Prevention of Postischemic Canine Neurological Injury Through Potentiation of Brain Energy Metabolism by Acetyl-L-Carnitine

Robert E. Rosenthal, MD; Rita Williams, BA; Yolanda E. Bogaert, BS; Pamela R. Getson, PhD; and Gary Fiskum, PhD

Background and Purpose: Mechanisms of ischemia/reperfusion brain injury include altered patterns of energy metabolism that may be amenable to pharmacological manipulation. The purpose of this study was to test the effectiveness of postischemic acetyl-L-carnitine administration on potentiation of metabolic recovery and prevention of neurological morbidity in a clinically relevant model of complete, global cerebral ischemia and reperfusion.

Methods: Neurological deficit scoring as well as spectrophotometric and fluorescent assays of frontal cortex lactate and pyruvate levels were used in a canine model employing 10 minutes of cardiac arrest followed by restoration of spontaneous circulation for 2 or 24 hours.

Results: Dogs treated with acetyl-L-carnitine exhibited significantly lower neurological deficit scores (p=0.0037) and more normal cerebral cortex lactate/pyruvate ratios than did vehicle-treated control animals.

Conclusions: Postischemic administration of acetyl-L-carnitine potentiates normalization of brain energy metabolites and substantially improves neurological outcome in a clinically relevant model of global cerebral ischemia and reperfusion. (Stroke 1992;23:1312-1318)

Key Words • brain injuries • cerebral ischemia • metabolism • dogs

In the United States, 540,000 people die annually as a result of complications from myocardial infarction; 350,000 of these individuals die as a result of cardiac arrest before reaching the hospital.1 With the growth of emergency medical services throughout the country, prehospital cardiac resuscitation is attempted for many victims of cardiac arrest. However, even if an individual survives initial resuscitative efforts, ischemic damage to the brain occurring during cardiac arrest and reperfusion often results in neurological morbidity or mortality. In one recent study of 262 initially comatose survivors of cardiac arrest, for example, 79% of the patients had died within 1 year; the authors conclude that cerebral failure was the cause of death in 37% of cases.2

During periods of normal cerebral perfusion, viability of neuronal tissue is maintained through oxidative metabolism of glucose, with resultant production of adenosine triphosphate (ATP). Under aerobic conditions, more than 80% of the brain's ATP is generated by mitochondrial oxidative phosphorylation.3,4 The anoxia that accompanies complete cerebral ischemia results in complete cessation of aerobic metabolism, followed by rapid depletion of glucose as a result of anaerobic glycolysis.5 Inhibition of energy metabolism leads to an effectively complete elimination of ATP within the first several minutes of ischemia, resulting in a loss of neuronal ionic homeostasis with resultant cellular efflux of potassium, and influx of sodium and calcium.3,6 It has been suggested that calcium influx plays a major role in the activation of various degradative cellular processes that may eventually lead to neuronal death.7,8

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For brain tissue to recover from this ischemic insult, mitochondria must be rapidly reenergized during reperfusion so that ATP can once again be produced and ionic homeostasis restored. Restoration of normal energy metabolism may, however, be initially retarded due to the mitochondrial injury that occurs during even brief periods of ischemia.9 Recent evidence also demonstrates that neuronal oxidative metabolism may be suppressed long after initiation of reperfusion. It has been shown, for example, that activity of pyruvate dehydrogenase is suppressed for up to 4 hours after 20 minutes of cerebral ischemia in gerbils.10 This leads to a secondary, late failure of brain energy metabolism in this model, with increased levels of brain lactate and significantly lowered levels of brain ATP seen at 4 hours. To maintain neuronal viability after resuscitation from cardiac arrest, it appears imperative to promote rapid and prolonged cellular reenergization in an attempt to counteract the degradative processes begun during ischemia.

From the Departments of Emergency Medicine (R.E.R., G.F.), Biochemistry and Molecular Biology (R.E.R., R.W., Y.E.B., G.F.), and Pediatrics (P.R.G.), The George Washington University Medical Center, Washington, DC.

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Address for correspondence: Dr. Robert E. Rosenthal, The George Washington University Medical Center, 2140 Pennsylvania Avenue, NW, Washington, DC 20037.

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Theoretically, various pharmacological agents might serve to speed up reenergization after ischemic neuronal injury. One such agent, acetyl-L-carnitine, is a naturally occurring substance found in human tissues. It is formed in mammalian tissues through reversible acetylation of carnitine by carnitine acetyltransferase and is the most widely distributed short-chain ester of carnitine. Both acetyl-L-carnitine and carnitine acetylcarnitine are present throughout the central nervous system, demonstrating similar areas of concentration throughout the brain. It has been demonstrated in several different models that L-carnitine as well as acetyl-L-carnitine can increase the metabolic rate of mitochondria, thus improving mitochondrial oxygen utilization in these systems, making this a potentially useful drug to counteract some of the effects of ischemic injury to the brain. This concept is further supported by other in vitro studies indicating that carnitine can stimulate aerobic pyruvate metabolism, thereby inhibiting flux of pyruvate to lactic acid, a known potentiator of ischemic injury. Although some evidence suggests a protective role for carnitine derivatives administered during cardiac ischemia, the effects of carnitine esters on ischemic brain injury have not been investigated; ambiguous results have been reported for unesterified L-carnitine in a model of cerebral ischemia. This study was designed to examine the potential neuroprotective effects of acetyl-L-carnitine after resuscitation from cardiac arrest.

Materials and Methods

All animal experiments were conducted in accordance with guidelines established by the Institutional Animal Care and Use Committee of the George Washington University Medical Center. Thirty-six adult female beagles were initially anesthetized with 17.6 mg/kg Bio-Tal (thiamylal sodium for injection, USP). Prolonged anesthesia was then induced with an infusion of alpha chloralose (75 mg/kg). Antibiotic prophylaxis was provided with 250 mg i.v. ceftriaxone. After establishment of appropriate anesthesia, animals were intubated endotracheally with a 7-mm endotracheal tube and subjected to controlled ventilation on room air (Bennett Respiration Co. MA-1 ventilator, Los Angeles). Initial ventilator settings were as follows: FiO₂, 21%; tidal volume, 18 ml/kg; and respiratory rate, 20 breaths per minute. Ventilator settings were later adjusted to set Pco₂ between 25 and 35 mm Hg (IL 1302 blood gas analyzer, Instrumentation Laboratories, Italy). After endotracheal intubation, muscle paralysis was induced and maintained with 0.1 mg/kg per hour i.v. pancuronium bromide. Cut-down catheters were placed in the left femoral artery and vein. The arterial catheter was attached to a pressure transducer for continuous monitoring of arterial pressure. The venous catheter was advanced through the inferior vena cava to the level of the heart for resuscitative drug delivery. An intravenous infusion of normal saline (3 ml/kg per hour) was started at this point and continued for the remainder of the experiment. A left lateral thoracotomy was then performed through the fourth intercostal space. The pericardium was incised and reflected. Systolic arterial pressure, diastolic arterial pressure, pulse, electrocardiogram, and rectal temperature were monitored continuously. Heating blanket and lights were used to maintain rectal temperature >37°C.

After preparation, dogs were separated into six experimental groups: 1) no ischemic controls (n=8); 2) those undergoing 10 minutes of ventricular fibrillation (VF) without restoration of spontaneous circulation (ROSC) (n=11); those undergoing 10 minutes of VF followed by ROSC for either 2 hours or 24 hours. Animals who underwent ROSC for 2 hours were treated with 3) standard intensive care (n=5) or 4) received an injection of acetyl-L-carnitine (ALCAR; Sigma Tau, S.p.A., Rome) at a dose of 100 mg/kg i.v. delivered immediately after ROSC (n=6). Animals who underwent ROSC for 24 hours were randomly assigned to either 5) standard intensive care (n=10) or 6) to receive ALCAR at a dose of 100 mg/kg i.v. delivered immediately after ROSC, followed by 50 mg/kg i.v. every 6 hours (n=8). Animals not receiving ALCAR received a similar volume of drug vehicle adjusted to the identical pH of dissolved ALCAR. The animal treatment team was blinded as to the identity of solutions administered to dogs undergoing 24 hours of ROSC.

After surgical preparation, cardiac arrest was induced with electrical train of currents (Harvard electronic stimulator model 340; Harvard Instruments, Dover, Mass.) applied directly to the epicardium of the left ventricle. The presence of cardiac arrest was verified by electrocardiographic rhythm consistent with VF in the presence of systolic arterial pressure <20 mm Hg. At the initiation of VF, artificial ventilation was discontinued. Ventricular fibrillation was allowed to continue without treatment for 10 minutes. At the end of 10 minutes of VF, ventilation was resumed with 100% oxygen at a respiratory rate of 28 per minute, simultaneous with the beginning of manual open-chest cardiopulmonary resuscitation (OCCPR) at a rate of 60 compressions per minute. Epinephrine (0.2 ml/kg of a 1/10,000 solution) and sodium bicarbonate (1 meq/kg) were injected at the beginning of resuscitation through the femoral central venous catheter. The OCCPR was continued for 3 minutes, at which point internal defibrillation was performed at 35 J. An arterial blood gas sample was measured immediately after ROSC and at regular intervals thereafter. Ventilator settings were adjusted to maintain Po₂>70<100 mm Hg and Pco₂>25<35 mm Hg.

In the 24-hour animals, following stabilization of vital signs, the chest was closed in four layers, a chest tube inserted, the chest cavity evacuated, and the chest tube connected to a Heimlich chest valve. Beginning 3 hours after ROSC, anesthesia and paralysis were maintained with a constant infusion of 0.1 mg/kg morphine sulfate per hour and 0.1 mg/kg pancuronium bromide per hour. The infusion was continued for 20 hours, at which point the pancuronium was discontinued. Between hours 20 and 23, animals were weaned from controlled ventilation as the paralytic agent wore off. Twenty-three hours after ROSC, animals were awakened by an intravenous injection of 0.6 mg/kg naltrexone. Animals were then tested for neurological deficit (0%, normal; 100%, brain death) through the use of a standardized 430-point neurological deficit scoring (NDS) system modeled after a widely accepted NDS system initially published by Bircher and used extensively in the literature as an indicator of posts ischemic neurological injury in
The NDS examination involved specific testing of levels of consciousness, respiratory pattern, cranial nerves, motor function, and behavior. Animals with neurological deficit scores of 0–30 were considered to have suffered mild neurological injury, with scores of 31–60 representing moderate injury and 61–100 severe neurological damage. Testing was performed by two individuals blinded to treatment protocol and trained to the criteria of the NDS system. Interrater agreement was r=0.96. At the completion of the NDS examination, animals were once again deeply anesthetized with 17.6 mg/kg thiamylal sodium and 40 mg/kg alpha chloralose and subjected to controlled ventilation as previously described.

Animals were excluded from consideration for any of the following reasons: Po2<60 mm Hg on room air ventilation before cardiac arrest, temperature <36°C before cardiac arrest despite heating blanket, systolic arterial pressure <60 mm Hg for >1 minute at any time, systolic arterial pressure <80 mm Hg for 10 minutes at any time, or death from nonneurological cause before completion of the experiment. A total of eight animals were excluded for one or more of these reasons.

At the end of the appropriate experimental period, the animals were placed in the prone position, the scalp and skull musculature surgically reflected, and a 5×3.5×1-cm-thick wedge of right frontal cortex was then removed and immediately immersed in liquid nitrogen and stored at −80°C. Samples (approximately 0.1 g) of frozen tissue were accurately weighed and homogenized in 3% perchloric acid with a Brinkman Polytron homogenizer (Westbury, N.Y.). Homogenates were briefly centrifuged and the supernatant used for metabolite assays. Lactate was assayed with a YSI Model 2300 Stat glucose and lactate analyzer (Yellow Springs, Ohio). Pyruvate was analyzed fluorometrically using the lactate dehydrogenase–linked assay according to the method of Lowry and Passonneau.25

All data values are mean±SE. Physiological data were compared among the preischemic and postischemic groups using analysis of variance, with post hoc tests for planned comparisons. The α error levels were adjusted for multiple comparisons where appropriate. The NDS of drug-treated dogs were compared with values from 24-hour vehicle-treated dogs through the use of an independent two-tailed t test. Values of p<0.05 were considered significant for all tests.

Results

Physiological variables for the various dog groups were compared immediately before the initiation of cardiac arrest and at 1 hour after restoration of spontaneous circulation. The results are displayed in Table 1. No significant differences were observed between dogs in the different experimental groups when assessed before or 1 hour after the ischemic episode. These results also indicate that intravenous administration of acetyl-L-carnitine after 10 minutes of cardiac arrest has no significant effect on blood pressure, heart rate, or body temperature.

The NDS was performed in all dogs that underwent 24 hours of intensive care after 10 minutes of cardiac arrest (Figure 1). The mean NDS for 10 vehicle-treated dogs was 48.4±5.4. None of 10 in this group demonstrated moderate to severe neurological injury. Although all dogs could breathe spontaneously, the majority showed marked alterations of their levels of consciousness. Several appeared totally unaware of external stimuli and demonstrated severe purposeless running motions on examination. Only one dog in this group had a neurological deficit score <30 (NDS=29).

In contrast, the average NDS at 24 hours for eight dogs that received ALCAR immediately after ROSC (22.3±5.2) was significantly lower than the average NDS measured for vehicle-treated dogs (p=0.0037). Most animals in this group demonstrated only minimal impairment of their levels of consciousness and appeared aware of external stimuli. Most could stand and walk ataxically. Two dogs in this group appeared essentially uninjured (NDS=4, NDS=8). Only one in this group demonstrated moderate-to-severe injury, with an NDS of 53.

The results of lactate and pyruvate measurements performed on acid extracts of fast-frozen samples of the frontal cortex excised from the brains of dogs in differ-
TABLE 2. Frontal Cortex Lactate and Pyruvate Levels After Cardiac Arrest in Dogs

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>n</th>
<th>Lactate (nmol g⁻¹ wet wt)</th>
<th>Pyruvate (nmol g⁻¹ wet wt)</th>
<th>Lactate/pyruvate (nmol/nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>1.9±0.2</td>
<td>126±7</td>
<td>16.3±2.2</td>
</tr>
<tr>
<td>10-Minute arrest</td>
<td>11</td>
<td>12.5±0.5*</td>
<td>52±7*</td>
<td>291±44*</td>
</tr>
<tr>
<td>2-Hour ROSC</td>
<td>5</td>
<td>4.3±0.2*</td>
<td>160±41</td>
<td>34.3±6.9</td>
</tr>
<tr>
<td>2-Hour ROSC+ALCAR</td>
<td>6</td>
<td>2.0±0.4t</td>
<td>197±24t</td>
<td>9.5±1.1t</td>
</tr>
<tr>
<td>24-Hour ROSC</td>
<td>4</td>
<td>4.5±2.1</td>
<td>96±23</td>
<td>49.8±22</td>
</tr>
<tr>
<td>24-Hour ROSC+ALCAR</td>
<td>5</td>
<td>2.5±0.9</td>
<td>184±21§</td>
<td>12.5±3.0</td>
</tr>
</tbody>
</table>

Values are mean±SEM. n, Number of dogs; ROSC, restoration of spontaneous circulation; ALCAR, acetyl-L-carnitine.

*p<0.01 compared with control group.
†p<0.01 compared with 2-hour ROSC group.
‡p<0.05 compared with control group.
§p<0.05 compared with 24-hour ROSC group.
gest that one or more aspects of aerobic metabolism remain abnormal after 2 hours of reperfusion and that anaerobic glycolysis remains somewhat elevated in response to these alterations. After 24 hours of reperfusion, lactate levels and lactate/pyruvate ratios were still high when compared with those of nonarrested animals; however, variability in these values was substantially greater than that observed after 2 hours of reperfusion. Nevertheless, these results indicate that abnormal patterns of cerebral energy metabolism persist over an entire 24-hour period of circulation following 10 minutes of cardiac arrest, suggesting a connection to profound neurological impairment evident after this period of reperfusion.

Several explanations could account for the abnormally elevated lactate/pyruvate ratios measured in the cortex of the resuscitated, non-drug-treated dogs used in this study. Any alteration in the ability of the tissue to generate ATP by aerobic metabolism would tend to accelerate glycolytic ATP production and, therefore, lactate accumulation. Such alterations could include an inhibition of electron transport chain activity16–18 or specific inhibition of mitochondrial enzymes such as pyruvate dehydrogenase.6 In addition to subcellular alterations that are evident from assays performed in vitro, conditions present in vivo after ischemia and reperfusion may further compromise the ability of the brain to undergo normal energy metabolism. Examples of such conditions include elevated levels of free fatty acids and fatty acylcoenzyme A (acyl-CoA) molecules that adversely affect mitochondrial activities such as oxidative phosphorylation.31,32 Aerobic metabolism of pyruvate can also be specifically retarded by an accumulation of intramitochondrial acetylcoenzyme A (acyt-

Several of the proposed bioenergetic mechanisms of ischemia/reperfusion brain injury may help explain how postischemic treatment of animals with acetyl-l-carnitine normalizes cortical lactate levels and, in turn, improves neurological outcome. It is quite possible that the hydrophobic acetyl moiety of acetyl-l-carnitine improves the ability of this drug to cross the blood–brain barrier and to be taken up by neuronal tissue, possibly through the γ-aminobutyric acid transporter.34 Once inside, the carnitine moiety could serve several beneficial roles. First, it could act to reduce the mitochondrial toxicity of fatty acyl-CoA molecules by forming the less-inhibitory fatty acylcarnitine compounds through carnitine acyltransferase reactions.35 The activities of the enzymes that catalyze these reactions with medium- and long-chain fatty acyl-CoA molecules are, however, relatively low in the brain, which is consistent with the relatively low utilization of fatty acids for energy production in this organ. Brain does possess a relatively high carnitine acetyltransferase activity caused by ammonium salts and the protective effect of glycine.36 Although it is not clear whether this is a direct effect of the drug or an indirect consequence of altered tissue energy metabolism.

In summary, this study has provided evidence that administration of acetyl-l-carnitine during restoration of spontaneous circulation after 10 minutes of cardiac arrest in dogs results in a significant improvement in the neurological outcome assessed 24 hours after the period of global cerebral ischemia. Normalization of cerebral cortex lactate levels and lactate/pyruvate ratios present at a very early stage (2 hours) of reperfusion suggests that acetyl-l-carnitine may at least partially exert its therapeutic effect by potentiation of aerobic energy metabolism and inhibition of glycolytic lactic acidosis. We conclude that these results warrant further verification of the clinical usefulness of acetyl-l-carnitine in the treatment of ischemic brain injury and that additional studies are necessary to elucidate the biochemical mechanisms of action of this drug.

References
This issue of Stroke includes a provocative study by Rosenthal et al that examines the use of acetyl-L-carnitine to reduce postischemic neurological injury. This study represents a new approach to normalization of cellular events that occur in cases of cerebral ischemia. Presumably, acetyl-L-carnitine acts in mitochondria by reversing altered energy metabolism. This neuroprotective mechanism of action is totally different from that of other cellular protective agents such as calcium channel blockers and excitatory amino acid antagonists.

The physiological role of carnitine in beta-oxidation of fatty acids has been known for many years. It is now apparent that carnitine and its acyl analogues are also involved in other physiological and pathological processes. These include shuttling acyl groups of acyl-coenzyme A between various tissue compartments, such as mitochondria, peroxisomes, and cytosol and, in certain pathological conditions, detoxifying non-metabolized acyl residues and buffering the intramitochondrial acyl-CoA/free CoA ratio.¹

Neuroscientists have shown interest in carnitine for almost 20 years. Engel and colleagues² were the first to report on human carnitine deficiency, describing both muscle carnitine deficiency and systemic carnitine deficiency.³ Mitochondrial dysfunction and lipid storage were characteristic features of both disorders. In the last 10 years, carnitine deficiencies have been described in patients with several different organic acidurias that are caused by inherited mitochondrial defects. Most involve the catabolism of fatty acids and/or branched chain amino acids, which are very similar metabolic processes. Fatty acids or deaminated amino acids are attached to CoA and then sequentially catabolized to acetyl-CoA.⁴ In patients with disorders such as medium-chain fatty acyl-CoA dehydrogenase deficiency and propionic acidemia, this breakdown is blocked and acetyl-CoA accumulates.⁵,⁶ The accumulation of acetyl-CoA has two deleterious effects. Since the total amount of mitochondrial CoA is constant, an increase in acetyl-CoA is accompanied by a decrease in free CoA. This has an adverse effect on enzymatic reactions that involve free CoA as a substrate. Additionally, high concentrations of acetyl-CoA are quite toxic and inhibit numerous mitochondrial enzymes.⁷

Carnitine is important in both the pathophysiology and treatment of these inherited mitochondrial disorders. In patients affected by these disorders, the accumulating acyl group is transferred from CoA to carnitine, diffuses out of the mitochondria, and is excreted in the urine. Consequently, many patients develop either a secondary carnitine deficiency or insufficiency.⁵,⁶

The beneficial effects of L-carnitine therapy were first demonstrated in a patient with propionic acidemia. The patient showed marked clinical improvement. In addition, mitochondrial metabolism greatly improved as revealed by analysis of the organic acids in the urine.⁸ L-Carnitine therapy for treatment of other patients with organic aciduria has also been associated with marked clinical and biochemical im-
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