

# Cyclosporin A Enhances Survival, Ameliorates Brain Damage, and Prevents Secondary Mitochondrial Dysfunction after a 30-Minute Period of Transient Cerebral Ischemia

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Received October 8, 1999; accepted April 12, 2000

Cyclosporin A (CsA) has been shown to be efficacious in protecting against ischemic injury after short periods (5 to 10 min) of forebrain ischemia. The present experiments were undertaken to study if a long period of forebrain ischemia (30 min), induced at a brain temperature of 37°C, is compatible with survival and if the brain damage incurred can be ameliorated by CsA. The results showed that animals subjected to 30 min of forebrain ischemia at a brain temperature of 37°C failed to survive after the first 24 h of recovery and showed extensive neuronal necrosis in all selectively vulnerable regions after 1 day of survival. CsA, when injected in combination with an intracerebral lesion to open the blood-brain barrier, markedly prolonged the survival time. CsA-injected animals also showed amelioration of histological lesions, an effect that was sustained for at least 4 days. Experiments with mitochondria isolated from the neocortex and hippocampus showed that state 3 respiratory rates decreased during ischemia, recovered after 1 and 3 h of recirculation, and then showed a secondary decline at 6 h. Administration of CsA prevented this secondary decline. Measurements of neocortical cerebral blood flow showed that there was no secondary hypoperfusion prior to secondary mitochondrial dysfunction, implying that changes in blood flow may not be responsible for the rapidly developing, secondary brain damage. The results thus demonstrate that if brain temperature is upheld at 37°C, a 30-min period of ischemia is not compatible with survival after the first day of recovery, and gross histopathological damage develops within that period. CsA was efficacious in prolonging animal survival, ameliorating brain damage, and preventing the secondary mitochondrial dysfunction. Since CsA blocks the mitochondrial permeability transition pore its action may, at least in part, be on mitochondrial integrity and function. © 2000 Academic Press

**Key Words:** cerebral ischemia; cyclosporin A; mitochondrial function; mitochondrial respiration; cerebral blood flow; pathology.

## INTRODUCTION

Most studies on therapeutic interventions in transient cerebral ischemia have been conducted on one of two major models: the global/forebrain ischemia model and the focal ischemia model, the former usually being induced by two- or four-vessel occlusion and the latter by middle cerebral artery (MCA) occlusion (18). As discussed elsewhere, these two types of ischemia usually differ with respect to the duration and density of ischemia; besides, focal ischemia usually is accompanied by dense ischemia in core areas and less dense ischemia in the so-called penumbra (37). These differences may explain why some pharmaceutical drugs are efficacious in one, but not in the other type of ischemia model. For example, at constant brain temperature the NMDA antagonist MK-801 (dizocilpine maleate) is poorly neuroprotective in forebrain/global ischemia, but clearly anti-ischemic in focal ischemia models (5, 6). Furthermore, CsA, provided that it can be made to pass the blood-brain barrier (BBB), is dramatically neuroprotective both in forebrain ischemia (44, 45) and in focal ischemia (28, 35, 46). FK506, another immunosuppressant, is clearly neuroprotective in focal ischemia (30), but its effect in forebrain ischemia seems to be less marked (14). Interestingly, in hypoglycemic coma CsA is neuroprotective while FK506 is not (16). Since CsA, but not FK506, blocks the mitochondrial membrane permeability transition (MPT) pore, a membrane channel which is opened under adverse conditions such as mitochondrial calcium accumulation and oxidative stress, the results suggest that mitochondrial failure is involved in reperfusion damage following brief periods of forebrain/global ischemia or following hypoglycemic coma (for discussion, see 16, 36, 38, 39). It has also been suggested that focal ischemia, possibly because of its duration, is accompanied by reactions involving an inflammatory or immunological cascade (23).

The present experiments were undertaken to study if the brain damage which is caused by a long period of forebrain ischemia can be reduced by treatment of CsA. To that end, we induced transient forebrain ischemia of 30 min duration. Such long periods of forebrain ischemia have been studied before (12, 19, 34), but the impact of the insult was lessened by the fact that the brain temperature was allowed to fall, increasing the resistance of the brain to the insult (13, 17). In the present experiments, both the body and the brain temperatures were maintained at 37°C during the ischemic insult, rendering the results comparable to those obtained in many studies of forebrain ischemia of brief duration.

## MATERIAL AND METHODS

### *Surgical Procedures*

Male Wistar rats (Simonsen Laboratory, Gilroy, CA), weighing 270–340 g, were fasted overnight prior to the operation day with free access to tap water. Anesthesia was induced by inhalation of 3.5% halothane in a mixture of N<sub>2</sub>O and O<sub>2</sub> (70:30). After intubation with a polyethylene tube (Intramedic PE 200, Clay Adams), the animals were connected to a rodent ventilator. Anesthesia was maintained with 1.5% halothane in N<sub>2</sub>O and O<sub>2</sub> (70:30) during the operation. The neck was incised in the midline. The common carotid arteries were isolated and loose threads were placed for subsequent occlusion. A flexible silicone catheter was inserted into the vena cava via the right jugular vein for withdrawal of blood. The tail artery was cannulated for monitoring of blood pressure and blood gases. A rectal thermometer was inserted and another thermometer was inserted subcutaneously on the skull to monitor the body and head temperatures, which were maintained at 37°C by a combination of a homeothermic blanket control unit and lamp heating. Electroencephalogram (EEG) needles were inserted into the temporal muscles on the skull to monitor the EEG activity during ischemia. Heparin (30 IU/kg) was injected iv before blood sampling. Samples were analyzed for arterial blood gases and pH on a blood gas monitor (278 blood gas system, Ciba Corning Diagnostic Corp., Norwood, MA) and for blood glucose concentration on a blood glucose meter/test strip (One Touch, Lifescan Inc., Milpitas, CA).

### *Experimental Protocol*

Four experimental series were included in the study.

*Experiment I.* To determine animal survival rate after ischemia and CsA treatment 24 rats were subjected to 30 min of forebrain ischemia and their mortality was recorded. Animal groups include vehicle

( $n = 6$ ), a low dose of CsA (10 mg/kg ip,  $n = 13$ ), and a high dose of CsA (50 mg/kg, iv,  $n = 5$ ). In the low-dose CsA group, brain needle lesions were stereotactically made 7 days before ischemia on two sites in the right hippocampus to increase the penetrations of CsA across the BBB. The placements of the needle lesions were at (i) 2.5 mm caudal to bregma, 1.2 mm lateral to midline, and 3.1 mm ventral to dura and (ii) 4.5 mm caudal to bregma, 3.6 mm lateral to midline, and 2.9 mm ventral to dura. It has been reported in the literature that brain lesion disrupts immediately the BBB function and the effect persists for at least 28 days (22). CsA was injected ip from 7 days before ischemia to 4 days after reperfusion in low-dose CsA group based on our previous protocol (27, 44, 45). Brain lesion and CsA pretreatment were also employed in Experiments II–IV. In a recent publication, Friberg *et al.* (16) used a high dose of CsA (50 mg/kg) to overcome the BBB to CsA and proved that a high dose of CsA prevented neuronal damage in rats subjected to hypoglycemic coma (16). Using the same protocol, we injected a high dose of CsA (50 mg/kg) through a tail vein 20 min after the induction of ischemia without brain needle lesion in a separate group (high-dose CsA group).

*Experiment II.* Since Experiment I showed that low-dose CsA increases animal survival, we conducted Experiment II to determine whether brain damage was reduced by CsA. Animals were subjected to 30-min transient ischemia and their brains were fixed with 4% formaldehyde after recirculation under 3.5% halothane anesthesia. The brains of vehicle-injected animals ( $n = 9$ ) were perfusion fixed after 24 h of reperfusion, while those from low-dose CsA animals were fixed after either 24 h ( $n = 6$ ) or 4–6 days ( $n = 7$ ) of recirculation. Rats treated with the high-dose CsA were unable to survive more than 36 h; thus they were not included in the histopathological analysis.

*Experiment III.* In order to determine whether mitochondria are the rate-limiting factor for recovery, we measured mitochondrial function in Experiment III. In this experiment, mitochondrial state 3 respiration (ADP and substrate present, ADP-stimulated oxygen consumption rate, nmol O<sub>2</sub>/min/mg protein) and state 4 respiration (after ADP was depleted, non-ADP-stimulated oxygen consumption rate, nmol O<sub>2</sub>/min/mg protein) were measured in mitochondria isolated from hippocampal and neocortical tissues and respiratory control ratios (RCR) were calculated as the ratio of state 3 respiration divided by “resting” O<sub>2</sub> consumption. Animals subjected to sham operation ( $n = 7$ ), to 30-min ischemia ( $n = 5$ ), and to 30-min ischemia plus 1, 3, or 6 h of reperfusion ( $n = 6$  in each time point) were included. To test whether CsA could preserve mitochondrial function, seven low-dose CsA-injected rats ( $n = 6$ ) were studied after 6 h of reperfusion. The

protocol for low-dose CsA injection was identical to those described in Experiment I.

*Experiment IV.* Previous study in the gerbil has shown that ischemia-induced secondary mitochondrial dysfunction was associated with a secondary cerebral hypoperfusion (1). To confirm whether the secondary deterioration of mitochondrial respiratory function is caused by secondary hypoperfusion and whether the neuroprotective effect of CsA is due its influence on cerebral blood flow (CBF), we monitored laser Doppler flow continuously in a neocortical area from 20 min before the induction of 30-min ischemia to 6 h after recirculation in vehicle-injected and low-dose CsA-injected animals ( $n = 4$  in each group). Low-dose CsA was injected as described in Experiment I.

### *Induction of Ischemia*

Following a 30-min steady-state period, reversible forebrain ischemia of 30 min duration was induced by a combination of bilateral carotid artery clamping and hypotension, as described before (26, 42). Animals for the mitochondrial study were decapitated under anesthesia at the predetermined endpoints, and those used for histopathological evaluation were detached from the ventilator when they regained spontaneous respiration and were then reanesthetized for perfusion-fixation. Rats used for CBF measurement were kept on ventilator during the entire experimental period. At the end of the experiments, the rats were sacrificed by iv injection of 2 ml saturated KCl.

### *Quantification of Brain Damage*

Animals were reanesthetized with 3% halothane, tracheotomized, and artificially ventilated. The thorax was opened and a perfusion needle was inserted into the ascending aorta. The brains were first rinsed with saline for 30 s and then perfusion-fixed with 4% formaldehyde buffered to pH 7.35. The brains were cut coronally in 2- to 3-mm-thick slices, dehydrated with a tissue processor (TP1050, Leica Instruments, Nussloch, Germany), embedded in paraffin with a histoembedder (Leica Instruments), sectioned with a Leica microtome, and stained with celestine blue and acid fuchsin. The sections were double-blind examined with light microscopy at 200 $\times$  magnification by direct visual counting of dead neurons. Surviving neurons were counted in the hippocampal CA1, CA3, and CA4 sectors and the percentage of dead neurons was calculated. Damage in the cingulate cortex, parietal cortex, and thalamus was scored on a four-grade scale where grade 0 meant no observed damage; grade 1, <10%; grade 2, 10–50%; and grade 3, >50% of cell death.

### *Measurements of Mitochondrial Respiratory Functions*

Mitochondria were isolated according to the method described by D'Alecy *et al.* (11). At predetermined endpoints, animals were decapitated and the brains were rapidly removed (within 60 s) and transferred to an ice-cold homogenization buffer which contained 0.32 M sucrose, 1 mM potassium-EDTA, and 10 mM Tris(hydroxymethyl) aminomethane (Trizma Base, Sigma, pH 7.4). The hippocampus and cortex of both hemispheres were dissected on ice. The samples were homogenized in 1 ml homogenization solution using a Potter-Elvehjem homogenizer (Kontes Glass Co., Vineland, NJ) and the mitochondria were separated by centrifugation. The homogenate was centrifuged at 3200g for 10 min. The resulting supernatant was removed and centrifuged at 10,000g for 15 min. The crude mitochondrial pellet was gently rinsed with fresh medium to remove fluffy or loosely adhering material and then resuspended and centrifuged again at 10,000g for 15 min. Respiratory activities of mitochondria in the mitochondrial suspension were measured polarographically with an oxygen microelectrode (Presearch Ltd., Hertfordshire, UK) in a closed and magnetically stirred glass chamber at 28°C (YSI Ltd., Hampshire, UK). The mitochondria (20  $\mu$ l) were added to the chamber with 400  $\mu$ l reaction buffer containing 100 mM KCl, 75 mM mannitol, 25 mM sucrose, 5 mM Tris-phosphate, 0.05 mM potassium-EDTA, and 10 mM Tris (pH 7.4). Substrates that consisted of 4  $\mu$ l of 0.5 M glutamate and 0.5 M malate (neutralized with KOH) were also added. Stimulated (+ADP) respiration was initiated by addition of 0.1 M ADP (typically 0.3  $\mu$ l followed by 0.6  $\mu$ l). Nonstimulated (–ADP) respiration was measured from tracings obtained after ADP was depleted and the rate of oxygen consumption had declined to a constant value. RCR was calculated as the ratio of stimulated to nonstimulated respiration (8). At the end of the measurements, an aliquot (0.2 ml) was removed and stored frozen (–20°C) for subsequent measurements of the protein content with the DC protein assay kit (Bio-Rad, Hercules, CA).

### *Cerebral Blood Flow Measurements*

A laser Doppler perfusion monitor (PeriFlux System 5000, Perimed AB, Stockholm, Sweden) was used to monitor CBF changes through the whole experimental period. With the help of an operating microscope, we carefully placed the laser Doppler probe on the dura, avoiding large vessels. The preischemic CBF level, when stable, was assigned a value of 100% and the value after KCl injection at the end of the experiment of 0%. Ischemic and postischemic CBF levels were obtained by deriving the percentage decrease or increase in CBF.

TABLE 1

Physiological Parameters Measured 5 min before Induction of Ischemia (Mean  $\pm$  SD)

Groups	Blood glucose (mmol/L)	PaCO <sub>2</sub> (mm Hg)	PaO <sub>2</sub> (mm Hg)	Arterial pH	MABP (mm Hg)	Head temp. (°C)	Body temp. (°C)
Experiment I, mortality							
Vehicle	4.3 $\pm$ 0.1	35.6 $\pm$ 4.0	128 $\pm$ 12	7.44 $\pm$ 0.01	120 $\pm$ 1	37.2 $\pm$ 0.1	37.4 $\pm$ 0.4
Low-dose CsA	4.9 $\pm$ 0.7	34.2 $\pm$ 3.1	126 $\pm$ 8	7.45 $\pm$ 0.03	120 $\pm$ 7	37.1 $\pm$ 0.2	37.0 $\pm$ 0.1
High-dose CsA	4.3 $\pm$ 0.3	38.5 $\pm$ 2.8	111 $\pm$ 5	7.43 $\pm$ 0.03	100 $\pm$ 2	37.2 $\pm$ 0.1	37.3 $\pm$ 0.3
Experiment II, histopathology							
Vehicle	4.3 $\pm$ 0.6	39.2 $\pm$ 6.6	112 $\pm$ 17	7.41 $\pm$ 0.06	114 $\pm$ 9	37.3 $\pm$ 0.3	37.4 $\pm$ 0.4
CsA, 1 day	4.5 $\pm$ 0.5	34.0 $\pm$ 1.9	123 $\pm$ 18	7.43 $\pm$ 0.03	125 $\pm$ 14	37.1 $\pm$ 0.1	37.2 $\pm$ 0.2
CsA, 4 days	4.7 $\pm$ 0.4	34.6 $\pm$ 2.0	113 $\pm$ 10	7.45 $\pm$ 0.03	111 $\pm$ 11	37.2 $\pm$ 0.1	37.2 $\pm$ 0.4
Experiment III, mitochondrial respiration							
Ischemia	4.5 $\pm$ 0.6	37.7 $\pm$ 3.5	112 $\pm$ 15	7.45 $\pm$ 0.03	110 $\pm$ 10	37.2 $\pm$ 0.2	37.2 $\pm$ 0.3
1-h recovery	4.6 $\pm$ 0.9	42.9 $\pm$ 4.2	110 $\pm$ 12	7.40 $\pm$ 0.02	110 $\pm$ 8	37.1 $\pm$ 0.1	37.2 $\pm$ 0.1
3-h recovery	4.5 $\pm$ 0.1	39.4 $\pm$ 9.2	108 $\pm$ 16	7.42 $\pm$ 0.05	113 $\pm$ 8	37.1 $\pm$ 0.2	37.3 $\pm$ 0.2
6-h recovery	4.4 $\pm$ 0.3	36.0 $\pm$ 2.8	124 $\pm$ 15	7.44 $\pm$ 0.03	109 $\pm$ 8	37.2 $\pm$ 0.2	37.1 $\pm$ 0.2
6-h recovery + CsA	5.1 $\pm$ 0.8	35.3 $\pm$ 3.2	111 $\pm$ 12	7.44 $\pm$ 0.03	116 $\pm$ 15	37.3 $\pm$ 0.3	37.1 $\pm$ 0.3
Experiment IV, CBF							
Vehicle	5.7 $\pm$ 1.4	39.3 $\pm$ 6.6	105 $\pm$ 12	7.42 $\pm$ 0.05	113 $\pm$ 13	37.3 $\pm$ 0.4	37.1 $\pm$ 0.1
CsA	4.7 $\pm$ 0.5	42.1 $\pm$ 7.3	115 $\pm$ 19	7.38 $\pm$ 0.05	114 $\pm$ 9	37.2 $\pm$ 0.1	37.1 $\pm$ 0.1

### Statistics

Data of physiological parameters, histopathological outcome in the hippocampal sectors, and mitochondrial respiration were analyzed by ANOVA followed by post Fisher's PLSD test. Graded histopathological data were analyzed with a nonparametric Mann-Whitney U test. Paired *t* test was employed for comparison of CBF values obtained during ischemia and those after reperfusion to their preischemic baseline values in vehicle and low-dose CsA group separately, and unpaired *t* test was used for comparison of CBF values between vehicle-injected and low-dose CsA-injected groups at identical sampling time. *P* < 0.05 was considered statistical significance for all the above-mentioned tests.

## RESULTS

### Physiological Parameters

Physiological parameters were well controlled in the experiments and they are given in Table 1. As shown in Table 1, mean blood glucose concentrations were 4.3–5.7 mmol/L. PaCO<sub>2</sub> was maintained close to 34–42 mm Hg, PaO<sub>2</sub> to 110–120 mm Hg, arterial pH to 7.38–7.45, and blood pressure to 100–120 mm Hg. Both core and head temperatures were controlled at 37.0–37.5°C. There were no significant differences between the experimental groups.

### Mortality

The mortality of each group is summarized in Fig. 1. Forebrain ischemia of 30 min duration was a severe

insult that resulted in 100% death in control group after 24–36 h of recirculation. When treated with a low dose of CsA, 2 rats died after 2 days of recovery but 11 of 13 rats survived more than 4 days. Of 11 rats with a low dose of CsA, 10 rats survived after 4 days and 1 survived after 6 days of recovery. Of all the animals treated with the high-dose CsA, 5 of 5 died within 36 h of recovery.

### Brain Damage

At a brain temperature of 37°C, transient forebrain ischemia of 30 min duration is such a severe insult that it resulted in nearly 100% of cell death in the hippocampal CA1, CA3, and CA4 sectors after only 24 h of

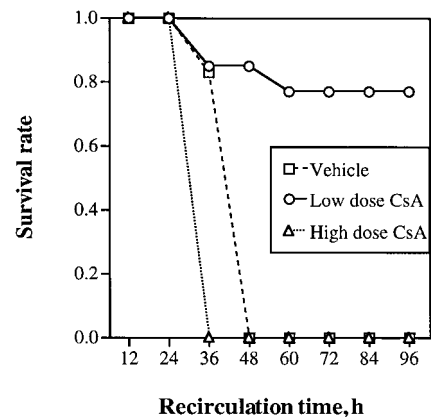
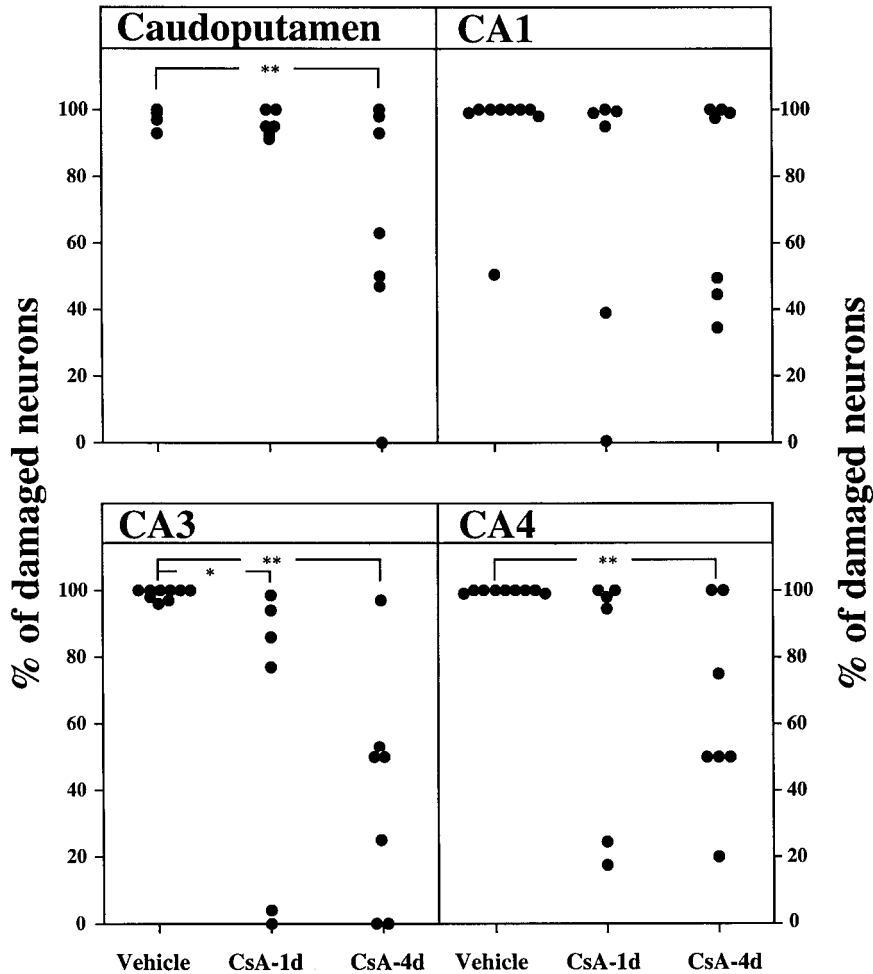


FIG. 1. Line graph shows effects of drugs on animal survival rates. While vehicle or high-dose CsA injection fail to increase the animal survival rate, low-dose CsA with needle lesion increased the animal survival for 4 days.



**FIG. 2.** Graphs showing percentage of damage in the areas of caudoputamen, hippocampal CA1, CA3, and CA4 sectors in vehicle (24 h) and low-dose CsA-treated (24 h and 4–6 days) animals. Each circle represents one animal. \* $P < 0.05$ ; \*\* $P < 0.01$ .

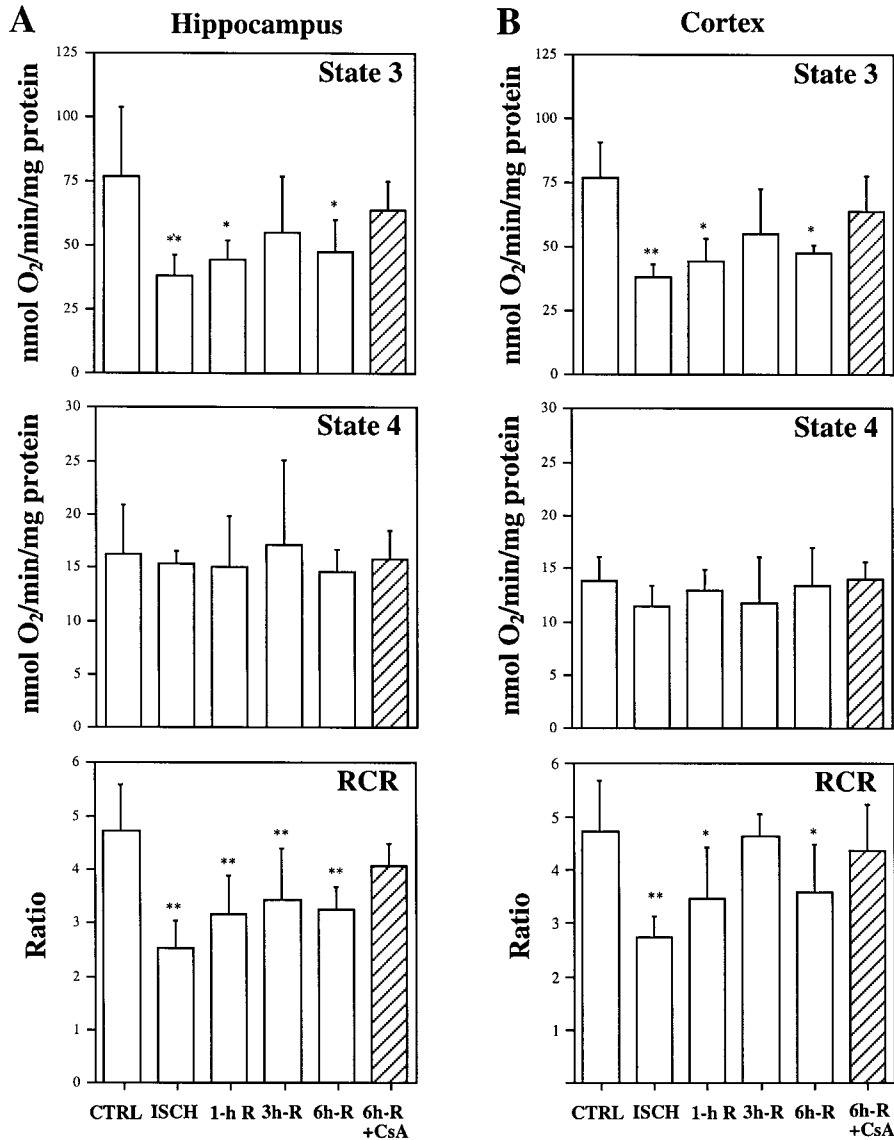
reperfusion (Fig. 2). Damage to the dentate gyrus region was also frequently observed. Close to 100% of the caudoputamen was recruited in the damage process (Fig. 2). Ischemia gave rise to pannecrosis and severe edema in the cingulate and parietal cortex and in the thalamus (Fig. 3). Thus, more than 70% of the neurons were damaged in the cingulate cortex, parietal cortex, and ventroposteriothalamic nuclei. This extensive brain damage was probably the cause of the short survival, necessitating perfusion-fixation after the first day of recovery.

CsA treatment reduced CA1 damage to less than 50% in two of six rats; however, the remaining four rats still had close to 100% damage. In the hippocampal CA3 sector, damage was reduced in CsA-treated animals from a mean level of 99 to 60% ( $P < 0.01$ ). In the CA4 sector, two animals in the CsA 1-day group had less than 30% of damaged neurons while the remaining four animals had 90–100% of damage. The damage in the neocortical areas and thalamus was moderately

ameliorated by CsA. Thus, damage was reduced to less than 50% in at least half of the animals (see Fig. 3).

The results thus demonstrate that CsA ameliorated the brain damage as assessed after 1 day of recovery. The question arose whether this was due to a delay in the maturation of the damage or if it involved a sustained sparing of neurons. To respond to this question, we perfusion fixed CsA-treated animals after 4 days of recovery. The results were as follows: in caudoputamen, damage was reduced from 99 to 64% ( $P < 0.001$ ), and in the hippocampal CA1 sector three rats had less than 50% of damage; however, the remaining four had nearly 100% damage, in the hippocampal CA3 sector one had close to 100% damage, four had 50% or below, and two had no observable damage. Similar effects of CsA were observed in the hippocampal CA4 sector. No infarction was observed in the parietal cortex in any of the CsA-treated animals. Five of seven rats had mild to moderate damage in cingulate cortex, and the remaining two rats had no observable damage. In the thala-

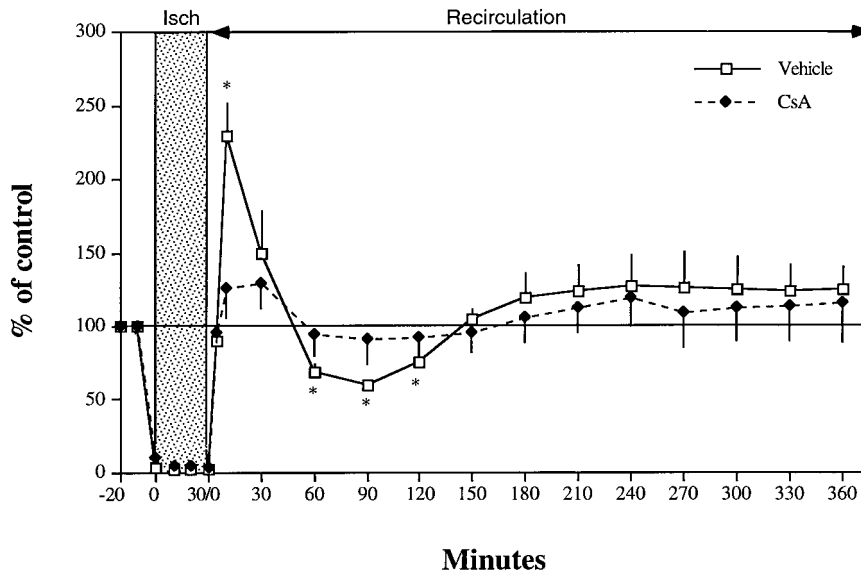




**FIG. 4.** Mitochondrial state 3 and state 4 respiratory rates, and RCR in brain tissue isolated from the hippocampus (A) and the neocortex (B). State 3 respiratory rate and RCR were initially reduced during ischemia and recovered gradually after 3 h of reperfusion. Secondary deterioration occurred after 6 h of reperfusion. CsA prevented secondary mitochondrial deterioration. \* $P < 0.05$ , \*\* $P < 0.01$  vs control.

ischemia is combined with a fall in brain temperature. However, when brain temperature is maintained at 37°C during ischemia, 30 min of global/forebrain ischemia must be considered long term since the insult is so severe that virtually no animals survive beyond 1 day after reperfusion. The primary objective of the study was to explore whether, in the absence of a decrease in brain temperature, resuscitation after long periods of ischemia may be followed by reasonable recovery of brain functions. To that end, a 30-min period of forebrain ischemia was induced at a brain temperature of 37°C. At that brain temperature, though, reversible forebrain ischemia of 30 min duration proved to be such a severe insult in rats that none of the

animals could survive more than 2 days after reperfusion. The question posed then was whether pharmacological intervention might improve recovery and ameliorate the brain damage incurred. According to previous publications from this laboratory, CsA is a potent neuroprotective agent against ischemia of the forebrain/global type (27, 44–46). Thus, CsA almost eliminated cell damage completely in the hippocampal CA1 sector after 7 or 10 min of forebrain ischemia in normoglycemic rats (44, 45) and prevented postischemic seizures after 5 or 10 min of ischemia in hyperglycemic animals (27). However, because of a low BBB permeability of CsA, probably due to active efflux mediated by P-glycoprotein (43), a cerebral needle lesion had to



**FIG. 5.** Line graph showing neocortical cerebral blood flow measured by laser Doppler. Preischemic baseline level was set to 100%. CBF was reduced to 5% during ischemia and increased about 230% of control values within 30 min after reperfusion. CBF was then reduced to 60–70% of control between 1 and 2 h of reperfusion. A mild but constant hyperemia was observed after 3–6 h of reperfusion. CsA injection tended to ameliorated the hyperemia and hypoperfusion observed in vehicle group; however, such amelioration did not reach statistical significance compared to vehicle group. \* $P < 0.01$  vs baseline.

be introduced to enhance the BBB permeability of CsA, given ip in a dose of 10 mg/kg. In the present experiments we first employed the same protocol for CsA delivery; as an alternative, however, we injected CsA iv in a higher dose (50 mg/kg), a dose which has been shown to effectively protect against hypoglycemia-induced brain damage (16). In this paradigm, CsA was given 20 min after the induction of ischemia. The results showed that only when the BBB was lesioned, could intravenously given CsA prolong animal survival after ischemia. Thus, animals treated with a low ip dose of CsA in combination with BBB breakdown survived for many days and could be fixed by perfusion after 4–6 days of recovery. However, animals treated with the high-dose CsA without BBB breakdown did not survive beyond the first 2 days of recirculation. It is not clear whether the rats died because of the toxicity of the high CsA dose or because of low efficacy due to poor penetration of CsA into the brain. Under physiological conditions, the brain uptake index for CsA is almost the same as for sucrose and mannitol (2). Although 50 mg/kg of CsA iv has been shown to be efficacious in ameliorating brain damage incurred in hypoglycemic coma (see above), this dose has been shown to be too toxic for rats to be tolerated when they were subjected to transient middle cerebral artery occlusion (46). Interestingly, though, a single injection of a low-dose CsA (10 mg/kg) into the carotid artery after reperfusion following 2 h of transient MCA occlusion markedly reduces infarct volume (46). Preliminary data from this laboratory also show that CsA penetration to

the brain is higher in rats with a brain needle lesion than those without a needle lesion (Uchino *et al.*, unpublished data).

Although animal survival rate and duration of survival were increased in CsA-injected animals in Experiment I, it was not clear whether the ischemic damage was reduced by CsA. Thus, we designed the second experiment in which we perfused rats in control and low-dose CsA groups and evaluated histopathological outcomes. Control animals showed nearly 100% damage in the CA1 and CA3 sectors, the caudoputamen, the cingulate and parietal cortex, and the thalamus after 24 h of recovery. Obviously, the extensive cerebral damage was the reason why survival time was brief. In addition to its effect in improving survival rate, CsA ameliorated brain damage in all brain regions except the CA1 sector. The results thus suggest that CsA, when allowed to pass the BBB, can markedly ameliorate the damage caused by transient ischemia of long duration.

Recent studies have shown that the mitochondrion plays a pivotal role in mediating ischemic cell damage in cardiac tissue (3, 4, 9, 20) and in the brain (for recent reviews from our own laboratory, see 32, 39). Under adverse conditions, such as mitochondrial calcium accumulation, and oxidative stress, mitochondria are believed to assemble a permeability transition pore, referred to as an MPT, in the inner membrane (3, 24, 47). The MPT is believed to cause disruption of mitochondrial membrane potential, cessation of ATP production, generation of oxidative reactive species, release of

cytochrome *c*, and, eventually, to cell death either through apoptotic or necrotic pathways. The MPT is inhibited by CsA (10, 15, 21). In order to assess if the mitochondrion is the rate limiting factor for recovery, we measured mitochondrial respiration in isolated mitochondria in both hippocampal and neocortical regions. Following a reduction of state 3 respiration and RCR during ischemia, the state 3 respiration and RCR returned close to normal after 3 h of recovery. This proves that recirculation leads to resumption of mitochondrial respiratory activities before secondary deterioration is observed in the form of delayed tissue damage. After 6 h of recovery, secondary mitochondrial failure developed. CsA prevented secondary mitochondrial failure in both the hippocampus and the neocortex, possibly by inhibiting MPT formation. Such secondary mitochondrial dysfunction has been previously observed after 2 h of recirculation following 30 min of global ischemia in the gerbil (1), after 6–24 h of reperfusion in the rat, and after 4–6 h of recirculation following 2 h of focal ischemia in the rat (25, 30, 40, 41). Further analyses revealed that, in the gerbil experiments, the immediate inhibition of mitochondrial function was due to the inhibition of complex I, complex II–III, and complex V activity, while the secondary mitochondrial dysfunction appeared to be due to inhibition of complex IV, i.e., cytochrome oxidase (1). However, no such inhibition was observed in rats subjected to long periods of focal ischemia (7).

Almeida and colleagues reported that CBF initially decreased during ischemia and recovered to control value after 30 min of recirculation and then fell again after 120 min of recirculation in gerbils subjected to 30 min of global ischemia (1). Thus, the secondary hypoperfusion might be, at least partially, responsible for secondary mitochondrial dysfunction. To confirm whether this is the case in our experimental paradigm, we monitored CBF by the laser Doppler technique. The results demonstrated that CBF was initially decreased during ischemia and that it was immediately increased over control value during the first 30 min of recirculation. A mild secondary decrease occurred after 60–120 min of perfusion. Then the CBF increased to 120–130% of control value and stayed at this high level after 6 h of recirculation. It is thus not likely that secondary CBF decrease is responsible for the secondary mitochondrial dysfunction which developed after 6 h of reperfusion. CsA injection tended to ameliorate the short-lasting hyperemia and hypoperfusion observed in vehicle group; however, such amelioration did not reach statistical significance when compare to vehicle group.

The present results demonstrate that the model used, i.e., 30 min of dense forebrain ischemia at preserved body and brain temperatures (37°C) yields severe brain damage, affecting large neocortical areas,

which is so massive and evolves so quickly that recovery is limited to 24–36 h. It is may be argued that lamp heating would have tended to maintain the cerebral cortex at 37°C but deeper tissues would have been less effectively temperature controlled and that may account for the massive damage in the neocortex. However, since severe damage was also observed in deep structures such as the thalamus, this possibility is unlikely. Besides, the major loss of heat from the ischemic brain is through the thin coverings over the neocortex (29). The CBF results obtained, and those pertaining to mitochondrial function, suggest that the damage incurred is more related to mitochondrial failure than to microcirculatory compromise. In this respect, the results seem akin to those obtained in focal ischemia of 2 h duration in rats (25, 30). A comparison between the present results and those reported by others for 30 min of forebrain ischemia in rats (31, 33), in which brain temperature was not maintained at 37°C, underscore the protective effects of the spontaneous fall in brain temperature which occurs in animals maintained at constant body temperature. The differences in neocortical damage are particularly impressive. The question arises if the protective effect of CsA is additive to that of the fall in temperature and if CsA is equally efficacious when administered in the recovery period after forebrain ischemia of long duration. Since the results may yield important information on the mechanisms of action of both CsA and lowered temperature, this issue is at present being studied in the laboratory.

## ACKNOWLEDGMENTS

The present study was supported by research grants from US Navy, Centaur Pharmaceuticals, and The Queen Emma Research Foundation.

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