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ORIGINAL PAPER

Effect of Acetyl-L-carnitine Used for Protection of Neonatal Hypoxic-Ischemic Brain Injury on Acute Kidney Changes in Male and Female Rats

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Abstract

Neonatal hypoxia-ischemia (HI) is a common cause of brain injury in infants. Acute kidney injury frequently occurs after birth asphyxia and is associated with adverse outcome. Treatment with acetyl-L-carnitine (ALCAR) after HI protects brain and improves outcome. Rat pups underwent carotid ligation and 75 min hypoxia on postnatal day 7 to determine effects of HI on kidney which is understudied in this model. HI + ALCAR pups were treated at 0, 4 and 24 h after HI. The organic cation/carnitine transporter 2 (OCTN2), transports ALCAR and functions to reabsorb carnitine and acylcarnitines from urine. At 24 h after injury OCTN2 levels were significantly decreased in kidney from HI pups, 0.80 ± 0.04 (mean ± SEM, $p < 0.01$), compared to sham controls 1.03 ± 0.04, and HI + ALCAR pups 1.11 ± 0.06. The effect of HI on the level of pyruvate dehydrogenase (PDH) was determined since kidney has high energy requirements. At 24 h after HI, kidney PDH/β-actin ratios were significantly lower in HI pups, 0.98 ± 0.05 (mean ± SEM, $p < 0.05$), compared to sham controls 1.16 ± 0.06, and HI + ALCAR pups 1.24 ± 0.03, $p < 0.01$. Treatment of pups with ALCAR after HI prevented the decrease in renal OCTN2 and PDH levels at 24 h after injury. Protection of PDH and OCTN2 after HI would improve energy metabolism in kidney, maintain tissue carnitine levels and overall carnitine homeostasis which is essential for neonatal health.

Keywords Hypoxia-ischemia · Acetyl-L-carnitine · Kidney · Neonatal · OCTN2 transporter · Pyruvate dehydrogenase

Introduction

Neonatal hypoxic ischemic encephalopathy (HIE) is a leading cause of neonatal brain damage resulting from birth asphyxia which occurs at a rate of approximately 1.5/1000 live births in developed nations, with substantially higher incidence in developing nations [1]. Mild therapeutic hypothermia remains the primary treatment option for infants with HIE, but death or significant neurological disability still occurs in 40–50% of treated infants [2]. Male infants are more likely to suffer from birth complications which result in HIE and suffer from worse outcomes after an insult [3].

A number of recent studies have documented the high prevalence of acute kidney injury in infants with HIE, and association of kidney injury with poor neurologic outcome and increased risk of mortality [4–7]. Up to 56% of infants with birth asphyxia also have acute kidney injury [8]. Recent studies report that 38% of the neonates treated with hypothermia still exhibit acute kidney injury, and asphyxiated neonates with acute kidney injury have poorer outcomes and increased mortality [7, 9, 10]. Infants with HI and acute kidney injury required longer mechanical ventilation and had longer stays in the NICU [10]. An acute kidney injury in the neonatal period can result in residual renal dysfunction and a greater risk of developing chronic kidney disease later in life [8]. There is very little research in models of neonatal hypoxia ischemia assessing effects on kidney. Xu et al. [11] recently reported swelling in renal cortex, membrane alterations in glomerular barriers, and changes in protein levels in kidney from rat pups at 24 h after HI. Despite the prevalence of kidney injury as a secondary injury in infants with HIE

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and the significant long-term implications of such damage, few studies have assessed changes in kidney or evaluated potential treatments for kidney injury in infants with HIE.

Acute ATP shortages and damage to energy metabolism pathways due to oxidative stress and other mechanisms contribute to both HIE [12] and acute kidney injury [13]. Pyruvate dehydrogenase (PDH), the key enzyme linking glycolysis to the tricarboxylic acid (TCA) cycle, is particularly vulnerable to damage from oxidative stress [14]. Loss of PDH would prevent glucose from being utilized via mitochondrial oxidative metabolism and exacerbate the initial ATP shortage that occurs after HI injury.

Acetyl-L-carnitine (ALCAR) is a naturally occurring ester of carnitine which consists of an acetyl moiety bound to an L-carnitine moiety and is naturally synthesized in low levels by the liver [15]. It enters cells primarily through the sodium-dependent transport protein organic carnitine/cation transporter 2 (OCTN2), one of a family of organic cation transporters [16], where it is metabolized in mitochondria to acetyl CoA and L-carnitine as shown in Fig. 1. Treatment with ALCAR is neuroprotective in animal models of brain injury including neonatal hypoxia ischemia [17–22], global cerebral ischemia [23], and pediatric traumatic brain injury [24]. Clinical studies show protection in a variety of conditions including Alzheimer's disease [25], depression [26], peripheral neuropathy [27], and improved neurological function in hepatic encephalopathy [28]. Our group has demonstrated that administration of ALCAR after HI in the postnatal day 7 rat pup decreased brain lesion size, improved mitochondrial function and improved motor function and learning [17–19, 21, 22].

It is critically important that potential treatments for HIE do not harm the body or exacerbate other complications of hypoxia ischemia, such as renal injury. The few studies that have been conducted show that ALCAR reduced oxidative damage in kidney in a carbon tetrachloride model of oxidative stress [29] and protected renal function against cisplatin-induced caspase-3 activity, apoptosis, and inflammation [30]. ALCAR may protect against injury through the conversion of its acetyl moiety to acetyl CoA and serve as an alternative metabolic substrate when glucose metabolism is impaired [15, 31, 32]. L-carnitine facilitates uptake of free fatty acids into the mitochondria for β-oxidation, potentially protecting against oxidative stress [33, 34]. These characteristics of ALCAR suggest that it could be protective against both HIE and related kidney injury.

OCTN2, the transporter for ALCAR, is found throughout the kidneys, but is most abundant in renal tubular epithelial cells, where the transporter functions to reabsorb L-carnitine and acylcarnitines from urine after glomerular filtration to maintain the plasma concentration of L-carnitine [16, 35]. It has been reported that the increases in serum L-carnitine during early development were positively correlated with the postnatal increases in OCTN2 expression in kidney [36]. A secondary carnitine deficiency can occur in infants with severe neonatal asphyxia [37–39].

Despite the prevalence of research regarding the protective effects of ALCAR on a variety of neurological and renal disorders, little research has involved the interaction between injury, ALCAR treatment, and its transporter, OCTN2. Few studies have discussed the effect of renal injury on metabolic changes and PDH in particular. Our study sought to determine the effect of neonatal hypoxic ischemic injury and subsequent ALCAR treatment on levels of the OCTN2 transporter and PDH in kidney.

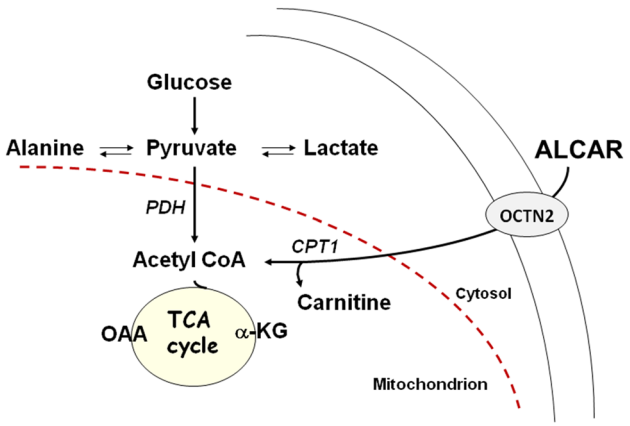

Fig. 1 ALCAR enters kidney via the OCTN2 transporter. In the mitochondria ALCAR can be cleaved by the enzyme carnitine acetyltransferase (CAT) to carnitine and acetyl CoA which can be metabolized to provide energy. The OCTN2 transporter in kidney releases carnitine into the circulation thus helping to maintain carnitine status

Materials and Methods

Animals

This study was approved by the University of Maryland Baltimore, Animal Care and Use Committee. All care and handling of rats were in compliance with the National Institutes of Health guidelines. Timed-pregnant female Sprague–Dawley rats were obtained from Charles River Laboratories (Frederick, MD, USA) and were housed in the central animal facility.

Surgery and Hypoxia-Ischemia

Seven day old rat pups were anesthetized with 3% isoflurane for induction (and 1.5% for maintenance) and underwent a modification of the Rice-Vanucci model of HI (right carotid artery ligation, 75 min 8% oxygen at 37 °C), or sham surgery [21, 22, 40]. After surgery pups were placed in an open

jar in a 37 °C water bath for 25 min to recover. Pups were returned to the dam for 60 min before the hypoxia. Hypoxia was performed in pre-warmed glass jars (2 pups per jar) in a 37 °C water bath. Jars were sealed, flushed with warmed gas mixture (8% oxygen and 92% nitrogen) at 1 L/minute and placed in a 37 °C water bath for 75 min. After hypoxia the pups recovered at 37 °C in room air for 2 h prior to being returned to the dam. Sham treated controls were anesthetized for 4.5 min and received an incision without any artery manipulation or hypoxia. Sham control pups were taken away from the dam for the same time as the HI pups, but did not undergo the operation or hypoxia. ALCAR treated animals received 3 doses of ALCAR (100 mg/kg in 8% sodium bicarbonate to control pH) subcutaneously immediately after HI, 4 h after, and 24 h after HI [24]. Control animals were injected with saline. ALCAR and saline treated animals were euthanized two hours after the final injection. After rat pups were euthanized the kidneys were removed, flash frozen in liquid nitrogen, and stored at -80 °C until usage.

Tissue Preparation

Renal cortex tissue samples were taken on dry ice, minced and placed in 400 µl immunoprecipitation assay lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with a cocktail of protease and phosphatase inhibitors (Roche Diagnostic, Indianapolis, IN) on ice for 25 min to begin homogenization. Tissue samples were fully homogenized by sonication and centrifuged for 10 min at 10,000×*g* to remove nuclear and cellular debris. The supernatant was removed from the pellet and aliquots frozen at − 80 °C until further use for western blots and protein determination. Protein concentrations were determined by the Pierce BCA microreagent assay [41].

Gel Electrophoresis and Transfer

Samples were supplemented with 10 µl 4× lithium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA) and deionized water to reach a desired protein concentration of 30 µg/40 µl solution. One lane in each gel was used for a combined protein standard (Novex® Sharp Pre-Stained Protein Standard, Invitrogen Carlsbad, CA) and biotinylated protein molecular weight ladder (Cell Signaling Technology, Danvers, MA). Tissue samples were incubated at 37 °C for 15 min to prepare for electrophoresis of membrane proteins [42] before being loaded onto a 4–12% polyacrylamide gel buffered with Bis-Tris-HCl, pH 6.4 and subjected to gel electrophoresis with MOPS SDS running buffer (Invitrogen, Carlsbad, CA) for 120 min at 120 V on ice. The gel was then transferred to a PVDF membrane in NuPAGE® Transfer Buffer (Invitrogen, Carlsbad, CA) for 60 min at 30 V.

Western Blots

The membranes were blocked with 5% skim milk for 1 h at room temperature prior to incubation with primary antibody. The rabbit polyclonal anti-OCTN2 primary antibody (Alpha Diagnostic, San Antonio, TX) was used at concentrations of 2 µg/ml in 2.5% skim milk overnight at 4 °C. Following primary antibody exposure, the membranes were washed in TBST (2.42 g Trizma-HCl, 8.01 g NaCl, 0.1% Tween20, pH 7.6) before being incubated in a donkey anti-rabbit IgG Horseradish Peroxidase linked F(ab')2 secondary antibody (GE Healthcare UK, Little Chalfont, Buckinghamshire, UK) for 1.5 h at a 1/3000 dilution in 2.5% skim milk at room temperature. For pyruvate dehydrogenase (PDH) the membranes were incubated with a mouse monoclonal anti-PDH E1-α subunit primary antibody (Abcam, Cambridge, MA) at a concentration of 2 µg/ml in 2.5% skim milk overnight at 4 °C. Following primary antibody exposure, the membrane was washed in TBST as described above and incubated in a goat anti-mouse IgG (H + L) HRP conjugate (Biorad, US) for 1.5 h at a 1/3000 dilution in 2.5% skim milk at room temperature. After washing, the membranes were treated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) and exposed with an aperture of .95 on a MultiImage II machine (Alpha-Innotech). After exposure, the membrane was stored at 4 °C until further analysis for β-actin.

For the β-actin Western blots the membranes were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL) for 15 min, washed, re-blocked in 5% skim milk for 1 h at room temperature, incubated with a mouse monoclonal anti-β-Actin primary antibody (SIGMA Aldrich, St. Louis, MO) at concentrations of 0.05 µg/ml in 2.5% skim milk for 1 h at room temperature, washed again, and incubated with goat anti-mouse IgG (H + L) HRP conjugate (Biorad, US) at a 1/3000 concentration in 2.5% skim milk for 1 h at room temperature. The membranes were then treated with ECL and exposed at a 2.5 aperture on the MultiImage II machine.

Statistical Analysis

Data are presented as mean ± SEM. Statistical significance was determined by one way ANOVA followed by Bonferroni's Multiple Comparison Test when ANOVA showed significant differences. A value of $p < 0.05$ was considered significant.

Results

Changes in OCTN2 from HI and ALCAR Treatment

As shown in Fig. 2, OCTN2 bands were observed at approximately 70–75 kDa, with an additional band at ~ 130 kDa

that is likely a dimer or more postranslationally modified form of the protein. These two bands are consistent with the major bands of 80 and ~ 160 kDa observed by Nagai et al. [43] although the lighter band at 60 kDa which they observed was not visible in our blots. OCTN2 bands at 70–80 kDa were observed by Tamai et al. [35]. As shown in Fig. 2, OCTN2 levels were significantly decreased at 24 h after injury in kidney from animals subjected to HI compared to shams and HI + ALCAR pups, $p < 0.01$. HI pups treated with ALCAR had OCTN2 levels comparable to kidney from sham controls. OCTN2/β-actin ratios were control, 1.03 ± 0.04; HI + Saline, 0.80 ± 0.04 and HI + ALCAR 1.11 ± 0.06. Renal OCTN2 decreased after HI but pups treated with ALCAR did not exhibit this decrease.

Changes in PDH Protein from HI and ALCAR Treatment

The PDH complex is comprised of several subunits. The target of the primary antibody used was the E1-α subunit of the complex, which has been shown to be vulnerable to oxidative damage [44]. As shown in Fig. 3, PDH E1-α subunit bands were observed at 40 kDa. PDH levels in kidney from HI + saline pups were significantly lower than PDH levels in sham control pups at 24 h after injury, $p < 0.05$. PDH E1-α levels in kidney from HI + ALCAR pups were comparable to levels in sham controls, and significantly higher than levels in HI + saline pups, $p < 0.01$. PDH/β-actin ratios were 1.16 ± 0.06 in kidneys from sham animals, 0.98 ± 0.05 for HI + saline animals, and 1.24 ± 0.03 for

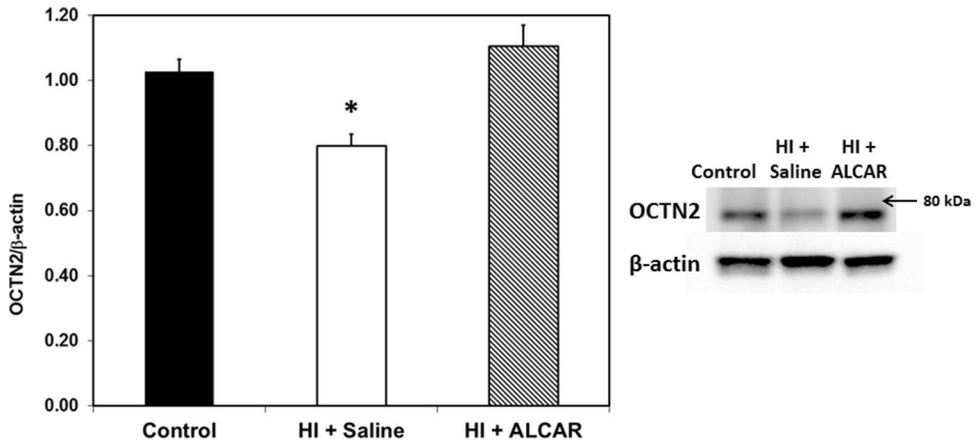

Fig. 2 Ratio of OCTN2 transporter to β-actin in kidney from sham control (black bar), HI + saline (white bar) and HI + ALCAR (hatched bar) rat pups 24 h after HI. OCTN2 was significantly decreased in HI + saline pups. Treatment with ALCAR after HI led to significantly increased OCTN2 levels compared to HI + saline pups. Western blot of proteins is shown on the right. Values are mean ± SEM for 13 controls, 14 HI + saline and 6 HI + ALCAR pups. Data were analyzed by ANOVA and Bonferroni post hoc test. *p < 0.01

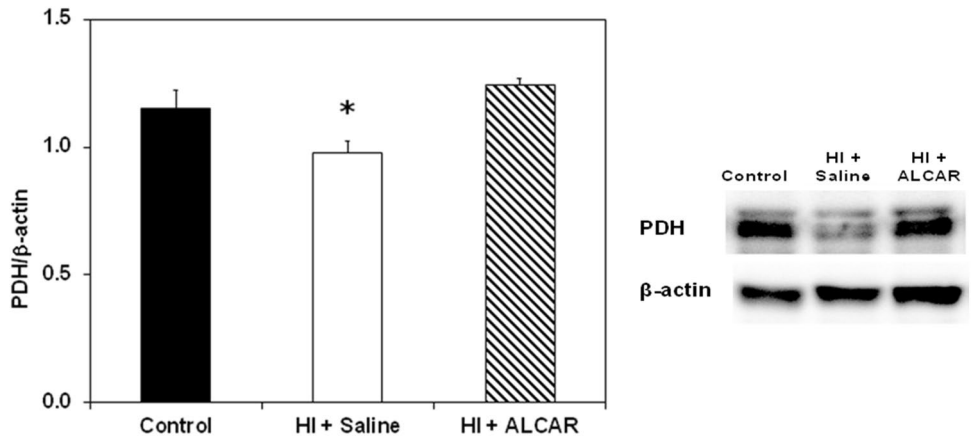

Fig. 3 Ratio of pyruvate dehydrogenase (PDH) to β-actin in kidney from sham control (black bar), HI + saline (white bar) and HI + ALCAR (hatched bar) rat pups 24 h after HI. Western blot of proteins is shown on the right. The primary antibody was against the PDH E1-α subunit. PDH levels in HI + saline pups were different than control ($p < 0.05$) and HI + ALCAR ($p < 0.01$) pups. Values are mean ± SEM for 14 controls, 14 HI + saline and 6 HI + ALCAR pups. Data were analyzed by ANOVA and Bonferroni post hoc test

HI + ALCAR animals. This suggests that HI may damage the E1-α subunit of PDH and that ALCAR may prevent this damage.

OCTN2 and PDH Levels in Male and Female Kidney

The ratios of PDH/β-actin and OCTN2/β-actin were determined in kidney from both male and female rat pups. No significant sex differences were observed in either OCTN2 and PDH levels as shown in Figs. 4 and 5, respectively. However, OCTN2 levels tended to be higher in kidney from rat pups treated with ALCAR after HI, compared to the HI + saline animals (Fig. 4). PDH levels tended to be higher in kidney from male rat pups treated with ALCAR after HI, compared to male pups treated with saline after HI (Fig. 5).

Discussion

The widely used Rice–Vannucci method of neonatal hypoxic ischemic brain injury was used in the current study [21, 22, 40]. Although alterations in brain after injury have been widely studied in this model, to our knowledge there is only one report of changes in kidney after HI in this model [11]. Xu et al. [11] recently reported swelling in renal cortex, membrane alterations in glomerular barriers, and changes in the expression of zonulin, occludin and aquaporin-4 in kidney from rat pups at 24 h after severe HI injury, and some attenuation of damage by an intraperitoneal injection of melatonin after injury. The present study shows that

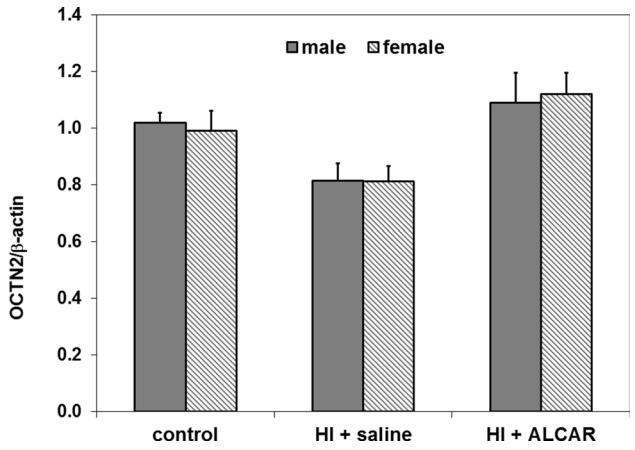

Fig. 4 Ratio of OCTN2 transporter to β-actin in kidney from male (solid grey bars) and female (hatched grey bars) in sham control, HI + saline and HI + ALCAR rat pups 24 h after HI. There were no significant differences in OCTN2 levels in kidneys from male and female rats under any of the conditions studied. Values are mean ± SEM for five male and eight female controls, five male and nine female HI + saline pups, and three male and three female HI + ALCAR pups. Data were analyzed by ANOVA

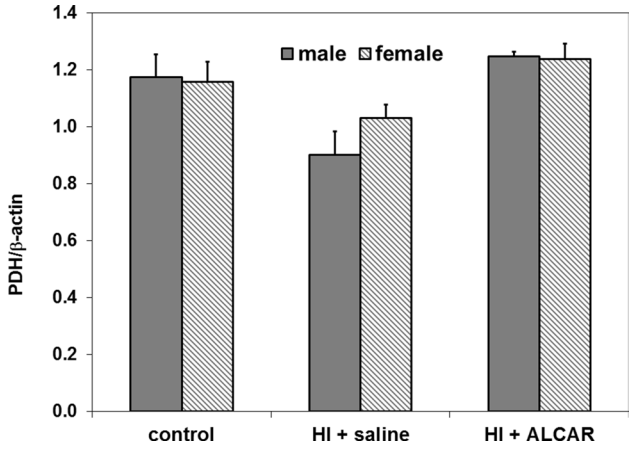

Fig. 5 Ratio of the PDH E1-α subunit to β-actin in kidney from male (solid grey bars) and female (hatched grey bars) in sham control, HI + saline and HI + ALCAR rat pups 24 h after HI. There were no significant differences in PDH levels in kidneys from male and female rat pups under any of the conditions studied. Values are mean ± SEM for five male and nine female sham controls, six male and eight female HI + saline pups, and three male and three female HI + ALCAR pups. Data were analyzed by ANOVA

a neonatal HI injury procedure that results in a moderate brain injury led to a decrease in renal proteins involved in carnitine homeostasis and energy metabolism. Treatment with ALCAR after HI, which attenuates brain injury and improves behavioral outcomes [17–19, 21, 22], prevented the loss of these key proteins in kidney. These results suggest that ALCAR, a proposed treatment for HIE, does not have an adverse effect on the kidney and thus could also be a potential treatment for kidney injury associated with neonatal HI.

A decrease in the renal OCTN2/β-actin ratio observed in HI + Saline animals 24 h after injury is consistent with reports of decreased OCTN2 levels in placental explants and cultured BeWo placental cells following 48 h of hypoxia [45, 46]; however, the mechanism leading to decreased OCTN2 levels is unclear. One possibility is through nitric oxide-mediated inhibition of OCTN2 expression. Schneider et al. [47] found that renal ischemia–reperfusion in adult rats and the subsequent increase in inducible nitric oxide synthase generated nitric oxide decreased the expression and levels of OCT1 and OCT2, two other proteins in the organic cation transporter family which exhibit significant similarity to OCTN2 [48].

Decreased OCTN2 levels are clinically relevant because of the role of OCTN2 in maintaining carnitine homeostasis [34]. Placental uptake of carnitine is needed to provide adequate levels during gestation. Thus, it is significant to note that OCTN2 levels were profoundly decreased in human placental tissue from mothers with preeclampsia [45]. Normal placental explants had decreased OCTN2 levels after 48 h of severe experimental hypoxia [45]. In young rat pups

renal OCTN2 levels are closely linked with serum L-carnitine levels [36]. Several groups have observed decreased free carnitine levels and increased long chain acylcarnitine levels in infants with HIE [37, 38, 49]. A decrease in renal OCTN2 levels leading to increased excretion of serum carnitine and/or decreased metabolism of acyl-carnitines could explain these differences.

In the present study, in animals treated with ALCAR after HI, the ratio of renal OCTN2/β-actin was comparable to that of the sham control animals at 24 h after injury, and significantly higher than the HI + saline animals. This suggests that OCTN2 levels respond to changes in levels of the substrate acetyl-L-carnitine. Furuichi et al. [50] observed a similar effect in skeletal muscle, where OCTN2 levels increased in response to increased intracellular carnitine levels. Prolonged (2 weeks) chemically induced depletion of carnitine led to severely decreased plasma and tissue carnitine levels and increased expression of OCTN2 in kidney [51]. In contrast, Gomez-Amores et al. [52] reported a decrease in kidney OCTN2 levels after long term (6 weeks) carnitine treatment. To our knowledge, the present study is the first to report an effect on OCTN2 transporter levels after ALCAR administration. Due to the antioxidant and possibly antiapoptotic effects of carnitine [53–55], it could be beneficial to maintain a high carnitine level in infants subjected to HI. This would be particularly important since infants are more vulnerable to carnitine deficiency due to immature carnitine biosynthesis pathways [20, 37]. ALCAR treatment could increase the level of carnitine in two ways, as a direct external source of supplemental carnitine and through raising the renal OCTN2 level, thus increasing the efficiency of carnitine reabsorption.

Decreased levels of PDH in the present study suggest potential damage to renal energy metabolism in rat kidney at 24 h after HI injury. Studies have identified damage to complex I of the mitochondrial electron transport chain in adult kidney during hypoxia [56], but to our knowledge, this is the first study to determine renal PDH levels in a model of neonatal HI. This enzyme, which catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA, NADH and CO_2, is the essential link between glycolysis and oxidative glucose metabolism. The E1-α subunit, which was determined in this study, is the regulatory subunit of the PDH enzyme complex [57], and is vulnerable to damage by oxidative stress [14]. Decreased PDH E1-α suggests the presence of oxidative damage in kidneys after HI. Damage to this metabolic enzyme has been shown in the brain as the result of global cerebral ischemia with hyperoxic reperfusion [44] and traumatic brain injury [58].

ALCAR treatment holds potential for reducing the effects of HI and oxidative stress injury on renal metabolism. No studies have determined the effect of ALCAR treatment on metabolism in the kidneys. However, Annadurai et al. [29] showed that ALCAR treatment after a model of oxidative stress reduced the level of lipid peroxidation and increased the amounts of antioxidants in kidney tissue. Another possible explanation for the protection of PDH levels after treatment with ALCAR is through the action of carnitine, which has been found to reverse depressed PDH activity and mitochondrial function in hypoxic myocardial cells, though the mechanism is not known [59]. These possibilities suggest that ALCAR does not just bypass PDH as an alternative source of energy; but may also play a role in protecting or restoring PDH-mediated glucose metabolism.

In summary, the results of the current study show that treatment with 100 mg/kg ALCAR after HI in the 7 day old rat pup prevented the loss of two key proteins in kidney at 24 h after injury. These results, in conjunction with studies demonstrating that ALCAR decreases lesion size in brain [21, 22], protects mitochondrial function [17, 22] and improves behavioral outcome [21] provide additional evidence supporting the potential of ALCAR as a treatment for neonatal hypoxia-ischemia.

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