Carnitine Treatment Inhibits Increases in Cerebral Carnitine Esters and Glutamate Detected by Mass Spectrometry After Hypoxia-Ischemia in Newborn Rats

Mark S. Wainwright, MD, PhD; Rohit Kohli, MD; Peter F. Whittington, MD; Donald H. Chace, PhD, MSFS

Background and Purpose—Cerebral ischemic insults disrupt normal respiratory activity in mitochondria. Carnitine plays an essential role in mitochondrial metabolism and in modulating excess acetyl-coenzyme A (acyl-CoA) levels. The effects of cerebral ischemia on carnitine metabolism are not well understood, although the newborn may be particularly vulnerable to carnitine deficiency. We used a newborn rat model of hypoxia-ischemia (HI) to test the hypothesis that HI alters acyl-CoA:CoA homeostasis and that this effect can be prevented by treatment with carnitine.

Methods—A total of 120 postnatal day 7 rats were subjected to 70 minutes of HI after treatment with 16 mmol/kg intraperitoneal L-carnitine or diluent. Carnitine, acylcarnitines, and excitatory amino acids were measured by mass spectrometry, and carnitine acetyl transferase activity, superoxide, and levels of the mitochondrial phospholipid cardiolipin (CL) were measured at 2- and 24-hour recovery.

Results—HI and hypoxia were associated with a significant increase in the ratio of acyl-CoA:CoA, which was prevented by treatment with carnitine. Carnitine treatment also prevented increases in glutamate, glycine, superoxide, and decrease of CL.

Conclusions—Carnitine metabolic pathways are compromised in HI and hypoxia. The protective effect of carnitine treatment on HI injury may be attributable to maintaining mitochondrial function. (Stroke. 2006;37:524-530.)

Key Words: hypoxia-ischemia, brain ■ carnitine ■ animals, newborn ■ glutamate

Disruption of mitochondrial function is a critical event in hypoxia-ischemia (HI) and stroke. Mitochondrial production of reactive oxygen species threatens neuronal survival by their ability to induce lipid peroxidation, protein oxidation, and DNA damage.

Carnitine is required for normal mitochondrial function. During metabolic stress, mitochondria accumulate acyl-coenzyme A (acyl-CoA), which is normally maintained in homeostasis with free CoA by the carnitine shuttle. High intramitochondrial acyl-CoA levels inhibit enzymatic processes involved in oxidative metabolism. Treatment with carnitine can correct the increased acyl-CoA:CoA ratio and permit normal functioning of mitochondrial enzymes. Oxidative damage to carnitine acetyl transferase (CAT) is associated with a loss of the mitochondrial phospholipid cardiolipin (CL), reduction in mitochondrial membrane potential, and a decrease in cellular oxygen uptake. The contribution of these processes to the compromise of mitochondrial function resulting from cerebral ischemia or HI is unknown.

Treatment with L-carnitine is associated with a reduction in neurologic injury after HI in the newborn rat through unknown mechanisms. L-Carnitine is an attractive therapy for perinatal asphyxia given the extensive pediatric clinical experience and its minimal toxicity. The newborn may be increased risk of cellular injury related to carnitine deficiency because of the immature development of carnitine biosynthetic pathways.

We used an established rat model of perinatal asphyxia with a combination of apoptotic and necrotic cell death in which the severity of injury varies with the duration of hypoxia. We tested the hypothesis that HI is associated with increased acyl-CoA:CoA ratio and that this imbalance can be overcome by treatment with exogenous carnitine. We also tested the hypothesis that carnitine acts as an antioxidant and attenuates degradation of mitochondrial CL after HI.

Materials and Methods

Animal Protocols

A total of 120 postnatal day 7 Wistar pups (Charles River, Wilmington, MA) were used in these studies. All experiments were performed in accordance with the relevant National Institutes of Health guidelines and approved by the institutional animal care and use committee of Children’s Memorial Hospital. Pups were housed with their dam in cages in the animal facility with a 12-hour light/dark cycle.

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From the Departments of Pediatrics (M.S.W., R.K., P.F.W.), Molecular Pharmacology and Biological Chemistry (M.S.W.), and Center for Drug Discovery and Chemical Biology (M.S.W.), The Feinberg School of Medicine, Northwestern University, Chicago, Ill; and Pediatrix Analytical (D.H.C.), Bridgeville, Pa.

Correspondence to Mark S. Wainwright, MD, PhD, Division of Neurology No. 51, Children’s Memorial Hospital, 2300 Children’s Plaza, Chicago, IL 60614. E-mail m-wainwright@northwestern.edu

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Induction of HI
We induced hypoxic-ischemic injury by unilateral carotid ligation followed by 70 minutes of hypoxia (8% oxygen/balance nitrogen). Rats were anesthetized with isoflurane (3.5% for induction and 1.5% for maintenance) and 50% oxygen-balance nitrogen. The right common carotid artery was ligated, and the animals recovered for 90 minutes with the dam before undergoing hypoxia. Sham-operated animals underwent neck incision and vessel manipulation without ligation or hypoxia.

Drug Administration
l-Carnitine inner salt was obtained from Sigma Chemical Co. and dissolved in saline as a 1.2-mol/L solution. Drug or diluent was administered via intraperitoneal injection at a dose of 16 mmol/kg 30 minutes before the induction of hypoxia.

Quantification of Cerebral Carnitine and Carnitine Esters and Amino Acids
At 2- and 24-hour recovery, rats were perfused with ice-cold PBS, pH 7.4. Hemispheres were divided at the midline and flash-frozen at −80°C before lipid extraction under methanol. Carnitine, carnitine esters, and amino acid levels were measured by electrospray tandem mass spectrometry (MS-MS).

Detection of Cellular Injury by Immunohistochemistry
To confirm the presence of degenerating neurons in frozen sections from the HI group after 24-hour recovery, the fluorescent dye Fluoro-Jade B (FJ-B) with concurrent 4',6-diamidino-2-phenylindole (DAPI) histochemistry was used. Standard immunohistochemical methods were used to identify cells expressing cleaved caspase 3 (Cell Signaling; 1:50).

CAT Enzyme Activity
CAT activity was measured at 2-hour recovery after HI by spectrophotometric detection of the reduced form of nicotinamide adenine dinucleotide (NADH) formed in the CAT-mediated conversion of acetyl-carnitine to l-carnitine using published methods.

Quantification of Superoxide Anion Production
Changes in superoxide levels after HI were measured in frozen brain sections by modifications of published methods using the fluorescent dye dihydroethidium (DHE; Molecular Probes). Five frozen sections (40 μm) from the genu to the body of the corpus callosum were incubated at 37°C for 30 minutes in a 15-μmol/L DHE solution. Three digital images of ethidium-stained cells were obtained from cortex in both hemispheres. Fluorescent intensity was quantified in grayscale images under identical imaging conditions by automated detection of the percentage area of each image above baseline image intensity (Metamorph; Fryer Corp). Specificity of DHE for superoxide was demonstrated by preincubation with polyethylene glycol-superoxide dismutase (Sigma; 50 U) quenching the ethidium signal.

CL Degradation After HI
The fluorescent dye 10-N-nonyl Acridine Orange (NAO; Molecular Probes) was used to detect changes in levels of the mitochondrion-specific phospholipid CL. Frozen sections were prepared after 2-hour recovery from HI as described for DHE, incubated for 45 minutes at 4°C with 15 μmol NAO, and fluorescence intensity quantified. Data are expressed as the percentage area of each image above the baseline threshold. To confirm the specificity of NAO for mitochondrial injury,
sections from sham-operated control animals were incubated for 15 minutes with 5 μmol/L of the mitochondrial toxin nitropropionic acid (NPA; Sigma) or diluent before incubation in NAO.

Data Analysis
Comparisons of intergroup differences for levels of carnitine, carnitine esters acid levels, CAT activity, DHE, and NAO fluorescence were performed by 1-way ANOVA with adjustment for multiple comparisons (GraphPad Prism version 4.00). All data are presented as mean±SEM. Statistical significance was assumed when P<0.05.

Results

Treatment with l-Carnitine Increases Free Carnitine Levels After HI
We measured free carnitine (FC) levels (Figure 1) in the nonischemic (left) and ischemic (right) hemisphere at 2 and 24 hours after HI (n=8 to 12 for all groups). Representative MS-MS spectra of acylcarnitines (ACs) from the ischemic hemisphere in vehicle (Figure 1A) and carnitine-treated rats (Figure 1B) are shown. At 2-hour recovery (Figure 1C), there was no difference in FC levels in vehicle-treated animals between hemispheres, which were not different from sham controls. Rats treated with l-carnitine showed a significant increase in FC levels in both hemispheres compared with both vehicle-treated or sham controls. These differences persisted at 24-hour recovery (Figure 1D).

Cerebral Available FC Is Increased by Administration of Carnitine
At 2-hour recovery (Figure 1E), the FC:total carnitine (TC) ratio in the right hemisphere of vehicle-treated animals at 2 hours was significantly reduced compared with sham-operated animals, consistent with a decrease in availability of FC in the HI group. There was no difference between the left (nonischemic) hemisphere in the vehicle group and sham controls. Rats treated with l-carnitine showed a significant increase compared with vehicle-treated animals in available FC levels in both hemispheres. These differences were not present at 24-hour recovery (Figure 1F).

AC:FC Ratio Is Increased After HI
There were significant differences in the AC:FC ratio between groups at 2-hour recovery (Figure 1G). The AC:FC ratio in vehicle-treated animals after HI was significantly increased in both hemispheres compared with sham-operated animals, consistent with disruption of mitochondrial metabolism and accumulation of acyl-CoA esters. In contrast, values in rats treated with l-carnitine were not significantly different from sham-operated animals. The ratio measured in both hemispheres of the vehicle group was also significantly increased compared with carnitine-treated animals. These differences were not present at 24-hour recovery (Figure 1H).

The increase in AC:FC ratio in the contralateral hemisphere was associated with cellular injury (Figure 2), shown by the presence of FJ-B–labeled neurons (Figure 2A), which were sparse compared with the staining in the ipsilateral cortex (Figure 2B) and were not present in sham controls (Figure 2C). Apoptotic cells were also detected in the left cortex (Figure 2D, inset). The extent of injury in the left hemisphere was subtle and was not proportionate to the increase in AC:FC ratio.

CAT Activity Is Not Altered After HI
There were no significant intergroup differences in CAT activity (values expressed in mmol/L±SEM; n=5 per group) at 2-hour recovery (Figure 3).
Glutamate and Glycine Levels Increase After HI
We measured changes after HI in transmitter (glutamate and glycine) and nontransmitter amino acids (tyrosine and valine; Figure 4). In vehicle-treated animals, there was a significant increase in glutamate levels in the right hemisphere compared with the left and compared with carnitine (right hemisphere) and sham controls after 2-hour recovery (Figure 4A). There were no interhemisphere differences in the carnitine-treated animals, which did not differ significantly from sham controls. Glycine levels after 2-hour recovery (Figure 4C) showed a similar pattern with the exception that levels were increased in the right hemisphere of the carnitine group compared with sham. There were no differences between groups for either glutamate (Figure 4B) or glycine (Figure 4D) at 24-hour recovery.

Nontransmitter Amino Acids Increase After HI But Are Not Affected by Treatment With L-Carnitine
In vehicle-treated animals at 2-hour recovery (Figure 4E), there was a significant increase in tyrosine levels in the right hemisphere compared with the left and with sham-operated animals. Treatment with carnitine did not prevent this increase. Tyrosine levels in right hemisphere of carnitine-treated animals were significantly increased compared with the left hemisphere and sham-operated animals. These differences were not present at 24-hour recovery (Figure 4F). A similar pattern was observed for changes in valine levels after HI (Figure 4G and 4H).

Superoxide Expression Is Increased After HI, and This Increase Is Attenuated by Carnitine
We used the fluorescent dye DHE to measure superoxide levels in brains from vehicle controls and carnitine-treated...
Figure 5. Changes in superoxide levels after HI. A through C representative fluorescent images of DHE-treated sections after 2-hour recovery HI (A and B) or sham operation (C). A, Right hemisphere (R) of vehicle (Veh)-treated animal. B, Treatment with carnitine during HI. Under identical imaging conditions, fluorescence is markedly decreased after carnitine treatment. C, Sham control. Inset, Superoxide dismutase treatment of frozen section after HI confirms specificity of ethidium for superoxide by showing a reduction in fluorescence. D, Superoxide levels after HI were quantified by changes in ethidium fluorescent intensity using commercial software (Metamorph). Data (percentage area above threshold intensity) expressed as mean±SEM; n=5 animals for all groups. *P<0.05 vs Veh R; #P<0.05 versus sham. Bar=100 μm.

Figure 6. Changes in CL levels after HI. A through C representative fluorescent images from of NAO-treated sections after 2-hour recovery after HI (A and B) or sham operation (C). NAO fluorescence is detected by excitation at 485 nm and emission at 530 nm in the presence of the mitochondrial specific phospholipid, CL. A, Right hemisphere of vehicle-treated animal. B, Treatment with carnitine during HI. Under identical imaging conditions, fluorescence is markedly increased after carnitine treatment, consistent with attenuation of mitochondrial injury. C, Sham control. Inset, Treatment of frozen section from sham control with the mitochondrial toxin, NPA confirms specificity of NAO for mitochondrial injury showing a reduction in fluorescence. D, CL levels after HI quantified by changes in NAO fluorescent intensity using commercial software (Metamorph). Data (percentage area above threshold intensity) expressed as mean±SEM; n=5 animals for all groups. L indicates left hemisphere; R, right hemisphere; Veh, vehicle treated; Carn, 16 mmol/kg IP L-carnitine; *P<0.05 vs Veh R. Bar=100 μm.

rats after 2-hour recovery from HI (Figure 5). In vehicle-treated animals, there was a significant increase in ethidium threshold area (values expressed as mean percentage area above threshold±SEM) in the ischemic compared with the nonischemic hemisphere. Values in both hemispheres of the vehicle group and the right hemisphere of the carnitine group were increased compared with sham controls. Animals treated with carnitine showed no difference between left and right hemispheres. DHE fluorescence was significantly reduced in the ischemic hemisphere of the carnitine-treated group compared with vehicle controls.

CL Levels Are Decreased After HI, and This Loss Is Attenuated by Carnitine

We used the fluorescent dye NAO to measure changes in levels of the mitochondrial membrane phospholipid and CL after 2-hour recovery from HI (Figure 6). In vehicle-treated animals, there was a significant decrease in NAO threshold area (values
expressed as mean percentage area above threshold±SEM) in the ischemic compared with the nonischemic hemisphere, consistent with degradation of CL after HI. Animals treated with carnitine showed no difference between hemispheres. NAO fluorescence was significantly reduced in the right hemisphere of vehicle-treated rats compared with the carnitine-treated group. To confirm the sensitivity of NAO for the detection of mitochondrial injury in the immature brain, we treated frozen brain sections from sham-operated animals with the mitochondrial toxin NPA. NPA-treated sections showed a significant reduction (53%) in CL levels compared with diluent controls.

**Discussion**

HI and hypoxia in the newborn are associated with a perturbation of acyl-CoA:CoA homeostasis. This increase in AC:FC ratio occurs in the left (hypoxic) and right (hypoxic-ischemic) hemisphere and is prevented by pretreatment with L-carnitine. This effect occurs without changes in activity of the CAT enzyme. The increase in the excitatory amino acid (EAA) levels in the ischemic hemisphere detected by 2 hours after HI is prevented by treatment with carnitine. Superoxide levels increase after HI, and this response is attenuated by treatment with carnitine. The decrease in levels of CL associated with HI is also prevented by treatment with carnitine.

The increase in the AC:FC ratio reflects changes in the intramitochondrial equilibrium between acyl-CoA and free CoA. Increase in this ratio indicates accumulation of acyl-CoA or reduction in FC and is associated with compromise of mitochondrial metabolism. The AC:FC ratio was increased in both hemispheres during HI but was not present in the animals treated with L-carnitine. Although we detected injury in the nonischemic hemisphere, the increase in AC:FC ratio in this hemisphere occurred without increase in EAAs. We have shown previously that treatment with carnitine reduces neurologic injury after HI in the newborn rat. The precise linkage between acyl-CoA homeostasis and neuronal injury remains to be determined. Our data imply that the mechanisms associated with carnitine neuroprotection include restoration of equilibrium between acyl-CoA and carnitine and preserved oxidative phosphorylation. Carnitine may serve to buffer intramitochondrial acyl-CoA moieties, which impair fatty acid oxidation, gluconeogenesis, and urea cycle metabolism.

The newborn may be at increased risk of carnitine deficiency. The FC:TC ratio is a sensitive indicator of the pool of available FC. We found a significant reduction in FC:TC ratio in the ischemic hemisphere of vehicle-treated rats after 2-hour recovery from HI compared with sham-operated animals and a similar trend in the nonischemic hemisphere, which did not reach significance. The prevention of this decrease by treatment with L-carnitine is consistent with the hypothesis that a combination of increase in acyl-CoA esters and decrease in available carnitine result in a state of “carnitine insufficiency” under conditions of metabolic stress, which can be overcome by administration of exogenous carnitine. This implies that the high AC:FC and FC:TC ratios reported in the neonatal period are consistent with a decrease in the availability of FC, placing the newborn at greater risk in conditions with high energy demand such as asphyxia.

Increases in EAAs are pivotal steps in cellular injury during ischemia. The mechanisms responsible for the attenuation of the rise in EAAs observed in the carnitine-treated rats are not clear. Carnitine inhibits glutamate-mediated toxicity in vivo by reducing the affinity of glutamate for its receptor and reducing activation of mitogen-activated protein kinase. Carnitine may also preserve glutamate transport. The reduction in EAA levels in this study may be associated with prevention of the compromise of neuronal or astrocyte glutamate uptake and metabolism associated with ischemia.

Other mechanisms may account for the effects of carnitine. Our results show that treatment with carnitine is associated with reduced superoxide expression after HI. Cell-based studies have also suggested attenuation of reactive oxygen species generation after treatment with carnitine. During aging, mitochondria undergo oxidative injury to CAT, which can be prevented by treatment with acetyl-l-carnitine. Our data suggest that in the initial period after HI, CAT activity is not altered despite the increase in superoxide levels.

Decrease in CL because of oxidative damage of the mitochondrial cristae has been proposed as a key event in apoptosis. Our data show a decrease in CL levels after HI and prevention of this effect by carnitine. We propose a model in which carnitine depletion or increased acyl-CoA after HI leads to an increase in mitochondrial superoxide levels, degradation of CL, and initiation of apoptosis. Our data imply an antiapoptotic role for carnitine as proposed previously.

There are currently no neuroprotective therapies in clinical use addressing mitochondrial dysfunction in cerebral ischemia. Our data demonstrate that HI and hypoxia are associated with alterations in acyl-CoA and carnitine homeostasis, and that this disequilibrium may be overcome by administration of exogenous carnitine, attenuating increases in superoxide and loss of CL. These data support the hypothesis that carnitine targets a novel mechanism involved in cerebral injury and lends support for the further investigation of carnitine as a therapy for acute brain injuries.

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**References**

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