Biochemical Pharmacology, Vol. 55, pp. 1853–1860, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00051-3

Restoration of Hepatic Cytochrome *c* Oxidase Activity and Expression with Acetyl-L-carnitine Treatment in spf Mice with an Ornithine Transcarbamylase Deficiency

*Yogesh R. Mawal, K. V. Rama Rao and Ijaz A. Qureshi**

Division of Medical Genetics, Sainte-Justine Hospital and University of Montréal, Montréal, Québec H3T 1C5, Canada

ABSTRACT. The sparse fur (spf) mutant mouse, with an X-linked ornithine transcarbamylase deficiency, is a model of congenital hyperammonemia in children. Our earlier studies indicated a deficiency of hepatic carnitine, CoA-SH, acetyl CoA, and ATP in spf mice. We have now studied the effects of a 7-day treatment with acetyl-L-carnitine (ALCAR) in the spf/Y mice on the activity and expression of the respiratory chain enzyme cytochrome *c* oxidase (COX; EC 1.9.3.1). We found decreased hepatic activity and expression of COX in the untreated hyperammonemic spf/Y mice, which was restored upon ALCAR treatment. Because COX is a mitochondrial membrane protein, we also carried out studies to explain the mechanism of ALCAR through its effect on membrane stability. Our results indicate a decrease of the mitochondrial membrane cholesterol/phospholipid molar ratio (CHOL/PL ratio) with the activity and expression of COX in untreated spf/Y mice. While ALCAR treatment normalized the ratios, it also restored the hepatic ATP production to normal. To study further if there was any effect of ALCAR on the mitochondrial matrix urea cycle enzymes, we measured the activity and expression of mutant ornithine transcarbamylase (OTC; EC 2.1.3.3) and normal carbamyl phosphate synthase-I (CPS-I; EC 6.3.4.16) in spf/Y mice. There was no general effect on the specific activities of the matrix enzymes upon ALCAR treatment, although their mRNA levels were enhanced. Our studies point towards the feasibility of an ALCAR treatment in conjunction with other treatment modalities, e.g. sodium benzoate and/or arginine, to improve the availability of cellular ATP and to counteract the effects of hereditary hyperammonemic syndromes in children. BIOCHEM PHARMACOL **55**;11:1853–1860, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. spf mice; congenital hyperammonemia; acetyl-L-carnitine; ornithine transcarbamylase; cytochrome *c* oxidase; liver mitochondria

The spf† mutant mouse is a model of congenital hyperammonemia with an X-linked OTC (EC. 2.1.3.3) deficiency in liver mitochondria [1]. A single base substitution in the OTC gene [2] in spf mice causes a feedback effect on mitochondrial urea cycle enzymes to produce hyperammonemia, hyperglutaminemia, and orotic aciduria [3]. Our earlier work on this animal model repeatedly indicated elevated levels of ammonia and glutamine in liver, plasma, and brain [4, 5]. We also reported a depletion of hepatic and cerebral mitochondrial energy metabolites including CoA-SH, acetyl CoA, and mitochondrial NADH/NAD^+ ratios, with a concomitant increase in the hepatic and cerebral lactate and cytosolic NADH/NAD^+ ratios, which results in hepatic and cerebral ATP depletion [4, 5]. We have also observed a secondary carnitine deficiency in spf mice, as the hepatic levels of free carnitine, short and medium chain acylcarnitines, and total carnitine are low compared with those of normal mice [6].

Based on the above studies, we hypothesized that congenital hyperammonemia in spf mice disturbs hepatic mitochondrial ATP production, possibly through an inhibition of the activity and expression of COX (EC 1.9.3.1), a respiratory chain enzyme. Treatment with ALCAR could restore the ATP levels through an induction of COX. Previous studies have shown that ALCAR has an inducing effect on the transcription of certain mitochondrial proteins in the rat brain and heart [7, 8]. ALCAR treatment was shown to restore the age-dependent decrease in cerebral COX activity and its subunit I mRNA expression [7], as well as the activity of COX in aged heart tissue [8]. It was also reported that ALCAR restored the age-dependent decrease in the activity of ATP-ADP translocase in the

* Corresponding author: Professor Ijaz A. Qureshi, Division of Medical Genetics, Hôpital Sainte-Justine, 3175, Côte Sainte-Catherine, Montréal, Québec, H3T 1C5, Canada. Tel. (514) 345-4931, Ext. 3587; FAX (514) 345-4766; E-mail: qureshii@ere.umontreal.ca.

† *Abbreviations:* ALCAR, acetyl-L-carnitine; COX, cytochrome *c* oxidase; COX-I, cytochrome *c* oxidase, subunit-I; CHOL/PL ratio, cholesterol/phospholipid molar ratio; CPS-I, carbamyl phosphate synthetase I; OTC, ornithine transcarbamylase; and spf, sparse fur.

Received 14 July 1997; accepted 5 January 1998.

heart of Fisher rats [8]. In addition, our earlier studies on spf mice indicated a restoration by L-carnitine of the levels of cerebral energy metabolites, including the decreased mitochondrial NADH/NAD^+ ratio and the overall ATP levels [9].

In the present study, we examined in spf mice the effect of congenital hyperammonemia on the enzymatic activity and mRNA expression of the respiratory chain enzyme COX, with and without ALCAR treatment. Our results indicate that the activity of COX and the mRNA expression of COX-I were decreased in untreated spf mice and that ALCAR treatment increased both its activity and mRNA expression. Because COX is a mitochondrial membrane protein, we also carried out studies to explain the mechanism of ALCAR on mitochondrial membrane stability, by measuring the membrane CHOL/PL ratio. We observed that ALCAR restored the disturbed CHOL/PL ratio. To study further if there was any effect of ALCAR on the mitochondrial matrix urea cycle enzymes, we measured the activity and expression of mutant OTC and normal CPS-I (EC 6.3.4.16) in spf mice. Our results indicated no general effect on the activities of matrix enzymes upon ALCAR treatment, whereas their mRNA levels were enhanced.

MATERIALS AND METHODS

Chemicals and cDNA Clones

Acetyl-L-carnitine was a gift from Sigma-Tau. All other chemicals used were of analytical grade and were obtained from either the Sigma Chemical Co. or the Fisher Scientific Co. The cDNA clones for OTC (from human liver), COX (subunit I, from human hippocampus), and β-actin (from fetal human brain) were obtained from the American Type Culture Collection.

Animal Breeding and ALCAR Treatment

The parent stock for the colony of spf mice was originally supplied by Dr. L. B. Russel of the Oak Ridge National Laboratories [1]. The spf/Y mouse breeding was carried out as reported earlier [9]. Male CD-I strain mice (Canadian Breeding Farms) were used as normal controls. ALCAR dissolved in saline was injected i.p. daily, at a dose of 4 mmol/kg body weight for 7 days, into 6-week-old male mutant (spf/Y), and normal (CD-I) mice. Untreated control groups for both male CD-I and spf/Y mice were injected with saline only [9]. The CD-I and spf/Y mice injected with saline were designated as CD-I saline and spf/Y saline groups, while the mice injected with ALCAR, were designated as CD-I ALCAR and spf/Y ALCAR groups. All animal experimentation was performed in accordance with the regulations of the Canadian Council for Animal Protection. At the end of treatment, all spf/Y and CD-I mice were killed by cervical dislocation, and the livers were removed and washed with PBS (0.01 M of phosphate-buffered saline, pH 7.2) prior to freezing in dry ice and storing at −70°.

Hepatic COX Activity Measurement

COX activity was measured using the spectrophotometric method of Hevner *et al.* [10]. Mitochondria were treated with 1% (w/v) deoxycholic acid, and the amount of cytochrome *c* oxidized was determined by measuring the change in absorbance at 550 nm. Protein content was measured, based on the method of Lowry *et al.* [11], using bovine serum albumin as standard.

Measurement of Hepatic Ammonia and Energy Metabolites

Livers were quickly removed and frozen in liquid nitrogen. They were then transferred into preweighed tubes containing 5% (v/v) perchloric acid and homogenized. The homogenate was centrifuged at 3000 *g*, and the perchloric acid supernatant was neutralized with 2 M of K_2CO_3 to pH 7.0. The neutralized supernatants were used immediately for metabolite estimations. This method has been performed routinely in our laboratory and published previously [4, 5, 9]. Ammonia was determined by the ion-exchange method, followed by a colorimetric estimation of the isolated ammonia nitrogen with the phenol-hypochlorite reaction [12]. Glutamine was hydrolyzed to ammonia by glutaminase, and ammonia nitrogen was estimated as described above. CoA-SH, acetyl CoA, and ATP were measured by the method of Williamson and Corkey [13], and the values are expressed as nanomoles per gram liver.

Mitochondrial Membrane CHOL/PL Ratio

The liver mitochondria and the inner and outer membrane fractions were isolated according to the method of Visentin *et al.* [14]. Mitochondrial lipids were extracted according to the method of Folch *et al.* [15]. After lipid extraction, the cholesterol was determined using the enzymatic method of Siedel *et al.* [16]. The inorganic phosphorus in phospholipid was measured colorimetrically, after acidic digestion according to the method of Bartlett [17]. To assess the purity of the outer and inner mitochondrial membranes, we measured monoamine oxidase and COX activities as respective markers of outer and inner mitochondrial membranes. Monoamine oxidase activity in total mitochondria and in the inner and outer membranes was measured using the method of Schnaitman *et al.* [18].

Measurement of the Activity of Mitochondrial Matrix Enzymes

Hepatic OTC activity was measured using the protocol of Ceriotti [19] at pH 7.7. CPS-I activity was measured in 1:50 (w/v) liver homogenate using a colorimetric method, which

determined the amount of hydroxyurea formed by the conjugation of hydroxylamine and carbamyl phosphate [20].

Northern Slot Blot Analysis

RNA was extracted from the liver tissue using the Life Technologies "Triazol" method [21]. The integrity of each RNA sample and the accuracy of the quantification were verified by agarose gel electrophoresis and visualization of the 18S and 28S bands with ethidium bromide [22]. RNA (10 μg) was blotted onto the "Genescreen" membrane according to the procedure provided by the manufacturer (NEN Research Products). The labeling, prehybridization, hybridization, and washing of the membrane was performed as described by Sambrook *et al.* [23]. The "Genescreen" membrane was exposed to "Molecular Dynamics" PhosphorImager screens, and the individual blot intensity was calculated on a "Series 400 PhosphorImager" using "ImageQuant 3.2" software (Molecular Dynamics). The amount of the radioactivity hybridized to the specific RNA was adjusted for the housekeeping gene β-actin, so as to correct for any variations resulting from the RNA loading [24]. The relative mRNA abundance of the control CD-I mice was set at 1.0 as a standard, according to the method of Horiuchi *et al.* [25], to calculate the relative intensity.

Statistical Analysis

Data are presented as means ± SEM. ANOVA with Fisher PSLD was used to test for significant differences between different groups according to the "Statview®SE+Graphics" program on an Apple® Macintosh computer. The data were cross-checked using non-parametric statistical analyses (Mann Whitney) between different groups using the same program. Differences were considered significant when $P < 0.05$.

RESULTS

Induction of COX Activity and the mRNA Levels of COX-I with ALCAR Treatment

The specific activity of COX and its mRNA levels in both spf/Y and CD-I mice are shown in Fig. 1. The specific activity of COX was found to be significantly lower ($P < 0.01$) in the spf/Y saline group than in the CD-I saline group. The COX-I mRNA levels were also found to be significantly lower in the spf/Y saline group than in the CD-I saline group. ALCAR treatment in spf/Y mice significantly enhanced the COX activity and the COX-I mRNA levels ($P < 0.01$) as compared with the CD-I and spf/Y saline groups. No significant difference in COX mRNA levels was observed in CD-I mice upon ALCAR treatment.

Changes in the Ammonia and Energy Metabolite Levels upon ALCAR Treatment

Table 1 indicates significantly elevated ($P < 0.01$) levels of hepatic ammonia and glutamine and a significant decrease ($P < 0.01$) in the levels of hepatic CoA-SH, acetyl CoA, and ATP in the spf/Y saline group as compared with the CD-I saline group. A tendency to decrease in the hepatic ammonia and glutamine levels in the spf mice was seen upon ALCAR treatment, but this was not found to be significant. ALCAR treatment resulted in a significant increase ($P < 0.01$) in CoA-SH, acetyl CoA, and ATP levels in the spf/Y saline group, restoring the levels to those of the CD-I saline group (Table 1). No significant changes in the energy metabolites in the normal CD-I mice were observed upon ALCAR treatment.

Effect of ALCAR Treatment on the CHOL/PL Ratios in the Mitochondrial Fractions

The CHOL/PL ratios of the mitochondrial fractions of spf/Y and CD-I mice are shown in Table 2. The spf/Y saline group had significantly lower ($P < 0.01$) CHOL/PL ratios in both the inner and outer mitochondrial membrane fractions as compared with the CD-I saline group. We also found a significant decrease ($P < 0.01$) in the total mitochondrial CHOL/PL ratio of the spf/Y saline group as compared with the normal CD-I saline group. Upon ALCAR treatment, the CHOL/PL ratio showed a complete restoration in the inner mitochondrial membrane, while it showed a partial restoration in the total and outer mitochondrial membranes in spf/Y mice as compared with the CD-I saline group (Table 2). No significant difference in the CHOL/PL ratio was seen in the CD-I mice upon ALCAR treatment.

Effect of ALCAR Treatment on the Specific Activity and mRNA Levels of the Matrix Urea Cycle Enzymes

Table 3 indicates the levels of mutant OTC and normal CPS-I specific activity and expression in the spf/Y and CD-I mice, with and without ALCAR treatment. In the spf/Y saline group, a significantly lower (14% of normal, $P < 0.01$) mutant OTC activity at pH 7.7 was observed as compared with the CD-I saline group. The OTC mRNA levels in the same spf/Y mice were also significantly lower (23% of normal, $P < 0.01$) as compared with the CD-I saline group (Table 3). Upon treatment with ALCAR, no significant changes were observed in the mutant OTC specific activity, whereas a significant increase (37% of normal, $P < 0.01$) in the OTC mRNA level in ALCAR-treated spf/Y mice was seen as compared with spf/Y saline group. No significant change in the OTC specific activity and the OTC mRNA was seen in untreated CD-I mice upon ALCAR treatment (Table 3).

We found that normal CPS-I activity was significantly higher (22%, $P < 0.01$) in hyperammonemic spf/Y mice than in the control CD-I mice, and a significant change in the CPS-I mRNA levels in spf/Y mice was observed

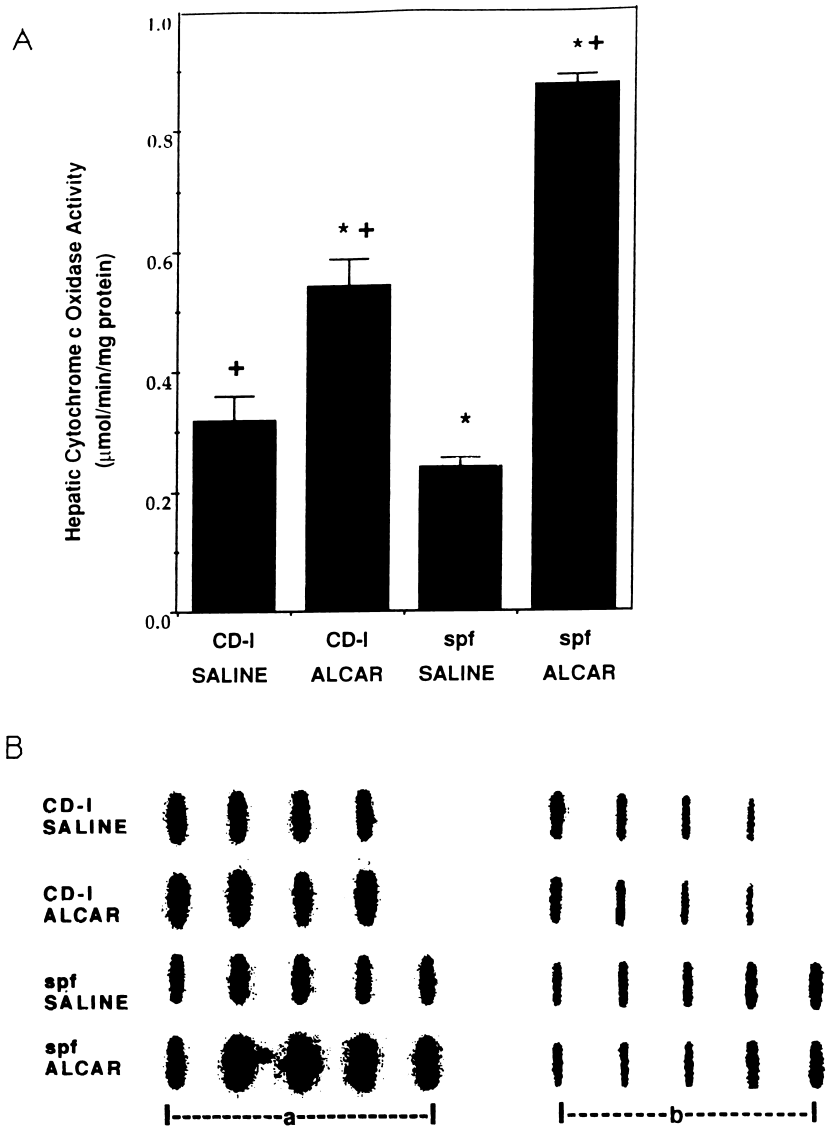

FIG. 1. Effect of ALCAR treatment on the specific activity of COX and its subunit I mRNA levels in the livers of congenitally hyperammonemic spf mice. (A) Enzyme specific activity. (B) mRNA abundance of COX-I. The CD-I ($N = 4$) and spf/Y ($N = 5$) control mice were injected with saline, while for the ALCAR treatment the CD-I ($N = 4$) and spf/Y ($N = 5$) mice were injected with ALCAR (4 mmol/kg of body weight) for 7 days. The enzyme specific activity measurement, total RNA isolation, northern blot analysis, and quantitation of the mRNA hybridized with COX-I and β-actin probe were carried out as described in Materials and Methods. The relative mRNA abundance of the control CD-I mice was set at 1.0 as a standard, according to the method of Horiuchi *et al.* [25], to calculate the relative intensity. The lanes marked (a) represent the extent of hybridization using ^{32}P-labeled COX-I cDNA as a probe, while the lanes marked (b) represent the extent of hybridization using ^{32}P-labeled β-actin cDNA probe in the corresponding mouse. The enzyme specific activity (means ± SEM) is expressed in μmol/min/mg of protein, whereas the quantity of mRNA hybridized (mean ± SEM) is expressed in cpm (10^3)/lane as observed in CD-I saline (106.9 ± 3.4), CD-I ALCAR (116.5 ± 8.1), spf/Y saline (94.1 ± 3.6) and spf/Y ALCAR (143.1 ± 8.0) groups. *Indicates significance at $P < 0.01$ as compared with the CD-I saline group; and (+) represents significance at $P < 0.01$ as compared with the spf/Y saline group.

upon ALCAR treatment as compared with the CD-I saline group (Table 3). The ALCAR treatment showed a tendency to decrease the CPS-I activity in the spf/Y mice, which was found to be not significant. However, we observed a significant increase (47%, $P < 0.01$) in the CPS-I mRNA levels in the spf/Y mice upon ALCAR treatment. In the CD-I mice, ALCAR treatment produced no significant change in the CPS-I activity but significantly increased their mRNA levels (80%, $P < 0.01$) (Table 3).

DISCUSSION

Significantly lower activity and mRNA expression of hepatic COX-I were found in hyperammonemic spf/Y mice as compared with normal CD-I mice (Fig. 1), which indicates a decreased oxidative phosphorylation, and a concomitant decrease in the cellular ATP (Table 1) in spf mice [4]. This could be due to the fact that the elevated ammonia might directly interfere with the transfer of reducing equivalents ($NADH/NAD^+$) from cytosol to the mitochondria via the

TABLE 1. Effect of ALCAR treatment on the levels of metabolites in the liver of congenitally hyperammonemic mutant (spf/Y) mice

	ATP	CoA-SH	Acetyl CoA	Ammonia	Glutamine
Treatment group			(nmol/g of liver)		
CD-I Saline	3922 ± 179	254 ± 15.9	52.8 ± 7.4	186 ± 17.6	638 ± 71.1
CD-I ALCAR	3824 ± 62*	236 ± 15.8*	53.2 ± 2.0*	169 ± 12.7*	683 ± 12.9*
spf/Y Saline	2538 ± 165†	186 ± 11.3†	29.6 ± 2.9†	316 ± 23.5†	2957 ± 263.1†
spf/Y ALCAR	3210 ± 109*†	260 ± 11.1*†	44.1 ± 1.6*†	305 ± 9.3†	2244 ± 213.6*†

ALCAR was administered at a dose of 4 mmol/kg of body weight, i.p. daily for 7 days, whereas in the control mice saline was administered. At the end of the treatment period, the 7-week-old normal (CD-I) and mutant (spf/Y) mice were killed by cervical dislocation, the livers were removed, and the ATP, CoA-SH, acetyl CoA, ammonia, and glutamine measurements were carried out as described in Materials and Methods. Values are means ± SEM ($N = 5$).

*Significantly different from the spf/Y saline group ($P < 0.01$).

†Significantly different from the CD-I saline group ($P < 0.01$).

malate-aspartate shuttle. At the cerebral level, hyperammonemia is known to disturb the transfer of reducing equivalents via the malate-aspartate shuttle by inhibiting the cytosolic malate dehydrogenase and aspartate aminotransferase activities [26]. The same phenomenon could also be occurring at the hepatic level. This condition would further result in the accumulation of the reducing equivalents in the cytosol and a depletion of their levels in the mitochondrion. This metabolic block in the transfer of reducing equivalents could further compromise the hepatic mitochondrial ATP production, possibly through substrate-depleted inhibition of the respiratory chain enzymes, e.g. COX, resulting in a subsequent feedback effect on their transcription. Our earlier report of depleted hepatic mitochondrial NADH/NAD^+ ratio [4] in conjunction with our present confirmation of depleted hepatic ATP (Table 1) in spf/Y mice would support the above argument. Moreover, we have already reported an inhibition of the respiratory chain enzyme COX at the cerebral level in spf mice [27]. The restorative effect of ALCAR on COX activity (Fig. 1) is consistent with a report by Paradies *et al.* [8], who showed that treatment of aged rats with ALCAR restored the reduced activity of the heart mitochondrial COX and the ATP-ADP translocase to the levels of young rats.

Apart from the direct effect of hyperammonemia on the malate-aspartate shuttle enzymes at the hepatic level in spf mice, the increased CPS-I activity (Table 3) may result in an excessive production of carbamoyl phosphate, which would condense with cytosolic aspartate to form orotate. The depletion of aspartate could also result in disturbances in the malate aspartate shuttle, since aspartate and malate are essential components involved in the transfer of reducing equivalents. This phenomenon could only be possible at the hepatic level in hyperammonemic spf mice, since the mitochondrial urea cycle enzymes including the CPS-I are only present in the liver and intestine but not in the brain and other organs.

A decrease observed in the levels of hepatic CoA-SH and acetyl CoA in the hyperammonemic spf/Y mice, as compared with the control CD-I mice (Table 1), is also a confirmation of our earlier work [4]. A mutation in the hepatic OTC compromises the ammonia detoxification and the production of urea in spf/Y mice, which results in elevated levels of hepatic and plasma ammonia and glutamine [4]. The observed decrease in free CoA levels might be explained on the basis of its increased utilization in the esterification of accumulated fatty acids. This condition could lead to sequestration of the hepatic levels of free CoA during the process of the detoxification of the accumulated fatty acids, which would result in a decreased channeling of the acetyl CoA into the tricarboxylic acid cycle. Our present data on the reduced hepatic levels of CoA-SH and acetyl CoA (Table 1) would support the above argument. A significant increase in CoA-SH and acetyl CoA levels upon ALCAR treatment in spf/Y mice (Table 1) could be due to the ability of ALCAR to deliver acetyl-CoA equivalents to the tricarboxylic acid cycle and facilitate the mitochondrial

TABLE 2. Effect of ALCAR treatment on the CHOL/PL ratio in the liver of the congenitally hyperammonemic spf/Y mice

Treatment group	Outer membrane	Inner membrane	Total mitochondria
CD-I Saline	0.229 ± 0.009 (4)	0.057 ± 0.001 (4)	0.289 ± 0.004 (4)
CD-I ALCAR	0.248 ± 0.005* (4)	0.063 ± 0.002* (4)	0.313 ± 0.004* (4)
spf/Y Saline	0.182 ± 0.005† (5)	0.034 ± 0.003† (5)	0.204 ± 0.007† (5)
spf/Y ALCAR	0.206 ± 0.004*† (5)	0.055 ± 0.005* (5)	0.241 ± 0.005*† (5)

ALCAR was administered at a dose of 4 mmol/kg of body weight, i.p. daily for 7 days, whereas in the control mice saline was administered. At the end of the treatment period, the 7-week-old normal (CD-I) and mutant (spf/Y) mice were killed by cervical dislocation, the livers were removed, and the CHOL/PL ratio measurement was carried out as described in Materials and Methods. Values are means ± SEM.

*Significantly different from the spf/Y saline group ($P < 0.01$).

†Significantly different from the CD-I saline group ($P < 0.01$).

TABLE 3. Effect of ALCAR treatment on the mitochondrial matrix enzyme OTC and CPS-I specific activities and mRNA expression levels in the liver of congenitally hyperammonemic spf/Y mice

Treatment group	Enzyme specific activity (μmol/hr/mg protein)		Relative mRNA abundance	
	OTC	CPS-I	OTC	CPS-I
CD-I Saline	78.4 ± 17.9	1.49 ± 0.08	1.00 ± 0.12	1.00 ± 0.07
CD-I ALCAR	60.2 ± 13.3*	1.62 ± 0.08	1.62 ± 0.41	1.80 ± 0.27*†
spf/Y Saline	10.8 ± 0.3†	1.83 ± 0.12†	0.23 ± 0.01†	0.87 ± 0.03
spf/Y ALCAR	10.3 ± 0.7†	1.53 ± 0.11	0.37 ± 0.04*†	1.28 ± 0.12*†

ALCAR was administered at a dose of 4 mmol/kg body weight, i.p. daily for 7 days, whereas in the control mice saline was administered. At the end of the treatment period, the 7-week-old normal (CD-I) and mutant (spf/Y) mice were killed by cervical dislocation, the liver was removed, and enzyme activity and mRNA abundance measurements were carried out as described in Materials and Methods. The relative mRNA abundance of the CD-I saline-treated mice was set at 1.0 as a standard according to the method of Horiuchi *et al.* [25] to calculate the relative intensity. Values are means ± SEM; $N = 4$ in the case of CD-I saline and CD-I with ALCAR treatment and $N = 5$ in the case of spf/Y saline and spf/Y ALCAR treatment.

*Significantly different from spf/Y saline group ($P < 0.01$).

†Significantly different from CD-I saline group ($P < 0.01$).

β-oxidation of fatty acids [28], thereby increasing the production of ATP in the spf/Y mice.

ALCAR treatment in spf/Y mice demonstrated no significant effect on the hepatic ammonia and glutamine levels (Table 1). These results are different from our earlier reports [27], wherein we observed a normalization of cerebral glutamine levels in spf/Y mice upon ALCAR treatment. This could be due to the direct effect of ALCAR on the cerebral glutamine-glutamate cycle involving glutamine synthetase and glutaminase. However, at the hepatic level, the bulk of ammonia derived from the nitrogen metabolism is primarily detoxified through urea synthesis. Hence, it could be possible that ALCAR may exert different effects on the ammonia metabolism in the two organs. Moreover, the spf mouse hepatocyte may be constantly subjected to ammonia and glutamine accumulation due to a mutation in the OTC gene, causing a reduced hepatic ammonia detoxification.

Decreased levels of ATP (Table 1) due to decreased COX activity (Fig. 1) could be caused by a disturbed oxidative phosphorylation in the electron transport chain, which may, in turn, result in generation of free radicals [29] in spf/Y mice. To investigate whether this could exert any effect on the hepatic mitochondrial membrane stability, we isolated the outer and inner membrane fractions of the spf/Y and the CD-I normal mice and determined the CHOL/PL ratios. The observed decrease in the hepatic mitochondrial membrane CHOL/PL ratio in spf/Y mice, as compared with the control CD-I mice (Table 2), could indicate an effect of hyperammonemia on membrane fluidity and viscosity, thereby causing instability of the mitochondrial membrane. The respiratory chain enzyme complexes, being membrane proteins, might also be affected as a result of membrane instability and may further compromise the mitochondrial ATP production, as observed in spf/Y mice (Table 1).

Upon ALCAR treatment, the CHOL/PL ratio increased significantly in the spf/Y mice (Table 2). The CHOL/PL ratio of the inner mitochondrial membrane in the spf/Y ALCAR group showed a complete restoration, while it showed a partial restoration in the total and outer mitochondrial membranes, as compared with the CD-I saline group. This effect could have been achieved by the effect of ALCAR in decreasing lipid peroxidation and exerting an effect on scavenging free radicals [30]. This would result in restoring the altered CHOL/PL ratio and normalizing the membrane fluidity to restore the decreased COX activity and expression, possibly along with other membrane proteins. In similar studies with isolated rat liver mitochondria, Bellei *et al.* [31] reported that L-carnitine could partially prevent the ammonia-induced swelling and loss of respiratory control in the mitochondria. These results are also similar to those of Visentin *et al.* [14], who studied a Reye's Syndrome rat model in which a restoration of the altered CHOL/PL ratio and the membrane viscosity by treatment with L-carnitine was observed. This effect of ALCAR on membrane stability may be synergistic with the restoration to normal of the mitochondrial reducing equivalents, thus enhancing the activity and expression of the respiratory chain enzymes as indicated by an effect on the COX activity and expression.

Horiuchi *et al.* [25], in a carnitine-deficient jvs mouse model, observed a suppressed expression of the urea cycle enzyme CPS-I; its levels were restored upon L-carnitine treatment. To examine if the mechanism of ALCAR treatment was similar to that of L-carnitine in our hyperammonemic spf/Y mice, we studied the specific activity and expression of the urea cycle matrix enzymes OTC and CPS-I. The spf/Y mice have a point mutation in the OTC gene, which may result in the synthesis of the mutant protein having lower specific activity as compared with control CD-I mice (Table 3). However, Dubois *et al.* [32], using western blot analysis, have reported that the amount of the mutant OTC enzyme protein increased by 144% in spf mice, as compared with the normal mice. In our present study, an increased translatable OTC mRNA level in the spf/Y mice (Table 3) did not result in increased specific activity of the mutant OTC protein, possibly due to either a degradation of the induced mutant protein or the possibility that the mutant OTC protein being synthesized may

be relatively inactive. We found significantly higher CPS-I activity in saline-treated spf/Y mice than in the control CD-I mice (Table 3), which could be a result of an increased post-translational modification in the CPS-I protein in spf/Y mice, so as to compensate for the reduced mRNA synthesis. Similar results were obtained by Dubois *et al*. [32], who speculated that the increase in CPS-I activity in the spf/Y mice as compared with the control CD-I mice could be a result of either an enhanced post-translational modification or a consequence of a better CPS-I and OTC physical interaction in the mitochondria. However, the increased levels of OTC and CPS-I mRNA upon ALCAR treatment in our present study (Table 3) are similar to the increase in mRNA contents of CPS-I and argininosuccinate synthetase in carnitine-deficient mice as reported by Horiuchi *et al*. [25]. They postulated that an L-carnitine-mediated hormonal effect on gene induction could be resulting in an enhanced expression of these enzymes. We, therefore, speculate that ALCAR may also be exerting the same mechanism in our spf mouse model. A significant increase observed in the OTC and the CPS-I mRNA levels in ALCAR-treated spf/Y mice, but no change in the specific activity, may also be due to either a degradation of the induced enzyme protein [32] or the result of a feedback inhibition.

In summary, our studies indicate that ALCAR treatment has an effect on enhancing the activity and expression of the membrane-bound respiratory chain enzyme COX in chronically hyperammonemic spf/Y mice, but had no significant effect on the activity of the matrix mutant OTC and normal CPS-I enzymes. ALCAR might achieve this specific effect by restoring the mitochondrial membrane CHOL/PL ratio and the mitochondrial membrane stability. This could have a positive effect on restoring the activity and expression of the membrane-bound respiratory chain enzyme COX. We also speculate that the mechanism of the ALCAR effect may be due to its ability to scavenge free radicals and restore mitochondrial membrane stability. Our results point towards the feasibility of ALCAR treatment as an adjunctive therapy with other treatment modalities, e.g. sodium benzoate and/or arginine, to improve the availability of cellular ATP to reduce the high ammonia levels so as to counteract the effects of hereditary hyperammonemic syndromes.

References

1. DeMars R, LeVan SL, Trend BL and Russell LB, Abnormal ornithine carbamoyltransferase in mice having sparse-fur mutation. *Proc Natl Acad Sci USA* **73:** 1693–1697, 1976.
2. Veres G, Gibbs RA, Scherer SE and Caskey CT, The molecular basis of the sparse fur mouse mutation. *Science* **237:** 415–417, 1987.
3. Qureshi IA, Letarte J and Ouellet R, Ornithine transcarbamylase deficiency in mutant mice. I. Studies on the characterization of enzyme defect and suitability as animal model of human disease. *Pediatr Res* **13:** 807–811, 1979.
4. Ratnakumari L, Qureshi IA and Butterworth RF, Effects of congenital hyperammonemia on the cerebral and hepatic levels of the intermediates of energy metabolism in spf mice. *Biochem Biophys Res Commun* **184:** 746–751, 1992.
5. Ratnakumari L, Qureshi IA and Butterworth RF, Effects of sodium benzoate on cerebral and hepatic energy metabolites in spf mice with congenital hyperammonemia. *Biochem Pharmacol* **45:** 137–146, 1993.
6. Michalak A and Qureshi IA, Tissue acylcarnitine and acyl-coenzyme A profiles in chronically hyperammonemic mice treated with sodium benzoate and supplementary L-carnitine. *Biomed Pharmacother* **49:** 350–357, 1995.
7. Gadaleta MN, Petruzzella V, Renis M, Fracasso F and Cantatore P, Reduced transcription of mitochondrial DNA in the senescent rat. Tissue dependence and effect of L-carnitine. *Eur J Biochem* **187:** 501–506, 1990.
8. Paradies G, Ruggiero FM, Petrosillo G, Gadaleta MN and Quagliariello E, Effect of aging and acetyl-L-carnitine on the activity of cytochrome oxidase and adenine nucleotide translocase in rat heart mitochondria. *FEBS Lett* **350:** 213–215, 1994.
9. Ratnakumari L, Qureshi IA and Butterworth RF, Effect of L-carnitine on cerebral and hepatic energy metabolites in congenitally hyperammonemic sparse-fur mice and its role during benzoate therapy. *Metabolism* **42:** 1039–1046, 1993.
10. Hevner RF, Liu S and Wong-Riley MTT, An optimized method for determining cytochrome oxidase activity in brain tissue homogenates. *J Neurosci Methods* **50:** 309–311, 1993.
11. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
12. Dienst SG, An ion exchange method for plasma ammonia concentration. *J Lab Clin Med* **58:** 149–153, 1961.
13. Williamson JR and Corkey BE, Assays of intermediates of the citric acid cycle and related compounds by fluorometric enzyme methods. *Methods Enzymol* **13:** 434–513, 1969.
14. Visentin M, Bellasio R and Tacconi MT, Reye syndrome model in rats: Protection against liver abnormalities by L-carnitine and acetyl-L-carnitine. *J Pharmacol Exp Ther* **275:** 1069–1075, 1995.
15. Folch J, Lees M and Stanley GHS, A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **226:** 497–509, 1957.
16. Siedel J, Hagele EO, Ziegenhorn J and Wahlefeld AW, Reagent for the enzymatic determination of serum total cholesterol with improved lipolytic efficiency. *Clin Chem* **29:** 1075–1080, 1983.
17. Bartlett GR, Phosphorus assay in column chromatography. *J Biol Chem* **234:** 466–468, 1969.
18. Schnaitman C, Ervin VG and Greenawalt JW, The submitochondrial localization of monoamine oxidase. *J Cell Biol* **32:** 719–735, 1967.
19. Ceriotti G, Optimal conditions for ornithine carbamyl transferase determination. A simple micromethod without deproteinization. *Clin Chim Acta* **47:** 97–105, 1973.
20. Pierson D, A rapid colorimetric assay for carbamyl phosphate synthetase I. *J Biochem Biophys Methods* **3:** 31–37, 1980.
21. Chomczynski P and Sacchi N, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162:** 156–159, 1987.
22. Arkins S, Rebeiz N, Biragyn A, Reese DL and Kelley KW, Murine macrophages express abundant insulin-like growth factor-I class I Ea and Eb transcripts. *Endocrinology* **133:** 2334–2343, 1993.
23. Sambrook J, Fritsch EF and Maniatis T, *Molecular Cloning: A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.
24. Adler GK, Chen R, Menachery I, Braley LM and Williams GH, Sodium restriction increases aldosterone biosynthesis by

increasing late pathway, but not early pathway, messenger ribonucleic acid levels and enzyme activity in normotensive rats. *Endocrinology* **133:** 2235–2240, 1993.
25. Horiuchi M, Kobayashi K, Tomomura M, Kuwajima M, Imamura Y, Koizumi T, Nikaido H, Hayakawa J-I and Saheki T, Carnitine administration to juvenile visceral steatosis mice corrects the suppressed expression of urea cycle enzymes by normalizing their transcription. *J Biol Chem* **267:** 5032–5035, 1992.
26. Hindfelt B, Plum F and Duffy TE, Effect of acute ammonia intoxication on cerebral metabolism in rats with portacaval shunts. *J Clin Invest* **59:** 386–396, 1977.
27. Rama Rao KV, Mawal YR and Qureshi IA, Progressive decrease of cerebral cytochrome C oxidase activity in sparse-fur mice: Role of acetyl-L-carnitine in restoring the ammonia-induced cerebral energy depletion. *Neurosci Lett* **224:** 83–86, 1997.
28. Rosenthal RE, Williams R, Bogaert YE, Getson PR and Fiskum G, Prevention of postischemic canine neurological injury through potentiation of brain energy metabolism by acetyl-L-carnitine. *Stroke* **23:** 1312–1318, 1992.
29. Nohl H and Hegner D, Do mitochondria produce oxygen radicals *in vivo*? *Eur J Biochem* **82:** 563–567, 1978.
30. Di Giacomo C, Latteri F, Fichera C, Sorrenti V, Campisi A, Castorina A, Russo A, Pinturo R and Vanella A, Effect of acetyl-L-carnitine on lipid peroxidation and xanthine oxidase activity in rat skeletal muscle. *Neurochem Res* **18:** 1157–1162, 1993.
31. Bellei M, Battelli D, Guarriero DM, Muscatello U, DiLisa F, Siliprandi N and Bobyleva-Guarriero V, Changes in mitochondrial activity caused by ammonium salts and the protective effect of carnitine. *Biochem Biophys Res Commun* **158:** 181–188, 1989.
32. Dubois N, Cavard C, Chasse J-F, Kamoun P and Briand P, Compared expression levels of ornithine transcarbamylase and carbamylphosphate synthetase in liver and small intestine of normal and mutant mice. *Biochim Biophys Acta* **950:** 321–328, 1988.