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Acetyl-L-carnitine cytoprotection against 1-methyl-4-phenylpyridinium toxicity in neuroblastoma cells

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Abstract

Acetyl-L-carnitine (ALCAR) plays an integral role in the transport of long chain fatty acids across the inner mitochondrial membrane for oxidative phosphorylation. In non-human primates, administration of ALCAR was reported to prevent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurological injury to the substantia nigra. The present study investigates the effects of ALCAR against the toxicity of 1-methyl-4-phenylpyridinium (MPP^+), the neurotoxic metabolite of MPTP, in murine brain neuroblastoma cells. MPP^+, a potent mitochondrial toxin, induced a dose-dependent reduction in mitochondrial oxygen consumption and cell viability, corresponding to an accelerated rate of cellular glucose utilization. Treatment with ALCAR, but not L-carnitine, prevented MPP^+ toxicity and partially restored intracellular ATP concentrations, but did not reverse the MPP^+-induced loss of mitochondrial oxygen consumption. These data indicate that protective effects are independent of oxidative phosphorylation. ALCAR had a substantial glucose sparing effect in both controls and MPP^+-treated groups, demonstrating a potential role in enhancing glucose utilization through glycolysis. Antagonizing the entry of fatty acids into the mitochondria, with either insulin or malonyl CoA, did not interfere with ALCAR protection against MPP^+. On the contrary, insulin potentiated the protective effects of ALCAR. In conclusion, these data indicate that ALCAR protects against MPP^+ toxicity, independent of mitochondrial oxidative capacity or β-oxidation of fatty acids. In contrast, the protective effects of ALCAR appear to involve potentiation of energy derived from glucose through anaerobic glycolysis.

Keywords: Acetyl-L-carnitine; MPP; Parkinson's disease; Metabolism; Energy; MPTP

1. Introduction

ALCAR plays a significant role in aerobic metabolism through its function as a carrier of long chain fatty acids into the mitochondria for β-oxidation [1]. Furthermore, its acetyl group can form acetyl CoA, contributing to oxidative phosphorylation (OXPHOS) through enhanced energy substrate supply to the Kreb's cycle [2]. Although naturally occurring in mammalian tissue, exogenously administered ALCAR can cross the blood–brain barrier through the GABA uptake system [3]. Moreover, ALCAR administration can attenuate neurological brain injury associated with degenerative disorders, including axotomy, human immunodeficiency virus, diabetic neuropathy, and Alzheimer's disease (AD) [4–6]. In human AD patients, therapeutic administration of ALCAR is effective in reducing cognitive losses as determined by a mini-mental status and AD assessment scale [4]. In rats, oral administration of ALCAR can reduce age-associated changes in spatial and temporal memory, along with a localized reduction in oxidative damage to the hippocampus [7]. ALCAR administration can also improve cholinergic transmission in the hippocampus of aging rats, through positive effects on choline uptake, acetylcholine synthesis, and acetylcholine release [8]. Its therapeutic role for treatment of AD is also based on its ability to reduce the toxicity of amyloid beta [9]. While defining a role for ALCAR in treatment of AD has gained sizable interest and reached the point of human clinical trials

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Abbreviations: AD, Alzheimer's disease; ALCAR, acetyl-L-carnitine; DMEM, Dulbecco's modified Eagle medium; HBSS, Hank's balanced salt solution; FBS, fetal bovine serum; ATP, adenosine-5′-triphosphate; MCA, malonyl coenzyme A; ME, malic enzyme; MPP, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; OXPHOS, oxidative phosphorylation; PC, pyruvate carboxylase; PD, Parkinson's disease; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; PI, propidium iodide; Tris–HCl, tris(hydroxymethyl)aminomethane hydrochloride; UV, ultraviolet.

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[4,10], there are meager data that examine a potential therapeutic role or mechanism for ALCAR in the treatment of Parkinson's disease (PD).

There is evidence to support a hypothetical rationale for why ALCAR may benefit PD patients. The pathology of aging and PD involves the loss of mitochondrial OXPHOS capacity and/or inherited mitochondrial abnormalities [11–13]. ALCAR is reportedly effective in reducing age-dependent mitochondria functional decay, including restoring losses of mitochondrial membrane potential, cardiolipin content, metabolic oxygen (O_2) consumption, and β-oxidation of fatty acids [13–15]. Furthermore, it has been demonstrated that ALCAR can restore age-associated membrane depolarization and the loss of Na^+/K^+-ATPase activity in the striatum of rats [16]. Most compelling is the evidence reported by Bodis-Wollner *et al.*, indicating that administration of ALCAR to non-human primates can prevent MPTP toxicity [17]. However, there has been little follow up on Bodis-Wollner's original research. Since, the exact mechanism(s) of ACLAR neuroprotection is unknown; the current study was designed to investigate the neuroprotective properties of ALCAR against the direct actions of MPP^+ cytotoxicity *in vitro*.

2. Materials and methods

In the current investigation, Neuro-2A (N-2A) brain neuroblastoma cells were obtained from American Type Culture Collection. L-Glutamine, fetal bovine serum—heat inactivated (FBS), PBS, Dulbecco's Modified Eagle Medium (DMEM), Hank's balanced salt solution (HBSS), and penicillin/streptomycin were supplied by Fischer Scientific, Mediatech. All other chemicals and supplies were purchased from Sigma Chemical Co.

2.1. Cell culture

N-2A cells were chosen for this investigation because the original clone for this cell line was established from the brain of an albino mouse. In addition, *in vivo* albino mice display selective dopaminergic neurodegeneration in response to MPTP, similar to the pathological lesions that occur in PD [18,19]. Moreover, N-2A cells exhibit true neuronal morphology and are vulnerable to the toxic effects of MPP^+ *in vitro* [20,21]. In the current investigation, N-2A cells were cultured in DMEM containing phenol red, L-glutamine (4 mM), penicillin/streptomycin (100 units/0.1 mg/mL), sodium pyruvate (20μM), and FBS (10% v/v). The cells were grown at 37° in 5% CO_2/atmosphere, scraped and sub-cultured every 2–5 days. The experimental plating media consisted of DMEM without phenol red, containing L-glutamine (4 mM), sodium pyruvate (20 μM), FBS (1.8% v/v), and penicillin/streptomycin (100 units/0.1 mg/mL). For experiments, N-2A cells were plated at a plating density of $\sim 0.5 \times 10^6$ cells/mL. A stock solution of ALCAR was prepared in HBSS containing 5 mM HEPES, pre-adjusted to a pH of 7.4. Six dilutions of ALCAR were prepared to span a 1000-fold experimental dilution range. Solutions of MPP^+ were prepared fresh daily.

2.2. ATP assay

Somatic ATP was analyzed using a firefly bioluminescence procedure (Technical Bulletin No. BAAB-1 for Stock # FL-AA, Sigma). After treatment, the cells were prepared in a lysis buffer and frozen immediately at −80°. The lyses buffer contained DL-dithiothreitol (4 mM), EDTA (2 mM), Tris–HCl (20 mM), apoprotin (5 μg/mL), pepstatin A, and phenylmethylsulfonyl fluoride (200 μM) prepared in sterile HBSS (pH 7.4). The prepared firefly luciferase–luciferin (L–L) solution in glycine buffer was purchased from Sigma and was reconstituted with sterile deionized water. The working (L–L) solution contained luciferase (0.04 mg/mL), luciferin (0.26 mM), $MgSO_4$ (19.5 mM), EDTA (1.95 mM), glycine (195 mM), and 324 mM Tris–HCl (pH 7.4). The cell lysate was thawed slowly, and diluted in serum-free plating medium (v/v) 1:2. The L–L solution was added at 1.28% of the final sample volume. Luminescence was quantified using a Beckman LS 6500 Scintillation Counter (Beckman). The settings were set at: count, cpm; background, none; isotope, manual; quench, off with no chemiluminescence adjustment. A standard curve was generated by dilutions of ATP in serum-free plating medium and light units were plotted as cpm vs. ATP concentration. The data were expressed as percentage of live control.

2.3. Oxygen consumption determination

Mitochondrial O_2 consumption was assessed using the oxygraph respirometer (Clark Electrode, Hansatech Instruments Ltd). Quantification of dissolved O_2 concentration in the medium is indicative of cellular mitochondrial oxidative respiration. The electrode was calibrated with both air saturated deionized water (O_2 line) and deionized water containing sodium dithionite (N_2 line). Briefly, the cell sample supernatant or control blanks were loaded into the cell chamber jacket at 25°. After rate stabilization, a reading was taken and the data were established as nanomoles of O_2/mL and converted to percentage of control.

2.4. Cell viability evaluation

Cell viability was quantified using resazurin (alamar blue) indicator dye. Although there are a wide variety of colorimetric indicators for toxicity testing, we chose this assay for several reasons. In the past, resazurin has been utilized for detecting both viable yeasts and bacteria in milk [22,23]. Yeasts obtain energy from glucose often through fermentation, a process that is anaerobic and dependent upon substrate level phosphorylation [24]. Therefore, in the study of MPP^+, which exerts inhibitory

effects on OXPHOS, resazurin indicator dye can also detect cell viability by energy derived through substrate level phosphorylation (i.e. anaerobic glycolysis). This point warrants the use of this dye for *in vitro* toxicology studies, in particular with mitochondrial toxins, such as MPP^+ or rotenone [25]. Briefly, resazurin was prepared in PBS (0.5 mg/mL) and filtered through a 0.2 μm mesh filter flask. Viable cells convert the oxidized form of the dye (resazurin) to its reduced form (resorufin). The dye solution was added (15% v/v equivalent) to the samples. Samples were returned to the incubator for 6–12 hr. Quantitative analysis of dye conversion was measured on a microplate fluorometer—Model 7620 version 5.02, Cambridge Technologies Inc., set at 550/580 (excitation/emission) wavelengths.

2.5. Cell death evaluation

Cell death was evaluated using propidium iodide (PI) fluorescence stain [26]. Briefly, a PI stock (1 mg/mL) solution was prepared in HBSS and stored at 0°. A final working concentration of PI (7 μg/mL) was achieved by diluting the stock solution in HBSS. The reagent was added (15% v/v equivalent) to the samples. Samples were returned to the incubator for 15 min and then analyzed on a Nikon Eclipse TE300 inverted microscope with a SPOT cooled CCD color digital camera (Diagnostic Instruments) using a green filter cube. PI is quantified at 536 nm (green) excitation and 620 nm emission. Images were captured using SPOT Imaging Software Version 2.2 and analyzed by IP Lab Scientific Processing Software (Scanalytics).

2.6. Glucose determination

Glucose was quantified using a glucose oxidase–peroxidase-linked enzyme reaction at 12 hr post-treatment. The profile of glucose utilization was taken at 12 hr in order to observe the metabolic profile occurring prior to full consumption of glucose. Briefly, the enzymatic assay contained equal volume of glucose oxidase (20 U/mL) [27] and the chromogenic solution, which contained a final working concentration: 1 mM vanillic acid, 500 μM 4-aminoantipyrine, and 4 U purpurogallin/mL horseradish peroxidase—Type II [28]. Both solutions were prepared in distilled water containing 10 mM HEPES, pH 5.1. The enzyme reagent was added to each sample (10% v/v) and incubated for 5 min at 25°. Glucose was quantified at 490 nm on a UV Microplate Spectrophotometer—Model 7600 version 5.02 (Cambridge Technologies Inc.). Controls and blanks were run simultaneously. The experimental medium, HBSS, and PBS contained glucose; therefore, the standard curve (0.01–10 mM) was prepared in buffered distilled water. Protein was determined using the Lowry protocol [29], and the data were expressed as μM/mg protein or volume equivalent.

2.7. Lactic acid determination

Determination of lactic acid was achieved by an enzymatic assay (Procedure No. 735, Sigma Chemical Diagnostics). Experiments involving lactate determination were established in low serum experimental plating medium due to interference by FBS at higher levels. Briefly, lactic acid

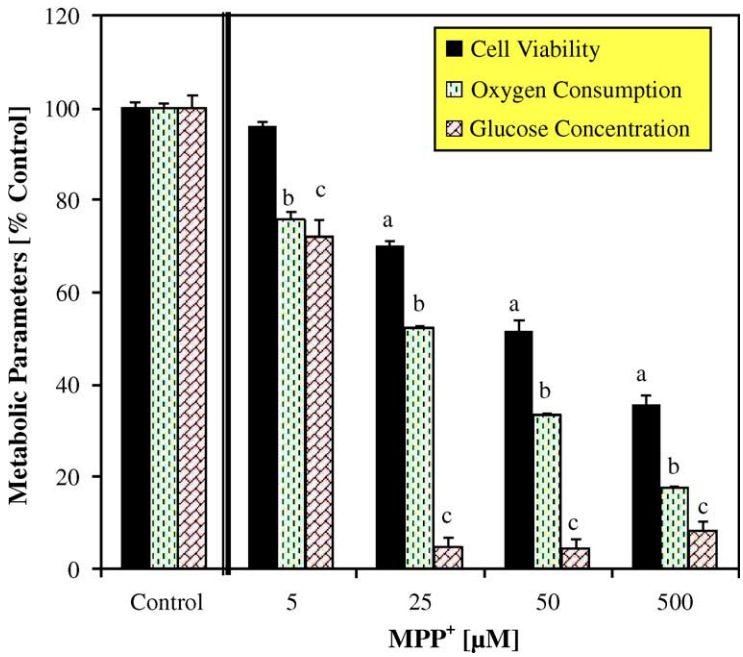

Fig. 1. Concentration-dependent cell death by MPP^+. N-2A cells were treated with varying concentrations of MPP^+ for 24 hr at 37°. The data represent percent control for cell viability, O_2 consumption, and residual DMEM glucose concentration relative to the media control blank without cells. The data are expressed as the mean ± SEM, N = 4. Significance of difference from the control was determined by a one-way ANOVA, followed by a Tukey's mean comparison post hoc test. [Viability (a) = $P < 0.00$], [O_2 consumption (b) = $P < 0.001$], [glucose concentration (c) = $P < 0.001$].

was converted to pyruvate and H_2O_2 by lactate oxidase. The reaction was coupled to peroxidase-linked oxidative condensation of a chromogen and H_2O_2. The lactate reagent consisted of lactate oxidase (400 U/L) and horseradish peroxidase (2400 U/L) (Sigma), prepared in 18 MΩ water containing 1 mM vanillic acid and 500 μM 4-aminoantipyrine. At 12 hr, the lactate reagent was added and samples were incubated for 8 min at 37°. Lactate was

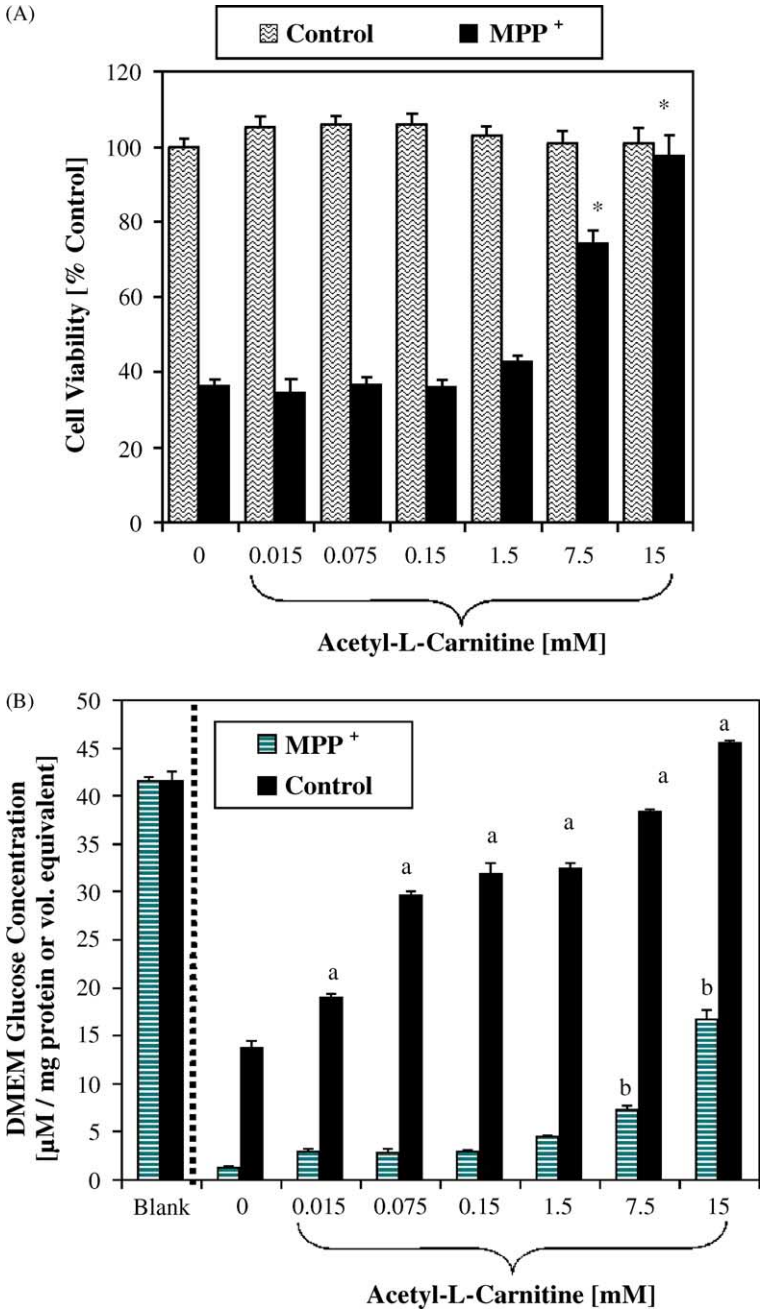

Fig. 2. (A) The effects of ALCAR on MPP^+ toxicity. N-2A cells were treated with MPP^+ (500 μM) ± a variation in ALCAR for 24 hr at 37°. The data represent cell viability as percent control and are expressed as the mean ± SEM, N = 4. Significance of difference from the live or MPP^+-treated controls was determined by a one-way ANOVA, followed by a Tukey's mean comparison post hoc test. $^*P < 0.001$. (B) The effect of ALCAR on cellular glucose consumption. N-2A cells were treated with ±MPP^+ (500 μM) ± a variation in ALCAR concentration for 12 hr at 37°. The data represent residual DMEM glucose concentration (μM/mg protein or volume equivalent: blank). The data are expressed as the mean ± SEM, N = 4. Significance of difference from the live or MPP^+-treated controls was determined by a one-way ANOVA, followed by a Tukey's mean comparison post hoc test. [Control (a) = $P < 0.001$], [MPP^+ (b) = $P < 0.001$]. (C) The effects of ALCAR on cellular lactic acid production. N-2A cells were treated with ±MPP^+ (500 μM) ± a variation in ALCAR concentration for 12 hr at 37°. The data represent lactic acid concentration (μM/mg protein or volume equivalent: blank). The data are expressed as the mean ± SEM, N = 4. Significance of difference from the live or MPP^+-treated controls was determined by a one-way ANOVA, followed by a Tukey's mean comparison post hoc test. [Control (a) = $P < 0.001$], [MPP^+ (b) = $P < 0.001$]. (D) The effects of ALCAR and glucose on MPP^+ toxicity. N-2A cells were treated with MPP^+ (500 μM) ± ALCAR or glucose for 24 hr at 37°. The data represent cell viability as percent control and are expressed as the mean ± SEM, N = 4. Significance of difference from the MPP^+-treated control was determined by a one-way ANOVA, followed by a Tukey's mean comparison post hoc test. $^*P < 0.001$.

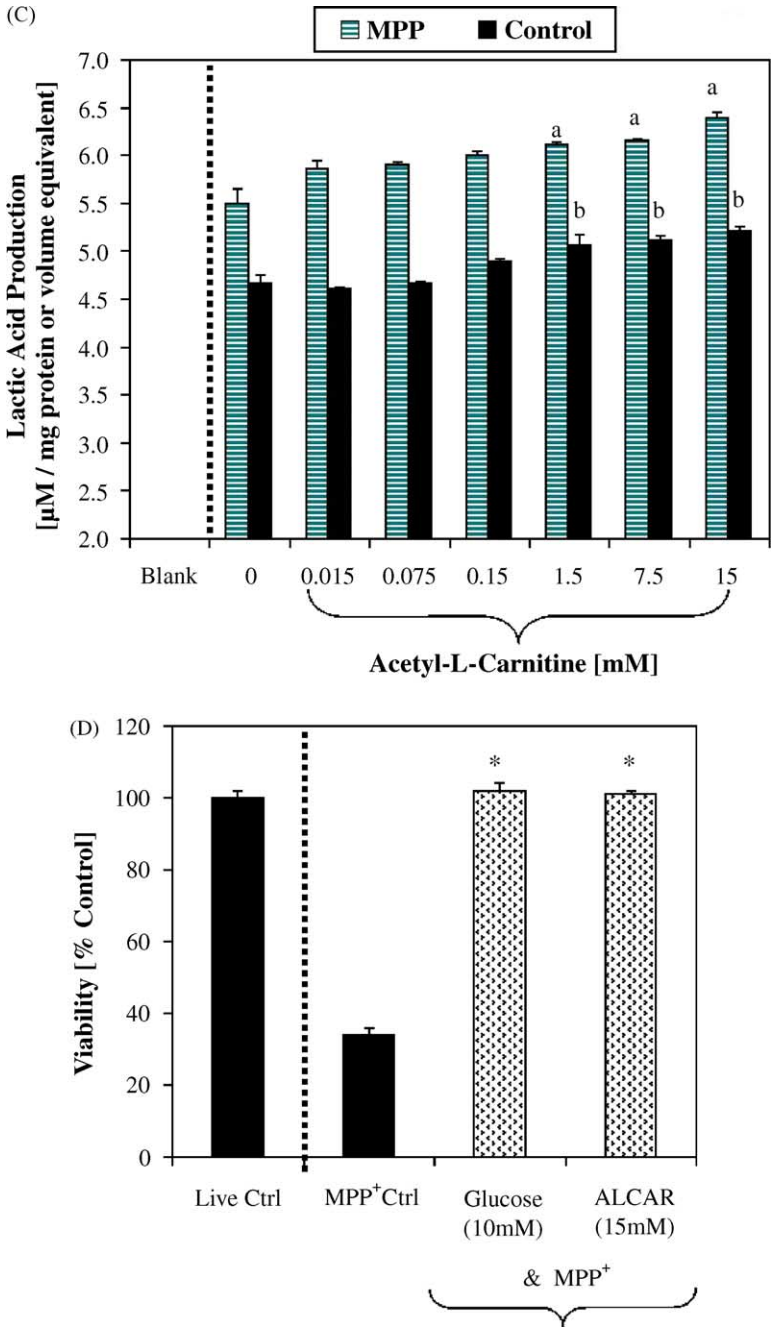

Fig. 2. (*Continued*).

quantified at 490 nm on a UV Microplate Spectrophotometer—Model 7600 version 5.02 (Cambridge Technologies Inc.). A lactic acid standard curve was established in low serum (0.18%) DMEM plating media minus phenol red. Protein was determined using the Lowry protocol [29], and the data were expressed as µM/mg protein or percent control.

2.8. Data analyses

Statistical analysis was performed using Graphpad Prism version 3.0, Graphpad Software Inc. Data were expressed as the mean ± SEM for each group. Significance of difference between the groups was assessed using a one-way ANOVA, followed by a Tukey's mean comparison post hoc test.

3. Results

N-2A cells were treated with varying concentrations of MPP^+ for 24 hr in order to establish the time–concentration-dependent toxicity (Fig. 1). MPP^+ (500 µM) was effective in reducing cell viability and O_2 consumption, with corresponding depletion of glucose. Subsequently,

the effects of ALCAR on MPP^+ toxicity were determined (Fig. 2A). N-2A cells were exposed to 500 μM MPP^+ ± varying concentration of ALCAR. Concentrations of up to 15 mM ALCAR were non-toxic and effective in blocking MPP^+ toxicity (from 36 ± 2% to 98 ± 6%, $P < 0.001$). On the other hand, L-carnitine was not effective in providing protection against MPP^+ (data not shown). Moreover, ALCAR was effective in reducing glucose utilization and slightly augmenting lactic acid production in both control and MPP^+-treated groups (Fig. 2B and C). These data indicate that effects of ALCAR may be the result of potentiating anaerobic glucose utilization. Pretreatment with both glucose (10 mM) and ALCAR (15 mM) had similar effects in blocking MPP^+ toxicity (Fig. 2D). Next, we examine the effects of ALCAR on cell death (Fig. 3A and B). Treatment with MPP^+ (500 μM) was effective in increasing PI staining to 287 ± 1.8% of live controls ($P < 0.001$). Treatment with ALCAR (15 mM) was effective in reducing cell death and restoring PI staining to 94 ± 6.5% of live controls. The metabolic changes that accompanied the neuroprotective effects of ALCAR against MPP^+ were examined simultaneously (Fig. 4). The concentration at which ALCAR became effective in reducing cell injury corresponded to even further reduction in cellular O_2 consumption, indicating an enhanced anaerobic state. On the other hand, the increase in somatic ATP (34 ± 1% of live controls) paralleled the increase in viability (Fig. 2A), indicating heightened anaerobic energy production. ALCAR plays a critical role in mitochondrial fatty acid β-oxidation, which yields reducing equivalents to the electron transport for OXPHOS. In order to examine what role this may play in the protection against MPP^+, the effects of malonyl CoA and insulin were examined (Table 1). Insulin and malonyl CoA (both serve to downregulate fatty acid entry into the mitochondria) were ineffective in blocking ALCAR protection. These data indicate that ALCAR is protective against MPP^+ through supporting anaerobic glucose metabolism, rather than its role in aerobic oxidation of fatty acids.

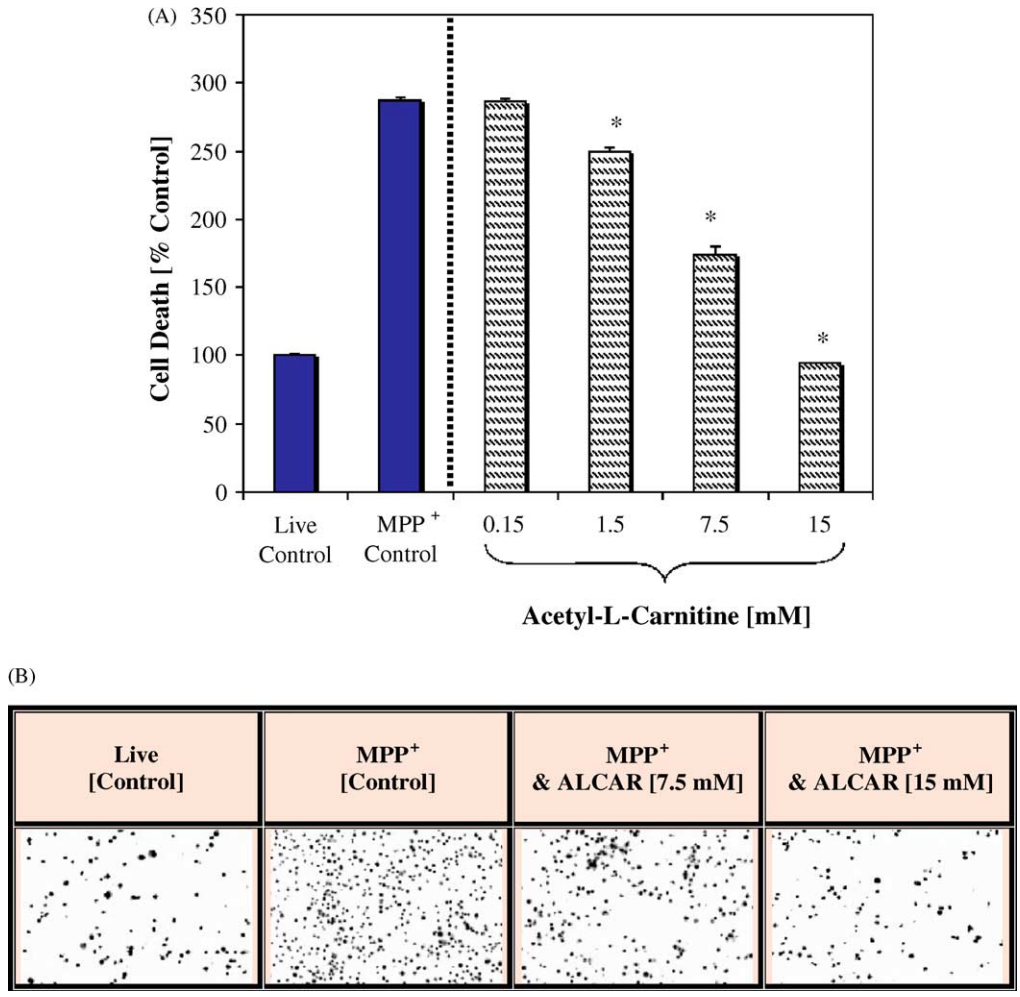

Fig. 3. (A) and (B) The effects of ALCAR on MPP^+-induced cell death. N-2A cells were treated with MPP^+ (500 μM) ± a variation in ALCAR concentration for 24 hr at 37°. Cell death was quantified using PI fluorescent staining and intensity values for each photograph in panel B were determined by densitometry. The data are expressed as the mean intensity per pixel ± SEM, N = 60,000 pixels. Significance of difference from the MPP^+-treated controls were determined by a one-way ANOVA, followed by a Tukey's mean comparison post hoc test. $^*P < 0.001$.

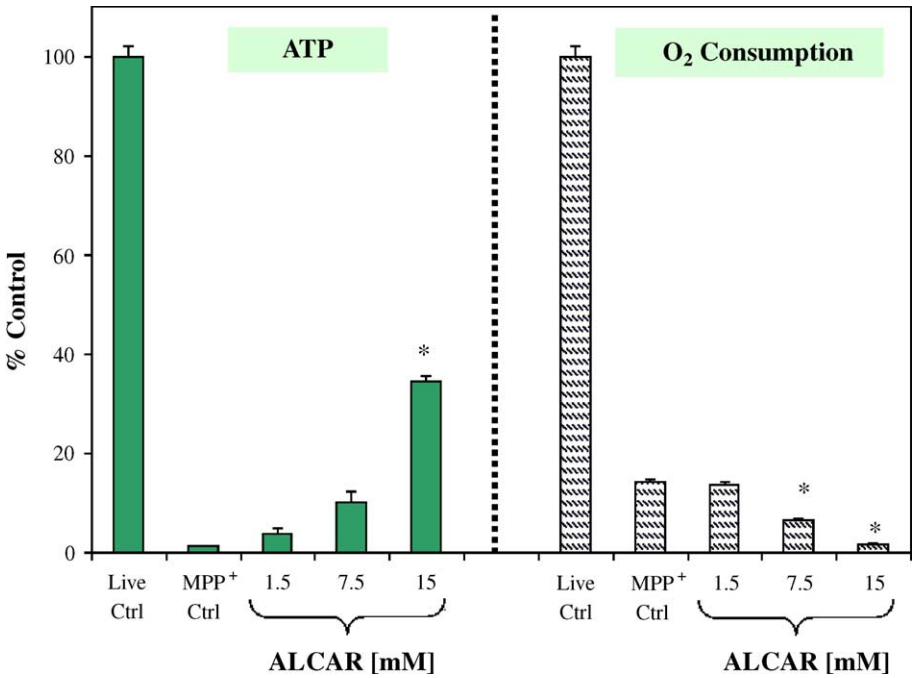

Fig. 4. Cellular ATP concentration and O_2 consumption were analyzed during the protection of ALCAR concentration against MPP^+. N-2A cells were treated with MPP^+ (500 μM) ± a variation in ALCAR for 24 hr at 37°. The data represent percent live controls and are expressed as the mean ± SEM, N = 4. Significance of difference from the MPP^+-treated controls were determined by a one-way ANOVA, followed by a Tukey's mean comparison post hoc test. $^*P < 0.001$.

Table 1
The effects of malonyl CoA and insulin on ALCAR rescue against MPP^+

INS (U/mL)	Viability (% control)			
	INS	INS and MPP^+	INS and ALCAR	INS, MPP^+, and ALCAR
0	100 ± 4	61 ± 3	100 ± 3	100 ± 4
0.01	99 ± 1	55 ± 4	112 ± 3	117 ± 3**
0.05	101 ± 0	49 ± 3	116 ± 1*	118 ± 1**
0.1	105 ± 1	51 ± 3	113 ± 3	117 ± 4**
1	106 ± 1	48 ± 4	113 ± 2	123 ± 2***
5	106 ± 2	41 ± 5*	103 ± 5	121 ± 2***
10	107 ± 1	39 ± 2**	98 ± 2	122 ± 2***
MCA (mM)	MCA	MCA and MPP^+	MCA and ALCAR	MCA, MPP^+, and ALCAR
0	100 ± 6	67 ± 3	100 ± 4	100 ± 3
0.001	104 ± 3	75 ± 4	96 ± 3	105 ± 4
0.005	103 ± 2	77 ± 3	102 ± 7	107 ± 5
0.01	97 ± 3	70 ± 3	100 ± 4	103 ± 8
0.1	104 ± 4	64 ± 3	103 ± 2	102 ± 5
0.5	96 ± 3	78 ± 4	109 ± 4	107 ± 2
1	104 ± 4	69 ± 1	103 ± 3	105 ± 7

INS, insulin; MPP^+, 1-methyl-4-phenylpyridinium (500 μM); MCA, malonyl CoA; ALCAR, acetyl-L-carnitine (15 mM). N-2A cells were treated with MPP^+ (500 μM) ± ALCAR with variation in insulin or malonyl CoA concentration for 24 hr at 37°. The viability data represent (% live control) and are expressed as the mean ± SEM. Significance of difference from the control was determined by a one-way ANOVA, followed by a Tukey's mean comparison post hoc test.
$^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

4. Discussion

The present study indicates that ALCAR is protective against MPP^+ toxicity *in vitro*. These findings are consistent with previously reported data in similar models where ALCAR is neuroprotective against other mitochondrial inhibitors, such as rotenone, 3-nitroproprionic acid, *p*-(trifluoromethoxy)phenylhdyrazone [30], and cyanide [31]. The protection of ALCAR in the presence of mitochondrial toxins or during cerebral ischemia [32] suggests that its administration may assist in sustaining neuronal energy supplies in the absence of O_2 or a malfunction of mitochondrial O_2 utilization, as in the case with PD [11–13].

It is well known that MPP^+ mediates its toxic effects primarily by inhibiting NADH: ubiquinone oxidoreductase in complex I of the electron transport chain [33]. This event impedes electron transport function and attenuates the receipt of reducing equivalents utilized for OXPHOS. Subsequently, the rapid loss of ATP leads to attenuation of membrane Na^+/K^+-ATPase activity, neuronal depolarization, and a degenerative cycle of apoptotic or excitotoxic cell death [34–36]. Preventing the loss of ATP is a foremost critical factor in antagonizing the *direct* toxic effects of MPP^+ [37]. Therefore, protection against MPP^+ can be accomplished by maintaining cellular energy supplies, through providing ample glucose to fulfill the demands of glycolysis [37–39]. The data presented in this study indicate that ALCAR may exert protection through maximizing cellular glucose efficiency under both normal and MPP^+-treated conditions.

It is also important to note that the model used in this study may or may not represent a neuroprotective mechanism for ALCAR on energy metabolism in normal mammalian tissue. Neuroblastoma cell lines contain neuronal properties merged with the characteristics of an immortal tumor. Clearly, the data in this study indicate that a large quantity of lactic acid is being produced by N-2A cells, indicating a compensatory anaerobic metabolic requirement. These findings are in alignment with the metabolic aberration of cancer cells. Possibly, the energy metabolism of immortal cell lines, such as N-2A cells, may be more representative of cancer cells than that of normal tissue. Therefore, future studies will be required to determine if protective effects of ALCAR against mitochondrial toxins can be demonstrated in primary cultures or *in vivo*.

Previously, several *in vivo* studies have substantiated the positive effects of ALCAR on glucose metabolism. For example, administration of ALCAR can potentiate glucose utilization by the brain, yielding higher resistance to neuronal insults. In a study using ^{14}C-labeled glucose injected in rats, ALCAR reduced the quantity of $^{14}CO_2$ released from [U-^{14}C]glucose [40], indicating that less glucose is required for cerebral metabolism. ALCAR administration can also lead to a concomitant increase in newly synthesized proglycogen, indicating a more efficient glucose storage capacity of the brain [40]. Further, administration of ALCAR provides restorative effects on energy metabolism, and is effective in preventing age-related neuronal deterioration in the cortex, hippocampus, striatum, and thalamus of rats [16]. The positive effects of ALCAR on carbohydrate metabolism are also thought to involve its contribution to mitochondrial cardiolipin content. Cardiolipin is a controlling component of pyruvate transport into the mitochondria for oxidative metabolism [41]. *In vivo*, administration of ALCAR is effective in attenuating age-related metabolic energy decay and can restore losses in cardiolipin, parallel to positive effects on membrane potential and elevated cellular O_2 consumption [14]. The positive effects of ALCAR on neurons extends beyond sustaining energy levels. Acetyl-L-carnitine arginine amide (ST-857) can stimulate sympathetic neurite outgrowth in rat pheochromocytoma (PC12) cells similar to that of nerve growth factor [42] and ALCAR can prevent the age-related loss of NGF mRNA in several brain regions [43].

From the findings in this study, we believe that ALCAR does not potentiate glycolysis, but acts as an energy fuel, thereby causing less glucose to be consumed, or more glucose to be available. Recently, we have found that acetyl groups may contribute toward the production of glycolytic metabolic energy intermediates, which can drive production of ATP.[1] Preliminary studies indicate that this cascade appears to involve the conversion of acetyl CoA to acetic acid and subsequent formation to phosphoenolpyruvate (PEP) or pyruvic acid, both which can drive anaerobic glycolysis. This could provide an explanation for the protective effect of ALCAR against mitochondrial toxins, where the energy is wholly dependent upon anaerobic glycolysis [30,31]. We have recently reported that neuroblastoma cells have a partial gluconeogenic cascade, with ability to readily convert selected substrates, such as malic acid and PEP, into glycolytic energy intermediates [44] or glucose. Conversely, we found that neuroblastoma cells appear to lack the gluconeogenic enzymes to catabolize almost all of the ketogenic or gluconeogenic amino acids and glycerol into energy. The partial gluconeogenic pathway in neuroblastoma may involve the metabolic activity of three enzymes: malic enzyme (ME), PEP carboxykinase (PEPCK), and pyruvate carboxylase (PC), in addition to the enzymatic pathway for reverse glycolysis [44]. These findings are somewhat in agreement with evidence describing localization of specific gluconeogenic enzymes in neurons of the brain. For example, central nervous system neurons have a high requirement for PEPCK, PC, and ME in order to regenerate tricarboxylic acid cycle intermediates to compensate for the loss of alpha-ketoglutarate used for synthesis of glutamate and GABA synthesis [45,46]. Further, in the rat brain, expression and activity of ME and PEPCK have been detected in whole brain homogenates, primary cultures of astrocytes, synaptosomal fractions, primary cultures of cortical neurons [47], cortical synaptic terminals, and cerebellar granule cells [48]. And, while PC is primarily known to exist exclusively in glia, there is evidence to support that PC activity may also occur in central nervous system neurons [49]. However, future research will be required to investigate the function of neuronal PC.

In summary, ALCAR appears to contribute to enhanced efficiency of glucose utilization by neuroblastoma *in vitro*. Future research will have to clearly define: (a) if glucose requirements are lower in the presence of ALCAR, (b) if ALCAR contributes directly to the production of glycolytic metabolic intermediates, or (c) if *de novo* glucose

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production is fostered through gluconeogenesis in the presence of ALCAR. With the dual nature of ALCAR contributing to glucose efficiency within the brain, concomitant to potentiation of nerve growth factors, it may be worthwhile to examine its potential as a therapeutic agent for PD.

Acknowledgments

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