimen to achieve “anesthesia.” However, they allowed paralysis to wear off and the animals to ventilate spontaneously during the latter hours of the 20-h observational period. In the development of their model, nonischemic sham-operated animals who were not paralyzed but were “anesthetized” as described in Methods did not demonstrate “escape” behavior (R. Rosenthal, personal communication, February 1996).

In our own preliminary studies, control, isoflurane-control, and ischemic animals were monitored continuously during the postarrest period using an A-1000 electroencephalographic device (Aspect Medical Systems, Inc., Newton, MA). Based on specific criteria enumerated in the appendix (see Web Enhancement), the control animals were determined to be at least deeply analgesic and, based on the degree of electroencephalographic suppression, were most likely surgically anesthetized.

Because the animals in the two control groups were neurologically undamaged and presumably would require more anesthetic, bolus injections of morphine

sulfate were given for defined criteria, although this potentially could have confounded the results. The control group was given an average total of 259 ± 50 mg of morphine, the isoflurane-control group was given 138 ± 7 mg, the isoflurane-ischemic group was given 155 ± 94 mg, and the ischemic group was given 67 ± 39 mg.

Exclusion Criteria

Animals were excluded from the study if any of the following occurred: (1) temperature dropped to less than 35.5°C at any time or was below 36°C at the time of arrest; (2) MAP was lower than 60 mmHg at any time or was lower than 80 mmHg for 10 continuous min; (3) more than 2.5 L of intravenous fluid was given over the duration of the experiment; (4) estimated blood loss was greater than 400 ml; (5) arterial oxygen tension (PaO_2) declined to less than 60 mmHg before arrest; or (6) death occurred before awakening of the animal at 20 h post-arrest.

Synaptosome Preparation

Synaptosomes were prepared from the frontal cerebral cortices, the hippocampus, and the striatum using a modification of the method of Dunkley *et al.*¹⁰

Ca^{2+} Measurements and [^3H]Dihydropyridine

Binding

Purified synaptosomes were loaded with $5 \mu\text{M}$ of the Ca^{2+} -sensitive fluorophore Fura-2 acetoxymethyl ester (Fura-2/AM, Molecular Probes, Inc., Eugene, OR) in the incubation buffer at 37°C for 40 min. Measurements of intrasynaptosomal Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) were carried out as previously described and are reported as mean \pm SD.⁷ The binding of the dihydropyridine [^3H]isradipine to synaptosomes was performed at 37°C as previously described to quantitate the number of L-type Ca^{2+} channel binding sites.⁶

CaMKII Western Blot Analysis of Brain Extracts

One hemisphere of the brain was removed and rapidly chilled in ice-cold 0.32-M sucrose. The hippocampus was dissected and frozen at -80°C until used. Brain tissue was homogenized in 10 volumes (ml/g) of 1% (w/v) sodium dodecyl sulfate containing 10 $\mu\text{g}/\text{ml}$ leupeptin. Protein concentrations were determined for each sample by the bicinchoninic acid method¹¹ using bovine serum albumin as a standard. Equal amounts of homogenate protein were solubilized in sodium dodecyl sulfate-sample buffer and separated in duplicate by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide) as described elsewhere.¹² After electrophoresis, the proteins were transferred to 0.2- μm nitrocellulose (Schleicher and Schuell, Keene, NH) in 25 mM Tris, 193 mM glycine, 20% (v/v) methanol (pH 7.8–8.4) at 100 mV for 1.5 h. Immunoblotting with purified rabbit

antibody raised against purified rat brain CaMKII (1:1,000)¹³ was performed by a modification¹⁴ of the method of Burnette¹⁵ using 2.5% dry milk (Carnation, Glendale, CA) in 10 mM potassium phosphate (pH 7.4), 150 mM NaCl, 0.05% (v/v) Tween 20, and 0.02% (w/v) Na azide as blocking solution. Immunolabeled proteins were detected using ^{125}I -protein A (0.1 $\mu\text{Ci}/\text{ml}$ in blocking solution; Amersham, Piscataway, NJ), identified by their relative molecular weights, and quantified by scanning and analysis with a PhosphorImager autoradiography system (Molecular Dynamics, Sunnyvale, CA). Arbitrary intensity units from three independent experiments (expressed as mean \pm SD) were normalized and analyzed.

CaMKII Activity Measurements

CaMKII activity was measured using the Upstate Biotechnology assay kit (Lake Placid, NY). Ten micrograms of the P2 fraction of synaptosomes isolated from the cortex, striatum, and hippocampus of dog brains from each group were incubated for 10 min at 30°C in 20 mM morphilinosulfate at pH 7.2 in the presence of 25 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1.5 μM calmodulin, and 1 mM CaCl_2 .¹⁰ The phosphorylation of the peptide KKALRRQETVDAL (100 μM) by $\gamma^{32}\text{ATP}$ (1 mM, 10 μCi) was assayed by liquid scintillation counting.

Statistics

Differences among treatment groups with respect to temperature, MAP, and heart rate were determined by analysis of variance, and differences at specific time points were evaluated by *t* tests with the Bonferroni correction. Linear regression was employed to evaluate the relations between NDS and temperature and between NDS and CaMKII content. The F test was employed to evaluate whether the slope of the regression fits differed from zero. The Mann-Whitney rank sum test was used to compare NDS among treatment groups. CaMKII content differences among treatment groups were evaluated using one-way analysis of variance with Newman-Keuls multiple comparison test. SIGMA-Stat (San Rafael, CA) and GraphPad (San Diego, CA) software were used for statistical analysis.

Results

Neurologic Outcome

The NDS for each animal in each group and the median NDS for each group are shown in figure 1. The control and isoflurane-control groups had essentially normal neurologic function. The ischemic group had a median NDS of 43.8%, indicative of severe neurologic deficit, compared with 22.6% for the isoflurane-ischemic group ($P < 0.05$; Mann-Whitney rank sum test). The NDS for the ischemic group was comparable to the value of

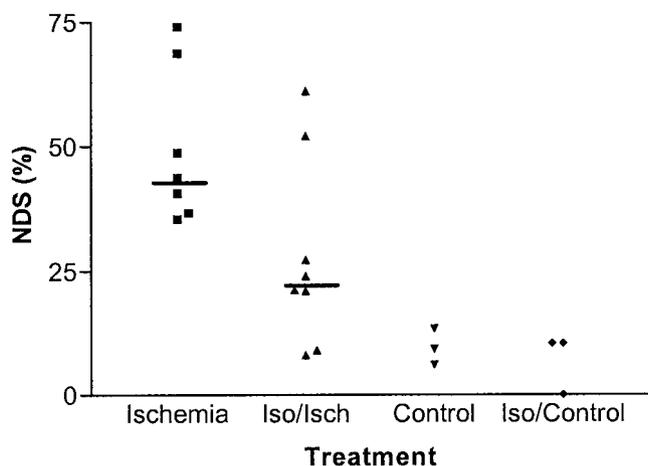


Fig. 1. The effect of treatment on neurologic deficit score (NDS). The average NDS value for each animal is shown as a function of the treatment group. The median values for the ischemic and isoflurane (Iso)-ischemic (Isch) groups are demonstrated by horizontal bars. The ischemic group is significantly different from the isoflurane-ischemic group ($P < 0.05$, t test).

$48.4 \pm 5.4\%$ reported by Rosenthal *et al.*⁸ using a similar canine cardiac arrest model.

Survival

To complete the study, 12 animals in the ischemic group and 13 animals in the isoflurane-ischemic group were required. Seven animals in the ischemic group and eight animals in the isoflurane-ischemic group completed the study. Three of the five animals in the ischemic group that did not complete the study were excluded because of excessive bleeding and the need for more intravenous fluid than permitted by the exclusion criteria; another died during the neurologic examination, possibly from inadequate neuromuscular blocker and opioid reversal; and another was excluded from the study because the epinephrine requirement for resuscitation and maintenance exceeded that of the exclusion criteria. Two animals in the isoflurane-ischemic group could not be resuscitated; one required an amount of epinephrine that exceeded the exclusion criteria; one developed a fatal arrhythmia; and one became hypotensive following a thiopental and morphine overdose at 13 h and died. Ischemic animals received an average total of $128 \pm 84 \mu\text{g}$ of epinephrine during the resuscitation, whereas isoflurane-ischemic animals received $23 \pm 30 \mu\text{g}$ of epinephrine.

Temperature

The pulmonary artery temperature (mean \pm SD) at arrest was identical for the ischemic (36.9 ± 0.6) and isoflurane-ischemic (36.9 ± 0.5) groups, and the temperature from the initiation of fibrillation to the end of fibrillation was unchanged. There were no statistically significant temperature differences between the ischemic and isoflurane-ischemic groups in the 18 h after

resuscitation. However, there was a statistically significant increase in temperature over time in all groups ($P < 0.0001$). To examine the possible confounding effect of temperature on NDS, the average temperature from arrest to 6 h later was calculated for each animal in both the ischemic and the isoflurane-ischemic group, and the NDS for each animal was plotted as a function of that temperature (fig. 2). A linear regression of the data resulted in a correlation coefficient of 0.41. The slope was not statistically significantly different from zero, suggesting that, in our study design, the neurologic outcome was not affected by the postarrest elevation in temperature.

Mean Arterial Pressure and Heart Rate

The MAP and heart rate profiles following resuscitation are demonstrated in figure 3. The difference in MAP between the ischemic group and the isoflurane-ischemic group is not statistically significantly different ($P > 0.05$). The average MAP was maintained above 100 mmHg after cardiac arrest in all groups studied. Conversely, heart rate was highest in the ischemic group and different from the average heart rate for the isoflurane-ischemic group ($P < 0.01$). Both the ischemic and isoflurane-ischemic group heart rates were statistically significantly higher than either control group heart rate over a similar time span.

Synaptosomal Ca^{2+} Uptake

The $[\text{Ca}^{2+}]_i$ transient measured on depolarization with KCl was similar to that reported by us for normal rat synaptosomes.⁷ There is no statistically significant difference in peak $[\text{Ca}^{2+}]_i$ regardless of treatment group: control $299 \pm 24.5 \text{ nM}$ ($n = 12$); isoflurane-control $265 \pm 28.0 \text{ nM}$ ($n = 6$); ischemic $268 \pm 28.1 \text{ nM}$ ($n = 15$); and

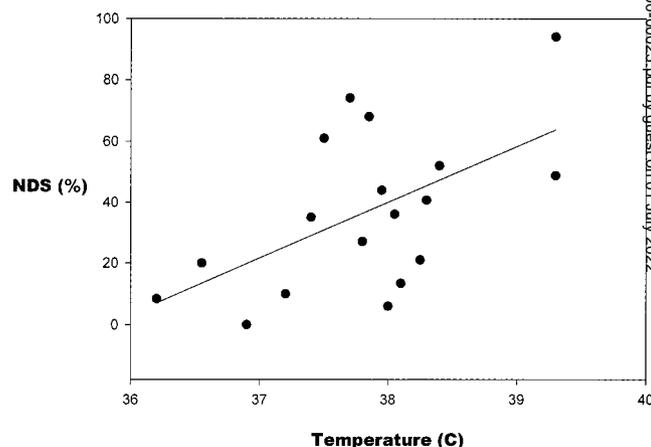


Fig. 2. The relation of neurologic deficit score (NDS) to the mean PA temperature during the first 6 h of reperfusion. The average pulmonary NDS of each animal, regardless of treatment group, is plotted as a function of the mean temperature for the first 6 h of reperfusion for the respective animal. The data were fit by linear regression analysis, yielding a correlation coefficient of 0.41; the slope is not statistically significantly different from zero.

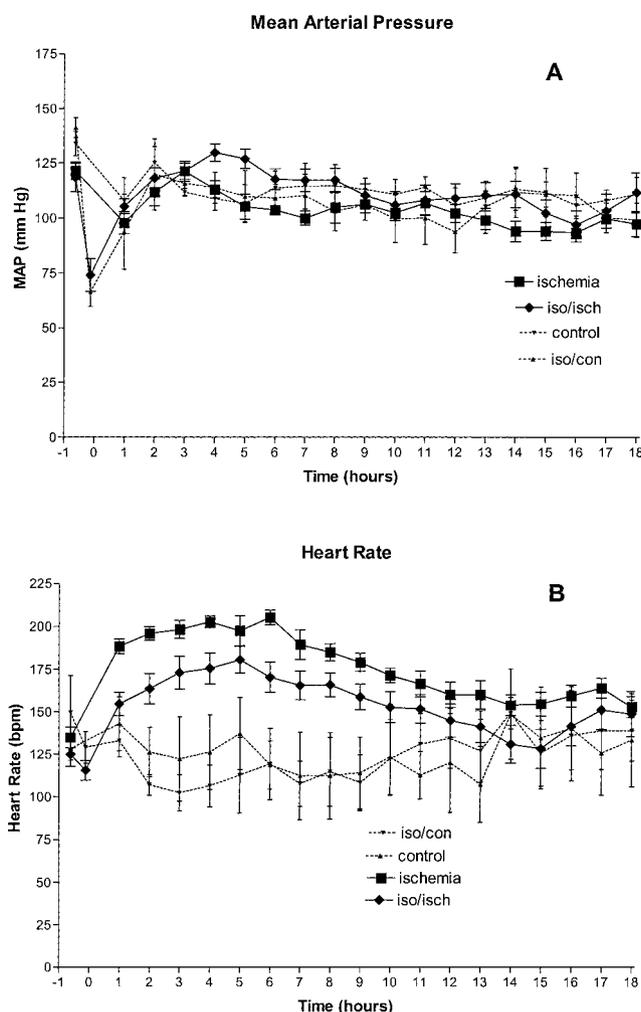


Fig. 3. (A) Mean arterial pressure (MAP) during reperfusion. The average MAP \pm SD of each group is plotted as a function of reperfusion time. MAP for the ischemic group is not statistically significantly different from that of the isoflurane (iso)-ischemic (isch) group, $P > 0.05$. (B) Heart rate during reperfusion. The average heart rate for all animals in each group is plotted as a function of the reperfusion time. Each data point is the mean of the value for all animals in the group \pm SD. The heart rate of the ischemic group is statistically significantly different from the heart rate of the isoflurane-ischemic group ($P < 0.001$, one-way analysis of variance and Bonferroni multiple comparison test). Con = control; bpm = beats/min.

isoflurane-ischemic 291 ± 17.4 nm ($n = 15$). Dihydropyridine equilibrium binding studies demonstrated no differences among treatment groups.

CaMKII Western Blot Analysis of Brain Extracts

Three independent measurements of duplicate samples of homogenate from the hippocampus of each animal were analyzed for amounts of the 50-kDa α -subunit of CaMKII (CaMKII α) and the 60-kDa β -subunit of CaMKII (CaMKII β). Representative immunoblots from each group are shown in figure 4A. The amount of CaMKII β in the ischemic group was found to be decreased compared with the control and the isoflurane-control groups ($P < 0.001$ and $P < 0.01$, respectively)

and compared with the isoflurane-ischemic group ($P < 0.05$). The content of CaMKII β in the isoflurane-ischemic group was also different and was lower than that of either control group ($P < 0.01$). Mean CaMKII β levels were reduced by approximately 52% after ischemia compared with control but by only approximately 32% in isoflurane-ischemic animals compared with controls (fig. 4B). CaMKII α was statistically significantly reduced in samples from the ischemic group compared with control and isoflurane-control groups, but the difference between the ischemic group and the isoflurane-ischemic group was not significant. This in part relates to the smaller magnitude in the CaMKII α antibody signal combined with the same large variability noted in all measurements of CaMKII in the isoflurane-ischemic group. However, the pattern of response for CaMKII β was similar to that of CaMKII α .

CaMKII Activity Measurement

Figure 5 demonstrates the effect of ischemia in the presence and absence of isoflurane on the activity of CaMKII in the cortex, hippocampus, and striatum. The results of the activity measurements agree with that of the immunoblot, indicating decreased CaMKII activity in the brains from ischemic animals, with preservation of activity in those animals that had been pretreated with isoflurane before the ischemic episode. The three areas of the brain examined appeared equally susceptible to injury by ischemia. Statistical analysis was not performed because the data demonstrated the results from only a subset of the animals: three control animals, one isoflurane-control animal, five ischemic animals, and three isoflurane-ischemic animals.

CaMKII and NDS

The relation between NDS and the mean amount of CaMKII β in the hippocampal homogenate of each animal is plotted in figure 6. Linear regression of the data yields a correlation coefficient of 0.81 ($r^2 = 0.66$). The slope of the line was statistically significantly different from zero ($P < 0.001$), suggesting a strong inverse relation between the amount of CaMKII β and the neurologic deficit.

Discussion

We found that isoflurane was neuroprotective in a canine model of cardiac arrest in which the animal was exposed to a clinically relevant concentration of isoflurane for 30 min before cardiac arrest. In other models of global and focal ischemia, the full pathologic effects of an ischemic event did not reach completion until day 3-5 after the ischemic insult.¹⁶ We limited our study to a 20-h reperfusion period, mainly for practical reasons. The 20-h reperfusion period required continuous cardio-

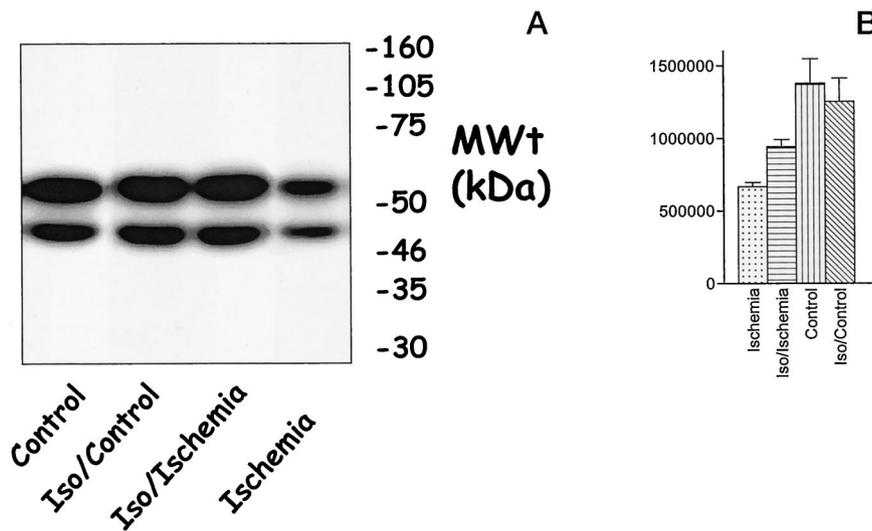


Fig. 4. (A) Immunoblot of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) in dog hippocampus. A representative immunoblot is shown for one dog from each group. Each lane represents 20 μg of total protein separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 10% acrylamide. The upper band (60 kDa) is the β -subunit (CaMKII β), and the lower band (50 kDa) is the α -subunit (CaMKII α). The numbers on the right indicate the migration molecular weight markers in kilodaltons (kDa). (B) CaMKII content in the hippocampus. The mean \pm SD of CaMKII β content in the hippocampus is plotted by treatment. The values are the average of three independent determinations of ischemic ($n = 7$), isoflurane (Iso)–ischemic ($n = 8$), control ($n = 3$), and isoflurane–control ($n = 3$) animals. $P < 0.001$ ischemic versus control, and ischemic versus isoflurane–control; $P < 0.01$ isoflurane–ischemia versus control, $P < 0.05$ isoflurane–ischemia versus isoflurane–control, and $P < 0.05$ ischemia versus isoflurane–ischemia (one-way analysis of variance and Neuman-Keuls multiple comparison test).

vascular and respiratory monitoring. In fact, survival for the entire 20-h period after the ischemic event was not assured; only seven of 12 ischemic animals and eight of 13 isoflurane–ischemic animals survived to neurologic evaluation. The neurologic damage incurred was sufficiently debilitating, especially in the ischemic group, that survival beyond 20 h would have required intensive care and constant supervision.

The cardiac arrest model we used has been employed by Rosenthal *et al.*,⁸ who demonstrated that 10 min of normothermic fibrillatory arrest and 20 h of intensive care results in a severe and reproducible neurologically damaged animal with an NDS comparable to that which

we observed here after 8 min of arrest. Our arrest time was limited to 8 min because in preliminary experiments, longer arrest times resulted in extremely poor survival. Safar *et al.*,¹⁷ using a closed-chest canine model of cardiac arrest and resuscitation, demonstrated that 12 min of cardiac arrest and 6 h of intensive care resulted in an NDS of 50% and 55% in two groups of animals. In the group in which blood pressure was elevated and hemodilution and heparinization were employed for the first 6 h after arrest, the NDS improved to 22% by 24 h. In their other group, without those combined treat-

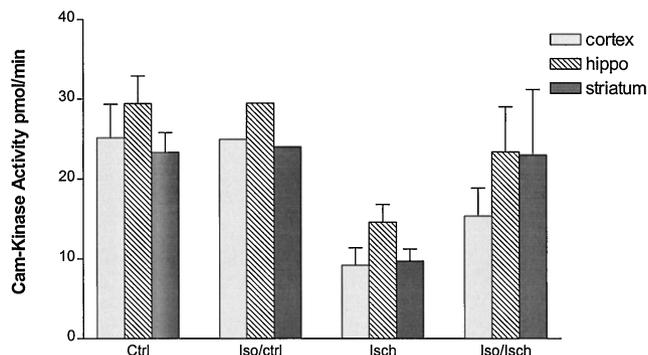


Fig. 5. Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) activity in three areas of the brain as a function of treatment group. Activity is expressed as the rate (in picomoles/minute) of the phosphorylation of a synthetic peptide. Ten micrograms of the P2 fraction of a synaptosomal preparation was incubated at pH 7.2, 30°C for 10 min. Each bar represents the mean activity \pm SEM for three determinations from three control (ctrl) animals: one isoflurane (Iso)–control animal, five ischemic (Isch) animals, and three isoflurane–ischemic animals. Hippo = hippocampus.

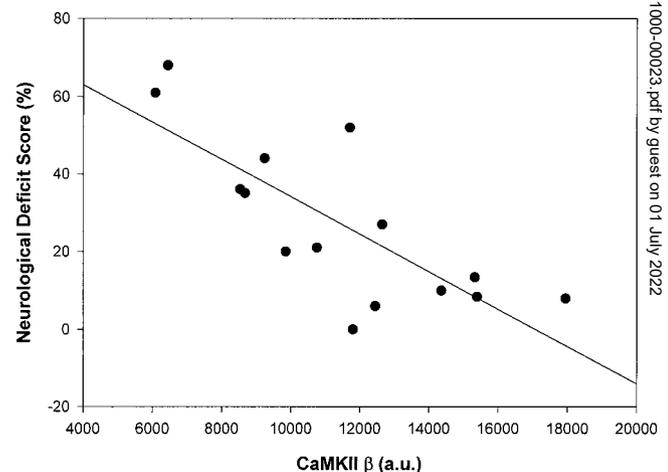


Fig. 6. Relation of neurologic deficit score to hippocampal β -subunit of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII β) content. The average neurologic deficit score is plotted as a function of the mean value of the CaMKII β content for each animal, regardless of treatment group. The data were fit by linear regression, yielding a slope of -6.57×10^{-7} and an intercept of 99.12. The slope is statistically significantly different from zero ($P < 0.001$).

ments, the NDS was 63% by 24 h. In our two experimental groups, MAP greater than 100 mmHg and oxygenation were maintained. Mild hemodilution resulted from blood loss during surgery and fluid replacement, but we did not use heparin.

Although it is possible that the median NDS for the isoflurane-ischemic group could have increased (*i.e.*, neurologic outcome worsened) if the animals had been allowed to survive longer, that appears unlikely based on the time profile published by Safar *et al.*¹⁷ They demonstrated an improvement in the NDS from 22% at 24 h to 2.4% by the day 7 in the treatment group.¹⁷

Additional support for the possible persistence of the neuroprotective effect of isoflurane beyond the 20-h period studied is provided by the recent study of Miura *et al.*¹⁸ Near-complete ischemia in rats was induced by exsanguination to a MAP of 30 mmHg followed by bilateral carotid occlusion. The rats were exposed to 30 min of isoflurane before 10 min of ischemia and for 110 min after the ischemic event. The total isoflurane exposure time was considerably longer than that in our experiment and included 110 min of the reperfusion period. The rats were extubated and maintained in an oxygen-enriched atmosphere for 22 h and then returned to cages for 5 days. At that time, motor scores and histologic analyses were done. An improvement of total motor score and survival of hippocampal CA1 neurons in animals exposed to isoflurane anesthesia during 10 min of near-complete ischemia was observed. Miura *et al.*¹⁸ observed stable neuroprotection resulting from preischemic and postischemic treatment with 1.4% isoflurane; we observed, at a single time point, neuroprotection in a large animal model with preischemic treatment with 1.5% isoflurane.

The recent report by Kawaguchi *et al.*¹⁹ identifies more exactly under what conditions neuroprotection by isoflurane will actually occur and persist. In a focal ischemic model in the rat, Kawaguchi *et al.*¹⁹ showed that isoflurane delayed the development of cerebral infarction. Infarction volume at 2 days was significantly less in isoflurane treated animals but essentially identical in control and isoflurane treated rats at 7 days. However, selective neuronal necrosis, which occurs in neurons that have been moderately ischemic, was statistically significantly decreased at 7 days in the isoflurane-treated animals, demonstrating that isoflurane was effective as a neuroprotective drug in certain aspects of neuronal cell injury. Ischemic injury has been shown to involve at least two separate modes of cell death: Necrotic and apoptotic. Necrotic cell death is a direct and immediate result of severe ischemia (*i.e.*, the absence or limitation of essential substrates), whereas apoptotic cell death is slower in onset, different in mechanism, and apparently triggered by moderate ischemia.

To test the efficacy of isoflurane in our model, it was necessary to use α -chloralose for baseline anesthesia

before thoracotomy in all groups. α -Chloralose is often used in veterinary anesthesia to allow maintenance of cardiovascular reflexes. Although the ischemic group, without isoflurane but with α -chloralose, helped to identify the efficacy of isoflurane in the isoflurane-ischemic group, we cannot rule out the possibility that the positive neuroprotective results with isoflurane might have been more marked in the absence of α -chloralose or depended on the presence of α -chloralose.

Our hypothesis that isoflurane is a neuroprotective drug developed from an experiment we reported in 1992.⁴ Using the same animal model, we demonstrated that after 10 min of normothermic global ischemia, there was a 300% increase in the number of L-type Ca^{2+} channel antagonist binding sites in the cortex of the ischemic animals. Subsequently, halothane and isoflurane were found to decrease the number of L-type antagonist binding sites and also decrease the entry of Ca^{2+} through L-type channels.²⁰⁻²² Therefore, we postulated that pretreatment with isoflurane might prevent the marked increase in L-type Ca^{2+} channel antagonist binding sites, reduce the elevation in $[\text{Ca}^{2+}]_i$ that occurs with ischemia and reperfusion, and improve neurologic outcome. Our previous studies have shown that ligand binding with an L-type Ca^{2+} channel antagonist, although showing a 300% increase in the cortex and striatum immediately following cardiac arrest, showed no statistically significant difference 24 h after arrest compared to prearrest values. In data not shown, radioligand binding studies using the tritiated L-type Ca^{2+} channel antagonist isradipine as the ligand and synaptosomes obtained from the cortex and striatum of ischemic and isoflurane-ischemic animals demonstrated no statistically significant difference when compared with each other or with either of the control groups, as expected from our previous reports.⁵

We examined, by Western blot analysis and enzymatic activity, a protein involved in signaling and neurotransmitter release, CaMKII. CaMKII is a large enzyme that makes up 1% of the protein in the brain and 2% of the protein in the hippocampus.²³ It is found in neuronal cell bodies and dendrites, and exists in high density in postsynaptic nerve endings. It is critical for the regulation of neurotransmitter release and is known to phosphorylate many substrates in the neuron (*e.g.*, synapsin I) involved with neurotransmitter synthesis and release.²⁴ Others have demonstrated a loss of CaMKII activity and immunoreactivity after an ischemic episode. The loss occurs rapidly during and after ischemia and appears to correlate with the number of damaged or dead neurons. CaMKII has the unusual characteristic of losing its immunogenicity soon after exposure to an ischemic event, which appears to relate to a posttranslational change that occurs immediately after ischemia.²⁵⁻²⁶ Others have demonstrated that transgenic mice lacking CaMKII α develop larger cerebral infarcts

after an ischemic event compared with syngeneic mice with intact CaMKII.²⁷

The three elements that have been implicated in the pathophysiology of ischemic and reperfusion injury are elevated intracellular Ca²⁺, excitotoxicity (*i.e.*, the augmented release of neurotransmitters such as glutamate), and the generation of reactive oxygen species (ROS). Although our hypothesis was based on isoflurane's potential to decrease intracellular Ca²⁺, which we now question,²² it is also possible that isoflurane modified the elaboration of or the response to excitotoxic amino acids and attenuated the production of ROS. Schlame and Hemmings²⁸ have demonstrated the inhibition by halothane of glutamate release from isolated synaptosomes derived from normal rat cerebral cortices, and Larsen *et al.*²⁹ demonstrated a similar effect with isoflurane. Bickler *et al.*³⁰ showed that isoflurane pretreatment attenuated the magnitude of the Ca²⁺ transient after exposure of rat cerebral cortical slices to glutamate, and Eilers and Bickler³¹ showed that the baseline Ca²⁺ level was elevated by isoflurane. Nakashima and Todd, studying the effect of isoflurane in a rat global ischemic model, demonstrated that isoflurane had little effect on the rate or magnitude of ischemic elevation of interstitial glutamate increase.³² However, they showed that isoflurane slowed the onset of terminal depolarization by several minutes. Because excess glutamate release does not occur until the onset of terminal depolarization, our improved outcome with isoflurane might have resulted in part from a delay in excess glutamate release. The length of ischemia in our experiment was relatively brief, and if isoflurane did delay the onset of terminal depolarization, excess interstitial glutamate might have reached less toxic levels. In other systems (*e.g.*, the rat hippocampal slice), isoflurane has been shown to reduce glutamate release after energy deprivation.²⁹ It appears that isoflurane does have a number of effects on neurons to modify glutamate release and on the response of neurons to glutamate that may ameliorate ischemic and reperfusion injury.

Another concern is whether isoflurane might be directly or indirectly involved in minimizing the production of ROS that are produced during reperfusion. Little data exists to clarify the effect of isoflurane on the formation of ROS. In a cardiac model of reperfusion, pretreatment with either sevoflurane or isoflurane had no apparent effect on the amount of ROS produced. However, in the perfused liver exposed to hypoxic conditions, contemporaneous perfusion with 2 minimum alveolar concentration isoflurane resulted in a marked reduction in the generation of ROS as measured by the reduction of ferricytochrome c.³³ In a cerebral ischemia model, the effect of isoflurane on ROS has yet to be measured.

We examined the efficacy of isoflurane in a global cerebral ischemia model and found, in a randomized trial

in which the assessment of the outcome measurements, NDS and CaMKII, were blinded, that isoflurane improved neurologic function in association with a preservation of the important neuronal regulatory enzyme CaMKII β . Additional studies are needed to determine whether isoflurane pretreatment will result in persistent neuronal preservation and improved neurologic outcome beyond the 20-h period.

The authors thank Robert Rosenthal, M.D., and Gary Fiskum, Ph.D., University of Maryland Medical School, Baltimore, Maryland, for help in reproducing the animal model; Paul Greengard, Ph.D., and Andrew Czernik, Ph.D., Rockefeller University, New York, New York, for antibodies to CaMKII; Cory Brayton, D.V.M., Hospital for Special Surgery, New York, New York, for her advice in appropriate care of the animals; Margaret Peterson, Ph.D., Hospital for Special Surgery, for her assistance with statistical analysis; and Mr. Brent Pleimann, Hospital for Special Surgery, for his expert technical support.

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