We investigated the ability of postischemic insulin administration to modify the structural and neurobehavioral consequences of cerebral ischemia in rats. Forebrain ischemia was induced in fed rats by combining controlled systemic hypotension with bilateral carotid artery clamping for 10½ minutes. Following clamp release, one group of five rats was given insulin (2 IU/kg s.c. b.i.d.) for 1 week. An ischemic-control group of six rats received no postischemic treatment. A sham-ischemia group of rats was used as a behavioral control. Throughout the recovery period until sacrifice, the drinking water of all rats was supplemented with 25% glucose. Rats were trained on two water maze place navigation tasks 1–2 months after ischemia. Escape latencies and swim patterns were recorded. Performance in the insulin-treated group was better than that in the ischemic-control group (p<0.05) on both tasks and did not differ significantly from that of the sham-ischemia group. Improvement in behavior correlated with a significant reduction in CA1 hippocampal necrosis in the insulin-treated group (p<0.05). Our findings demonstrate that postischemic treatment with insulin improves neurobehavioral performance in addition to lessening ischemic neuronal necrosis.

(Treatment for cerebral ischemia should ideally reduce neurologic and cognitive dysfunction as well as preserve tissue morphology. Our recent finding that insulin-induced hypoglycemia following transient forebrain ischemia in rats significantly reduces structural brain damage1 prompted us to investigate the long-term behavioral effect of reducing postischemic blood glucose levels. The CA1 sector of the hippocampus is susceptible to ischemic insult.2 Memory function in turn is highly susceptible to CA1 neuronal loss.3-6 Cerebral ischemia can induce a persistent amnestic syndrome secondary to CA1 neuronal loss in rats7 and humans.3,8,9 Using water maze–based place navigation tasks, we studied the effect of insulin administration on postischemic spatial learning dysfunction in rats.)

Materials and Methods

The ischemia model we used was based on that of Smith et al10 and has been described.1 Seventeen male Wistar rats (Charles River Breeding Centre, St. Constant, Canada) weighing 250–450 g, which had been allowed free access to food and water, were anesthetized with 4% halothane in 2:1 N2O:O2. The rats were then intubated and ventilated throughout the procedure with a Starling-type ventilator (Harvard, Edenbridge, England). The ventral tail artery was cannulated and connected to a Statham transducer (P50, Gould, Cleveland, Ohio) for continuous monitoring of mean arterial blood pressure (MABP). A Silastic catheter (Silastic tubing, Dow Corning, Midland, Michigan) was inserted into the right jugular vein and positioned with its tip in the inferior vena cava. The common carotid arteries were exposed and dissected free of the carotid sheaths and sympathetic chains. Subcutaneous unipolar parietal electrodes were inserted for electroencephalogram recording during ischemia. Halothane was then stopped and 0.3 ml succinylcholine chloride (2 mg/ml; Sigma Chemical Co., St. Louis, Missouri) was given intravenously. Twenty minutes elapsed for physiologic stabilization. Trimethaphan camsylate (5 mg/ml, 0.4 ml i.v.; Roche, Etobicoke, Canada) was then given, and controlled exsan-
guination into a prewarmed 10-ml heparinized syringe was begun. When MABP reached 50 mm Hg, the common carotid arteries were clamped. Ischemia was timed from the onset of electrocerebral silence, which occurred within 10–15 seconds after carotid occlusion. Mean±SEM MABP was held at 50±5 mm Hg by withdrawal or reinfusion of blood through the central venous catheter. After 10½ minutes of electrocerebral silence, the carotid clamps were removed, the shed blood was reinfused, and the rats were allowed to awaken.

Arterial blood gases and pH were measured before and after ischemia using an automated blood gas analyzer (1304 pH/blood gas analyzer, Instrumentation Laboratories, Milan, Italy). Blood glucose concentrations were measured with Dextrostix (glucose oxidase reaction) in a reflectance glucometer (Ames glucometer, Ames Division, Miles Laboratories, Elkhart, Indiana). Core body temperature was maintained at 37° C using a thermistorregulated heating blanket (Harvard).

Rats were randomly assigned to one of three groups. All rats were given glucose intraperitoneally 20 minutes before induction of ischemia (Figure 1). During recovery from ischemia, the six rats in the ischemic-control group were given 1.5 ml s.c. saline. The five rats in the insulin-treated group were given 2 IU/kg s.c. insulin as a mixture of one third crystalline insulin (Sigma) and two thirds extended insulin zinc suspension (Ultralente, Connaught Laboratories, Swiftwater, Pennsylvania); the blood glucose levels of the insulin-treated rats were measured twice daily, and insulin was given as required to maintain blood glucose concentrations between 2.9 and 5 mM. In the six rats assigned to the sham-ischemia group, the ventral tail artery was exposed and resutured, the right jugular vein was tied off, and the common carotid arteries were exposed but not occluded.

The drinking water of all rats was supplemented with 25% glucose following recovery until sacrifice to simulate continued feeding since rats in previous experiments had shown a tendency to become spontaneously hypoglycemic during the first 2 days following ischemia due to poor feeding.

Rats developing seizures during the recovery period were not excluded from the study to avoid selection bias.

Spatial learning and memory were tested in a water maze using two place navigation tasks that have been described in detail.4,11 Briefly, a circular white swimming pool 146 cm in diameter x 45 cm in height was filled to a depth of 25 cm with water at 18° C rendered opaque by the addition of 1,000 g powdered skim milk. All behavioral testing was conducted by one of the authors who was blinded to rat group. Swim latencies were recorded with a stopwatch, and swim patterns were recorded from above on a video recorder. A 13 x 13-mm Plexiglas platform was placed 14 mm below the surface of the water so that it was invisible to the rat while swimming.

Both place navigation tasks used to assess spatial learning required the rat to learn the location of a hidden escape platform in relation to distal room cues. In the place task, the platform was hidden at the same location each day. In the learning set task, a more demanding test requiring the rat to form a new place response each day, the platform was repositioned daily according to a designated sequence.11 Four starting positions were used in both tasks, based on the four cardinal points (north, south, east, and west), and these cardinal points divided the pool into four quadrants.
Training with scoring was begun 6–8 weeks after ischemia. During the first 10 days, the place task was administered. Each rat was trained for two blocks of four trials each day (i.e., eight trials per day for 10 days). The escape platform was located in the center of the southwest quadrant of the pool each day. At the beginning of each trial, the rat was released facing the wall of the pool from one of the starting positions, according to a pseudorandom sequence that was different for each rat. All rats were exposed to all starting positions on each day of training. Having located the platform, the rat was permitted to remain on it for 10 seconds. If the rat swam directly to the platform, staying within an 18-cm-wide path from the starting position to the platform, performance was scored as correct. If the rat deviated from this route at any point, an error was scored for that trial. If the platform was not located after 60 seconds, the trial was ended by removing the rat from the water and an error was scored for that trial.

During the next 6 consecutive days, the learning set task was administered; the trials were administered in pairs, and each rat received 16 trial pairs per day. Six different platform locations were used (Figure 2, inset) and the platform was moved each day to one of these locations in sequence. On the first of each trial pair, having located the platform, the rat was permitted to remain there for 5 seconds. The second trial of a pair was given immediately after the 5-second stay on the platform, and the same starting position was used. At the end of the second trial, the rat was returned to a holding cage for approximately 5 minutes (during which the remaining rats were tested) before the next pair of trials, from a new starting position, was given. Errors and latencies were recorded for each trial. A trial was terminated and an error was scored for the trial if, after 60 seconds, a rat failed to reach the platform.

The rats were killed by perfusion-fixation via a transcardiac aortic catheter under halothane and nitrous oxide anesthesia 12–15 weeks postischemia. The systemic circulation was flushed for 30 seconds with normal saline, then the rat was perfused with 4% phosphate-buffered formaldehyde (pH 7.3) for 15 minutes. The brain was removed the following day and was cut coronally into 2.8-mm slices that were then processed overnight in graded ethanol and xylol. The slices were embedded in paraffin and subserially sectioned at 8 μm to obtain sections from the cerebral cortex, caudate, hippocampus, thalamus, brainstem, and cerebellum. The subserial sections were stained with 1% acid fuchsin and 0.1% cresyl violet and examined by light microscopy. Hippocampal neuronal necrosis was quantified by counting the morphologically normal CA1 pyramidal neurons throughout the septotemporal extent of the hippocampus. The number of viable neurons was subtracted from the total number normally present at each level to obtain percent CA1 neuronal necrosis for each rat.

Statistical significance was accepted at p<0.05. Physiologic data, including preischemic, intraischemic, and postischemic MABP, arterial blood gases, arterial pH, and blood glucose levels, for the ischemic-control and insulin-treated groups were compared using Student’s t test. Behavioral and neuropathologic data for all three groups were compared using analysis of variance and Student’s t test, respectively.

**Results**

No significant differences between the ischemic-control and insulin-treated groups in mean preischemic blood glucose levels, preischemic and postischemic PaO₂, PaCO₂, and arterial pH, clamp times, or intraischemic MABP were found (Table 1). Mean postischemic blood glucose level (averaged from 2 hours to 7 days postischemia) in the ischemic-control group (9.58±1.16 mM) was significantly higher than that in the insulin-treated group (4.16±0.04 mM) (t=5.21, p<0.01; Figure 1).

Behavioral performance of the rats in the insulin-treated group was significantly better than that of
TABLE 1. Physiologic Parameters in Rats Subjected to Transient Forebraln Ischemia

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=6)</th>
<th>Insulin-treated (n=50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_{aCO}_2$ (mm Hg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preischemia</td>
<td>131±9</td>
<td>146±11</td>
</tr>
<tr>
<td>Postischemia</td>
<td>133±8</td>
<td>133±15</td>
</tr>
<tr>
<td>$P_{aco}_2$ (mm Hg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preischemia</td>
<td>33±1</td>
<td>30±2</td>
</tr>
<tr>
<td>Postischemia</td>
<td>34±2</td>
<td>31±3</td>
</tr>
<tr>
<td>Arterial pH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preischemia</td>
<td>7.41±0.01</td>
<td>7.42±0.02</td>
</tr>
<tr>
<td>Postischemia</td>
<td>7.36±0.03</td>
<td>7.37±0.03</td>
</tr>
<tr>
<td>Clamp time (min:sec)</td>
<td>10:38±5</td>
<td>10:34±4</td>
</tr>
<tr>
<td>Mean arterial blood pressure (mm Hg)</td>
<td>50.8±1.0</td>
<td>49.7±2.0</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preischemia</td>
<td>15.1±1.4</td>
<td>15.6±1.2</td>
</tr>
<tr>
<td>2 hr postischemia</td>
<td>10.58±2.10</td>
<td>3.93±0.57</td>
</tr>
<tr>
<td>Mean (Days 1–7)</td>
<td>9.16±1.03</td>
<td>4.21±0.99</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

On the learning set task, there were significant group effects for errors and latency. The mean latency to locate the platform and the mean error score per trial for the three groups are shown in Figure 2. The ischemic-control group was significantly impaired on both swim latency ($F_{1,9}=5.67$, $p<0.05$) and errors ($F_{1,9}=13.54$, $p<0.01$) compared with the insulin-treated and sham-ischemic groups, which did not differ from each other on either measure. All three groups showed improved performance on the task across trials ($F_{1,135}=8.94$, $p<0.01$) and across days ($F_{5,41}=4.14$, $p<0.01$).

The brains of all rats were examined after they had completed behavioral testing. Damage was restricted to the insulin-treated and ischemic-control groups and was largely confined to the hippocampus (Figure 3). Necrotic neurons were still visible even 12–15 weeks postischemia as acidophilic outlines in the region of the CA1 pyramidal cell band. CA1 neuronal necrosis was associated with dendritic phagocytosis in the stratum radiatum of the hippocampus. At levels showing minimal neuronal loss and in which acidophilic neurons could not be identified, dendritic phagocytosis was used as a semiquantitative marker of CA1 neuronal death. At levels showing greater neuronal necrosis, the remaining pyramidal neurons were counted and subtracted from the total pyramidal cell counts normally present at each level. The hippocampal CA3 pyramidal cells and dentate granule cells were uniformly unaffected. Mean±SEM CA1 cell loss (18.5±13.3%) in the insulin-treated group was significantly less than that in the ischemic-control group (58.2±7.7%; $t=2.4$, $p<0.05$). Neuronal loss was largely confined to the septal region of the CA1 sector of the hippocampus in the ischemic-control group, where a significant gradient in neuronal loss was found between the septal (81.4±8.4%)
and the temporal (28±18.5%) hippocampus. In the insulin-treated group, however, this gradient was not significant.

Extrahippocampal telencephalic damage was absent in all but one ischemic-control rat, which showed scattered neuronophagic figures in the suprolateral convexity of the parietal cortex. Focal infarcts of the mesencephalic tectal regions and the substantia nigra pars reticulata were found in three ischemic-control rats (two of which had had seizures during the recovery period) and in two insulin-treated rats (both of which had had seizures).

Discussion

Our results demonstrate that insulin, given early during the recovery period following transient forebrain ischemia, significantly reduces ischemia-induced deficits in place learning. The behavioral improvement indicates that the reduction in hippocampal CA1 necrosis associated with postischemic insulin administration is of sufficient magnitude to lead to an objective improvement in brain function.

Rats subjected to forebrain ischemia develop severe damage to the CA1 region of the hippocampus. The hippocampus plays an important role in memory and learning functions, and rats demonstrate impaired acquisition and performance of learning tasks following hippocampal injury. A similar amnestic syndrome often occurs in humans following transient global brain ischemia as a consequence of a selective loss of pyramidal cells in the CA1 field of the hippocampus.

Spatial learning and memory can be effectively examined in the Morris water maze. Severe and persistent deficits in place navigation are found after hippocampal ablation and ischemic injury.

The mechanism of selective neuronal death following ischemia is beginning to be understood. Experimental evidence has linked an ischemia-induced release of the excitatory amino acid glutamate to the development of selective necrosis of CA1 pyramidal cells. Ischemia-induced release of glutamate apparently occurs in humans as well and may underlie the selective damage seen in the human hippocampus after ischemic insults.

Insulin, which crosses the blood–brain barrier by receptor-mediated transcytosis, inhibits spontaneous depolarization of CA1 hippocampal neurons through its action as a central inhibitory neuromodulator. The administration of insulin following transient brain ischemia might thus reduce CA1 neuronal necrosis by inhibiting excitotoxin-mediated neuronal necrosis.

Ischemic damage was almost entirely confined to the hippocampus whereas, in a previous study using the same model, we found a high incidence of selective neuronal necrosis and infarction in other brain regions. The apparent discrepancy in the extent and pattern of damage found in our two studies is due to the different inclusion criteria used. In the first study we included all rats irrespective of survival. In the present study, however, we by necessity included only indefinitely surviving rats. The differing inclusion criteria would have resulted in a bias against more extensively damaged rats in the present study, due to postischemic mortality. This likely explains the differing degrees and pattern of ischemic damage found in the two studies.

Focal infarction of midbrain nuclei (substantia nigra pars reticulata, superior and inferior colliculi) were seen in five rats in this study, in four of which postischemic seizures occurred. We have previously reported a significant correlation between postischemic audiogenic seizures and focal brainstem infarcts. Flurothyl-induced epilepsy characteristically produces infarction of the substantia nigra. Midbrain structures (reticular formation, inferior colliculus) play essential roles in the elaboration and propagation of audiogenic seizures. The focal infarcts of midbrain structures seen in these rats is thus likely a manifestation of postischemic epilepsy rather than of ischemia per se.

The improvement in behavioral performance that occurred in association with postischemic insulin administration has important clinical implications and provides a basis for further experimental studies. Such studies will need to define the maximum postischemic interval during which insulin administration remains therapeutically effective, the optimal postischemic insulin dosage, and its mode of action in reducing ischemic brain damage.

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