

Mechanisms of Ischemic Neuroprotection by Acetyl-L-carnitine

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ABSTRACT: Acetyl-L-carnitine is a naturally occurring substance that, when administered at supraphysiologic concentrations, is neuroprotective in several animal models of global and focal cerebral ischemia. Three primary mechanisms of action are supported by neurochemical outcome measures performed with these models and with *in vitro* models of acute neuronal cell death. The metabolic hypothesis is based on the oxidative metabolism of the acetyl component of acetyl-L-carnitine and is a simple explanation for the reduction in posts ischemic brain lactate levels and elevation of ATP seen with drug administration. The antioxidant mechanism is supported by reduction of oxidative stress markers, for example, protein oxidation, in both brain tissue and cerebrospinal fluid. The relatively uncharacterized mechanism of inhibiting excitotoxicity could be extremely important in both acute brain injury and chronic neurodegenerative disorders. New experiments performed with primary cultures of rat cortical neurons indicate that the presence of acetyl-L-carnitine significantly inhibits both acute and delayed cell death following exposure to NMDA, an excitotoxic glutamate antagonist. Finally, several other mechanisms of action are possible, including a neurotrophic effect of acetyl-L-carnitine and inhibition of mitochondrial permeability transition. While the multiple potential mechanisms of neuroprotection by acetyl-L-carnitine limit an accurate designation of the most important mode of action, they are compatible with the concept that several brain injury pathways must be inhibited to optimize therapeutic efficacy.

KEYWORDS: metabolism; mitochondria; oxidative stress; excitotoxicity; cardiac arrest; stroke

INTRODUCTION

Ischemic and hypoxic brain injury caused by stroke, cardiac arrest, and perinatal asphyxia leads to neurologic morbidity or mortality in over one million individuals in the United States alone each year. At this juncture, the only neuroprotective inter-

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ventions demonstrated to be efficacious in large, multicenter clinical trials include administration of tissue plasminogen factor (TPA) to improve cerebral blood flow in a small subset of thromboembolic stroke victims, or of mild systemic hypothermia following cardiac arrest and resuscitation. The quest for a neuroprotective drug effective in humans has been elusive and a complete failure. One possible reason why many of these trials were unsuccessful is that the drug dosage was severely limited by dangerous side effects. Many investigators have thus turned their attention to compounds that are normally present in humans and can be administered at relatively high doses without evidence of toxicity. Examples of such compounds that have been or soon will be tested clinically for various neurologic disorders include creatine, coenzyme Q, and acetyl-L-carnitine (ALCAR). We have focused on the use of ALCAR in animal models of both global and focal cerebral ischemia. Our results and those of other laboratories indicate that ALCAR is neuroprotective in both small and large animal models of global cerebral ischemia and in a rat model of permanent focal ischemia.¹⁻³ Studies are in progress to determine if ALCAR is also therapeutically effective in a neonatal rat model of hypoxia/ischemia and in a rat model of traumatic brain injury.

STIMULATION OF AEROBIC CEREBRAL ENERGY METABOLISM

Three separate studies using a clinically relevant canine cardiac arrest and resuscitation model have demonstrated neuroprotection by postischemic intravenous administration of ALCAR.^{1,4,5} Most importantly, ALCAR administration significantly improves neurologic outcome 23 h following cardiac arrest.¹ In this study, ALCAR (100 mg/kg) was administered immediately following resuscitation after 10 min of normothermic cardiac arrest, and 50-mg/kg doses were administered at 6, 12, and 18 h. Animals were randomized to drug- and vehicle-treated groups and neurologic examinations were performed by examiners blinded to the treatment protocol. This same investigation documented a decrease in the brain lactate level and lactate/pyruvate ratio in animals sacrificed at 2-h reperfusion. Similar results were obtained with immediate postischemic ALCAR administration using a rat 4-vessel occlusion model of global cerebral ischemia and reperfusion. ¹H- and ³¹P-NMR spectroscopy of brain tissue obtained at various reperfusion times indicated that ALCAR significantly reduces levels of lactate and inorganic phosphate and elevates ATP and creatine-phosphate, as compared to vehicle-treated animals.⁶ Collectively, these effects of ALCAR on levels of cerebral energy metabolites are consistent with the hypothesis that the acetyl component of ALCAR is metabolized in the brain, thereby promoting oxidative cerebral energy production and minimizing anaerobic glycolysis and lactic acidosis (FIG. 1). As peri-ischemic cerebral acidosis is generally considered to promote neuronal cell death by several different mechanisms, the metabolic mechanism of neuroprotection by ALCAR is the simplest explanation for its primary activity. This mechanism of action is particularly attractive in light of the finding by several laboratories that a profound reperfusion-dependent inhibition of the activity of the enzyme pyruvate dehydrogenase occurs in selectively vulnerable brain regions.^{5,7,8} This enzyme catalyzes the conversion of pyruvate to acetyl-CoA and represents the sole bridge between anaerobic and aerobic cerebral energy metabolism (FIG. 1). Indirect evidence in support of ALCAR metabolism as a mechanism of neuroprotec-

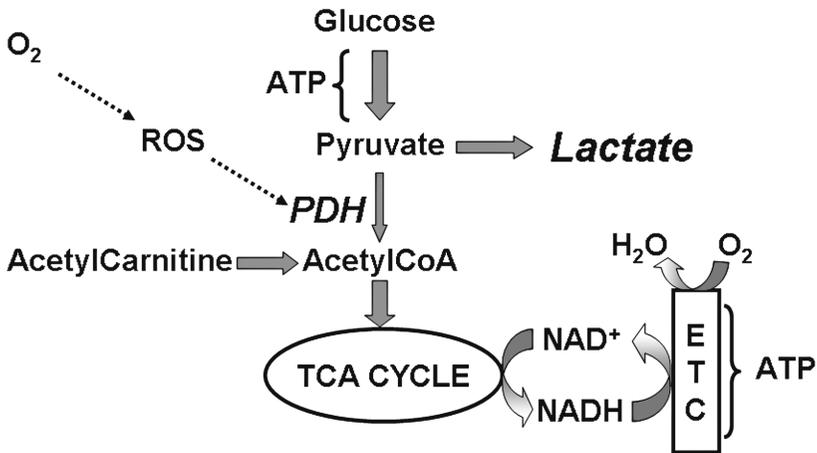


FIGURE 1. Possible metabolism of acetyl-L-carnitine (ALCAR) after cerebral ischemia. ALCAR may serve as an exogenous, alternative source of acetyl-CoA, thereby promoting aerobic energy metabolism via the electron transport chain (ETC), reducing tissue acidosis, and improving neurologic outcome after cerebral ischemia due to cardiac arrest or stroke. The pyruvate dehydrogenase complex (PDH) is a target of reactive oxygen species (ROS) and is inhibited following cerebral ischemia. Such inhibition may be responsible for chronically elevated brain lactate levels following ischemic episodes as this enzyme constitutes the bridge between aerobic and anaerobic cerebral energy metabolism.

tion is that neuroprotection is not observed in animals treated with free carnitine plus acetate.⁹ Nevertheless, pretreatment with free carnitine is neuroprotective in other animal models of neurodegeneration, including the 3-nitropropionic acid neurotoxicity model of Huntington's disease.¹⁰ It remains to be seen if carnitine or ALCAR pretreatment is neuroprotective for ischemic brain injury. Studies are in progress using NMR spectroscopy of brain tissue samples following infusion of ¹³C-ALCAR in sham-operated animals and in dogs following cardiac arrest to determine if the acetyl moiety of ALCAR is indeed metabolized to intermediates of aerobic energy metabolism in the adult brain and if its metabolism is accelerated in response to the metabolic roadblock created by postischemic inhibition of pyruvate dehydrogenase.

One observation suggesting that the metabolic effects of ALCAR may not be exclusively responsible for its postischemic neuroprotection is that, when administration of ALCAR to dogs following cardiac arrest is delayed by 30 min after resuscitation, no neuroprotection is observed, despite normalization of cerebral cortex lactate levels at 2-h reperfusion.¹¹ In addition to questioning the validity of the metabolic hypothesis, this finding and other results obtained with a rat global cerebral ischemia model indicate that, as with most other neuroprotective agents, there is a limited window of therapeutic opportunity for administration of ALCAR.¹² A limited window of opportunity has severely restricted effective treatment of ischemic stroke patients since the majority of patients are diagnosed several hours following the onset of the ischemic event. In contrast, cardiac arrest patients can potentially be treated almost immediately, as most successful resuscitations occur in the hands of para-

medics in the field or of physicians at hospitals. Considering the outstanding safety and tolerance profile of ALCAR, this agent may prove to be an exceptional candidate for neuroprotection clinical trials after cardiac arrest.

PROTECTION AGAINST OXIDATIVE STRESS

One study using the canine cardiac arrest model found that ALCAR treatment significantly reduces the level of protein carbonyl groups in the brains of dogs both at 2- and 24-h reperfusion after 10-min cardiac arrest.⁴ Protein carbonyl groups are a marker of oxidative tissue injury and increase significantly during ischemia/reperfusion. ALCAR likely protects against oxidative stress indirectly through its amelioration of tissue lactic acidosis, a condition that promotes the formation of

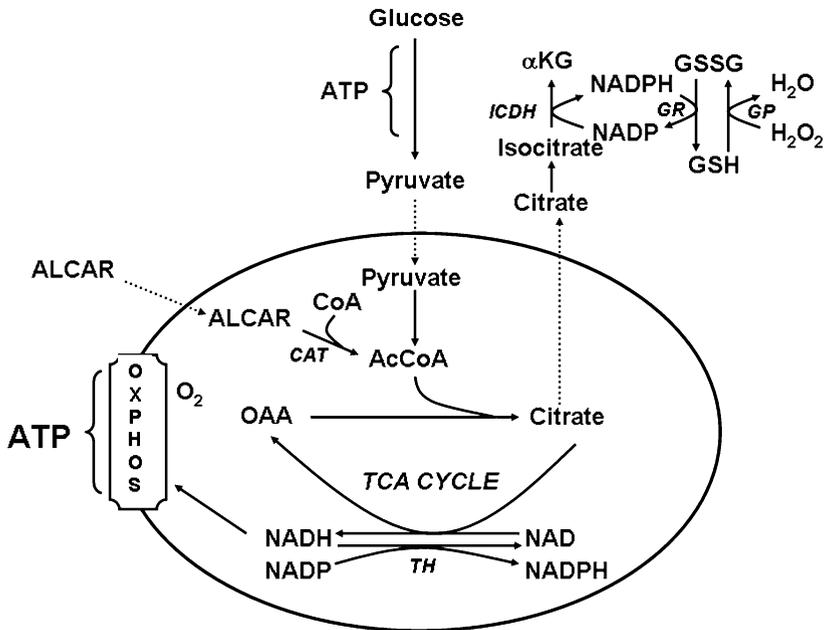


FIGURE 2. Possible shift in cellular redox state caused by ALCAR metabolism. ALCAR can be metabolized to acetyl-CoA by the carnitine acetyltransferase (CAT) reaction. Metabolism of acetyl-CoA by the tricarboxylic acid (TCA) cycle provides NADH that is then used for oxidative phosphorylation (OXPHOS) and for reducing intramitochondrial NADP to form NADPH via the transhydrogenase (TH) reaction. Elevation of intramitochondrial citrate results in transport out to the cytosol, where it is converted to isocitrate. The cytosolic isocitrate dehydrogenase (ICDH) reaction oxidizes isocitrate to form α -ketoglutarate (α KG) and NADPH. NADPH provides the reducing power for converting oxidized glutathione (GSSG) to reduced glutathione (GSH) via the glutathione reductase (GR) reaction. Reduced glutathione provides the reducing power for detoxifying H_2O_2 and organic peroxides via the glutathione peroxidase (GP) reaction. Intramitochondrial NADPH is also used to detoxify peroxides by an intramitochondrial glutathione reductase/peroxidase system (not shown).

reactive oxygen species. It is also possible that metabolism of ALCAR can cause a reduced shift in both the mitochondrial and cytosolic redox state, thus increasing the reducing power necessary for detoxification of reactive oxygen species, for example, H_2O_2 , via the glutathione peroxidase/reductase system, and for maintaining the normal redox state of protein sulfhydryl groups via the thioredoxin/thioredoxin reductase system (FIG. 2).

Another report established a strong trend toward improved levels of brain pyruvate dehydrogenase enzyme activity at 30-min and 24-h reperfusion in post-cardiac arrest animals treated with ALCAR compared to the drug vehicle.⁵ As evidence suggests that the loss of pyruvate dehydrogenase activity during reperfusion is due to oxidative stress, this finding can be interpreted as reflecting the indirect effects of ALCAR on brain protein oxidation. These results present the intriguing possibility that oxidative inactivation of pyruvate dehydrogenase occurs very rapidly during reperfusion, but that the inhibition is reversible upon administration of reducing power, for example, that potentially provided by metabolism of ALCAR.

ALCAR may also exert an indirect antioxidant activity through induction of one or more antioxidant genes. Primary rat cortical astrocyte cultures treated with ALCAR exhibit increased expression of the antioxidant enzyme heme oxygenase-1 and are protected against mitochondrial alterations and cell death caused by inflammatory cytokines.¹⁵ The effect of ALCAR on heme oxygenase expression is apparently mediated by increased expression of Nrf2, a transcriptional activating factor that can stimulate the expression of a wide array of antioxidant genes and even indirectly stimulates transcription of genes located on the mitochondrial genome.

ALCAR is also effective at inhibiting the oxidative stress and death caused by exposure of neural cells to A-beta-amyloid, a neurotoxic peptide that accumulates in the brain of Alzheimer's patients and is strongly implicated in the etiology of neurodegeneration.^{14,15} Treatment of multiple sclerosis with oral ALCAR for 6 months reduces several markers of oxidative stress that are typically elevated in the CSF of these patients.¹⁶

PROTECTION AGAINST EXCITOTOXICITY

ALCAR was reported in the mid-1990s to inhibit hippocampal neuronal cell death in response to glutamate exposure.¹⁷ Like many excitotoxicity experiments, conditions were used to maximize glutamate excitotoxicity. These conditions include the omission of magnesium and the inclusion of the coagonist glycine in the medium. We recently performed experiments with primary cultures of rat cortical neurons to determine if ALCAR is cytoprotective in the absence of added glycine and in the presence of magnesium, a physiological divalent cation normally present in the brain interstitium at ~1 mM concentration. The results of these experiments are shown in FIGURES 3 and 4. Exposure of cortical neurons at 10–14 days *in vitro* to the ionotropic glutamate receptor agonist *N*-methyl-D-aspartate (NMDA) at a concentration of 100 μ M for 30 min resulted in significant cell death compared to control cells subjected to a change of media, but not exposed to NMDA (FIG. 3). When 1 mM ALCAR was present together with NMDA, no significant cell death was observed. To test for the possibility that ALCAR only delayed the onset of cell death, a separate set of experiments was conducted where cell death was measured 24 h after the 30-min

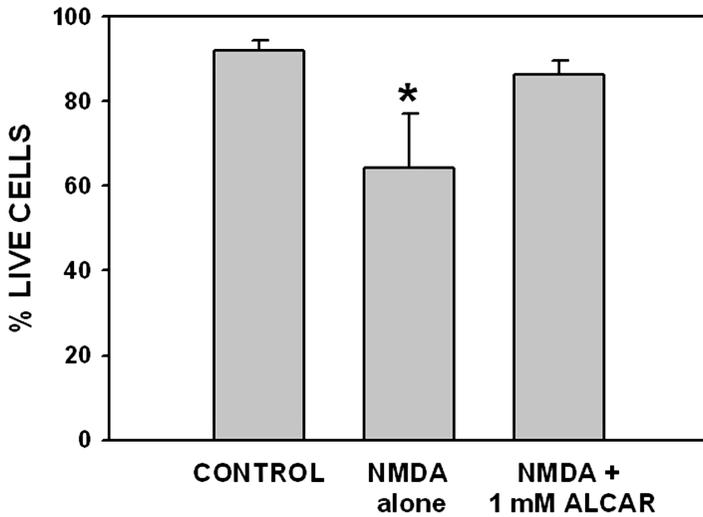


FIGURE 3. Protection by ALCAR against NMDA-induced acute neuronal death. Cultured cortical neurons (DIV 10–14) were exposed to 100 μ M NMDA for 30 min and immediately examined for cell death using the calcein-AM/PI ratio (live/dead). Exposure to NMDA resulted in significant neuronal cell death as compared to controls: $64.3 \pm 12.9\%$ live cells in the NMDA group vs. $91.8 \pm 2.4\%$ live cells in the control group ($*P = 0.007$ vs. control). In the presence of 1 mM ALCAR, cell death after exposure to NMDA was not significant: $86.2 \pm 3.4\%$ live cells ($P = 0.5$ vs. control). Differences between the mean were determined by ANOVA, and a Holm-Sidak test was applied for pairwise multiple comparisons. Results are shown as the mean \pm SEM for $n = 3$ experiments.

exposure to NMDA in the absence and presence of 1 mM ALCAR both during and after the NMDA treatment. The results described in FIGURE 4 indicate that, while significant cell death was also present at 24 h postinjury in the absence of ALCAR, no significant death was observed in its presence. The death observed with the live/dead assay reflects the additional cell death that ensues after NMDA is removed from the medium. These results confirm the results reported earlier for hippocampal neurons and extend them by demonstrating neuroprotection in the presence of magnesium and in the absence of glycine. Our findings also indicate that neuroprotection is observed with cortical as well as hippocampal neurons, two populations that are sensitive to cell death *in vivo* in response to insults (e.g., stroke) that involve excitotoxicity. Moreover, ALCAR cytoprotection is observed both during the acute phase of excitotoxicity and during the delayed phase after transient exposure to NMDA. Further studies are thus needed to determine if the antiexcitotoxic activity of ALCAR contributes to its neuroprotection in animal models of acute ischemic and traumatic brain injury.

Protection by ALCAR against excitotoxic cell death could be due to direct antagonism of glutamate receptors, to activation of gamma-aminobutyric acid (GABA) receptors that cause neuronal hyperpolarization and therefore resistance to NMDA receptor activation, or to inhibition of secondary events.¹⁷ These secondary events could include activation of the mitochondrial permeability transition that can cause

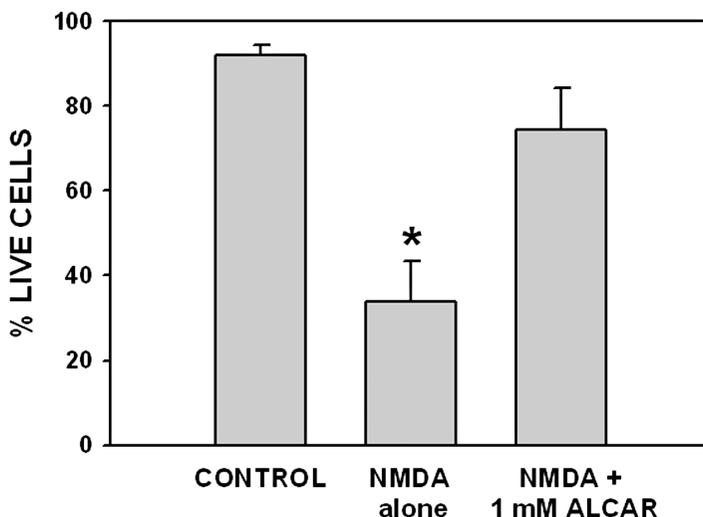


FIGURE 4. Protection by ALCAR against NMDA-induced delayed neuronal death. Cultured cortical neurons (DIV 10–14) were exposed to 100 μ M NMDA for 30 min and examined for cell death at 24 h postinjury using the calcein-AM/PI ratio (live/dead). Exposure to NMDA for 30 min followed by a 24-h recovery period resulted in significant neuronal cell death as compared to controls: 33.7 \pm 9.6% live cells in the NMDA group vs. 91.8 \pm 12.9% live cells in the control group (* P = 0.005 vs. control). In neurons treated with ALCAR during NMDA exposure and during the recovery period, cell death was not significantly different than controls (74.4 \pm 9.9% live cells; P = 0.09 vs. control) and significantly less than with NMDA in the absence of ALCAR (* P = 0.006 vs. NMDA). Differences between the mean were determined by ANOVA, and a Holm-Sidak test was applied for pairwise multiple comparisons. Results are shown as the mean \pm SEM for n = 3 experiments.

release of mitochondrial cytochrome *c* and stimulation of mitochondrial ROS production,¹⁸ and delayed neuronal calcium deregulation caused by opening of plasma membrane transient receptor potential (Trp) cation channels in response to the oxidative stress.¹⁹ Studies are in progress to test for these different possible effects of ALCAR.

OTHER MECHANISMS OF NEUROPROTECTION

Several other potential mechanisms of ALCAR neuroprotection are implicated based on studies performed both *in vitro* and *in vivo* with models other than acute neuronal cell death due to ischemia and excitotoxicity. For instance, exposure of cultured neural cells to ALCAR inhibits apoptosis caused by deprivation of serum or neurotrophic factors, suggesting that ALCAR may have an as yet undefined neurotrophic activity.^{20,21} Apoptosis that occurs following acute brain injury results either from initial intracellular alterations (e.g., elevated calcium or oxidative stress) or from the response to extracellular factors (e.g., inflammatory cytokines and “cell

death ligands”, such as Fas).²² Treatment of rats with carnitine and carnitine esters significantly lowers circulating levels of TNF α and interleukins using a lipopolysaccharide (endotoxin)-induced systemic inflammatory response.²³ It is thus possible that ALCAR may also reduce the inflammatory reactions in the brain following acute injury. Finally, it is also very likely that at least the carnitine component of ALCAR can “buffer” the toxic intracellular free fatty acids that are released by enzymes such as calcium-dependent phospholipase 2 during cerebral ischemia and trauma. The reduction in free fatty acids due to formation of fatty acyl carnitine esters inhibits their ability to induce the mitochondrial permeability transition²⁴ and appears responsible for the protection against anoxic death of hepatocytes afforded by carnitine.²⁵ While the multiple potential mechanisms of neuroprotection by ALCAR limit an accurate designation of the most important mode of action, they are compatible with the concept that several brain injury pathways must be inhibited to optimize therapeutic efficacy.

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