Research Article

Acetyl-L-carnitine-induced up-regulation of heat shock proteins protects cortical neurons against amyloid-beta peptide 1-42-mediated oxidative stress and neurotoxicity: Implications for Alzheimer's disease

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Funded by:

- NIH; Grant Number: AG-05119, AG-10836 to D.A.B

KEYWORDS

\( \text{A}\beta_{42} \) • Alzheimer's disease • acetyl-L-carnitine • inducible nitric oxide synthase • 3-nitrotyrosine • quercitin • reactive oxygen species • Zn protoporphyrin IX • N(G)-monomethyl-l-arginine

ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by loss of memory and cognition and by senile plaques and neurofibrillary tangles in brain. Amyloid-beta peptide, particularly the 42-amino-acid peptide (A\( \beta_{42} \)), is a principal component of senile plaques and is thought to be central to the pathogenesis of the disease. The AD brain is under significant oxidative stress, and A\( \beta_{42} \) peptide is known to cause oxidative stress in vitro and in vivo. Acetyl-L-carnitine (ALCAR) is an endogenous mitochondrial membrane compound that helps to maintain mitochondrial bioenergetics and lowers the increased oxidative stress associated with aging. Glutathione (GSH) is an important endogenous antioxidant, and its levels have been shown to decrease with aging. Administration of ALCAR increases cellular levels of GSH in rat astrocytes. In the current study, we investigated whether ALCAR plays a protective role in cortical neuronal cells against A\( \beta_{42} \)-mediated oxidative stress and neurotoxicity. Decreased cell survival in neuronal cultures treated with A\( \beta_{42} \) correlated with an increase in protein oxidation (protein carbonyl, 3-nitrotyrosine) and lipid peroxidation (4-hydroxy-2-nonenal) formation. Pretreatment of primary cortical neuronal cultures with ALCAR significantly attenuated A\( \beta_{42} \)-induced cytotoxicity, protein oxidation, lipid peroxidation, and apoptosis in a dose-dependent manner. Addition of ALCAR to neurons also led to an elevated cellular GSH and heat shock proteins (HSPs) levels compared with untreated control cells. Our results suggest that ALCAR exerts protective effects against A\( \beta_{42} \) toxicity and oxidative stress in part by up-regulating the levels of GSH and HSPs. This evidence supports the pharmacological potential of acetyl carnitine in the management of A\( \beta_{42} \)-induced oxidative stress and neurotoxicity. Therefore, ALCAR may be useful as a possible therapeutic strategy for patients with AD. © 2006 Wiley-Liss, Inc.

DIGITAL OBJECT IDENTIFIER (DOI)

10.1002/jnr.20877  About DOI

ARTICLE TEXT

Alzheimer's disease (AD) is a neurodegenerative disorder characterized pathologically by senile plaques, neurofibrillary tangles, and synapse loss (Katzman and Saitoh, [1991]). The major constituent of senile plaques is amyloid-beta peptide (A\( \beta_{42} \)), a hallmark of AD. AD is a neurodegenerative disorder associated with cognitive decline and aging. A\( \beta_{42} \) has been shown to induce protein oxidation and lipid peroxidation in vitro and in vivo (Yatin et al., [1999]; Butterfield et al., [2001]; Drake et al., [2003]). Both protein oxidation and lipid peroxidation have been found to be significantly increased in AD brain (Hensley et al., [1995]; Butterfield and Lauderback, [2002]; Castegna et al., [2002a], [b]). Consequently, A\( \beta_{42} \) has been implicated as a causative agent in AD (Butterfield et al., [2001]; Butterfield, [2003]) and has been proposed to play a central role in the pathogenesis of this disease, as a mediator of oxidative stress and...
The brain has a high oxygen consumption rate, abundant lipid content, and relatively low availability of antioxidant enzymes compared with other tissues (Coyle and Puttfarken, [1993]; Markesbery, [1997]), which makes this organ particularly susceptible to oxidative stress. To combat this vulnerability, the brain has evolved networks that detect and control different kinds of stress. Heat shock protein (HSP) response is one kind of such cellular stress response, which involves protection of cells from various forms of stress (Moss and Morimoto, [2004]). HSPs serve as molecular chaperones, and among the various HSPs, Hsp32 (also known as HO1), Hsp60, and Hsp72 have recently been demonstrated to play a protective role against brain oxidative stress (Kravets et al., [2004]). Several studies also suggest that Aβ-induced oxidative stress leads to apoptotic neuronal cell death that can be inhibited by antioxidants (Behl et al., [1994]; Sultana et al., [2004], [2005]). Several groups have studied the possible functions of a range of pharmacological agents and nutritional components that could be of therapeutic potential (Deleeye and Watson, [1991]; Silva et al., [2006]; Bauvois and Douzonne, [2006]). We used acetyl-L-carnitine (ALCAR) in the present study to determine its possible protective effects against Aβ42-induced oxidative stress in cortical neuronal culture. ALCAR is present in high concentrations in the brain and is involved in the production of acetylcholine (Dolezal and Tucek, [1981]). ALCAR has been shown to reverse age-related deficits in mitochondrial function (Hagen et al., [1998]; Paradies et al., [1999]).

Glutathione (GSH) is a tripeptide located in both the cytosol and the mitochondria and acts as a vital endogenous antioxidant to combat oxidative stress. GSH is found in millimolar intracellular concentrations in the brain (Cooper, [1997]). Aβ42 has been shown to deplete GSH levels in astrocytes (Abramov et al., [2003]). Additionally, GSH levels decrease with age, leaving neurons vulnerable to oxidative damage initiated by Aβ42 (Liu and Choi, [2000]). Consequently, Aβ42 may lead to depletion of GSH as an available antioxidant in neurons. Up-regulation of GSH could play a therapeutic role in AD (Butterfield et al., [2002]). Moreover, ALCAR has been shown to facilitate transport of the compound across the blood-brain barrier (Kido et al., [2001]) and elevates the nigral levels of GSH (Fariello et al., [1988]). Such up-regulation of GSH by other compounds has been shown to protect both synaptosomes and mitochondria against peroxynitrite-mediated oxidative stress (Drake et al., [2002]). In this study, we evaluated the role of ALCAR in cortical neurons as a protective therapeutic agent against Aβ42-induced oxidative stress and neurotoxicity.

MATERIALS AND METHODS

Chemicals

All chemicals were of the highest purity and were obtained from Sigma (St. Louis, MO) unless otherwise noted. Aβ42 was purchased from Anaspec (San Jose, CA), with HPLC and MS verification of purity. ALCAR (99.99% pure) was a generous gift from Sigma Tau Co. (Pomezia, Italy). Anti-HO1, anti-Hsp72, and anti-iNOS were obtained from Stressgen Biotechnologies, and anticytochrome C was from Sigma. The OxyBlot protein oxidation detection kit and caspase-3 antibody were purchased from Chemicon International (Temecula, CA). 4-Hydroxynonenal (HNE) was obtained from Cayman Chemical (Ann Arbor, MI). Anti-HNE was purchased from Alpha Diagnostic International (San Antonio, TX).

Cell Culture Experiments

Neuronal cultures were prepared from 18-day-old Sprague-Dawley rat fetuses. Aβ42 peptide was dissolved in sterile phosphate-buffered saline (PBS), pH 7.5, and preincubated for 24 hr at 37°C prior addition to cultures. The final concentration of the Aβ42 peptide in the cell culture was 10 μM, and its effects on the neuronal culture were measured after 24 hr of exposure. Inhibitors of HO1, Hsp72, and iNOS proteins [zinc protoporphyrin IX (ZnPPIX), quercetin, and L-NMMA (a nonisoform-specific nitric oxide synthase inhibitor), respectively] were added singly to the cell culture 1 hr before addition of ALCAR, which was added 2 hr prior to addition of Aβ42 peptide. The cells in the culture dishes were washed twice with PBS (pH 7.5) and incubated for 1 hr in CO2 incubator. The dark blue formazan crystals formed in intact cells were extracted with 250 μl of dimethyl sulfoxide, and the absorbance was read at 595 nm with a microtiter plate reader (Bio-Tek Instruments, Winooski, VT). Results were expressed as the percentage MTT reduction (Liu et al., [1997]) vs. control cells (untreated cells).

Determination of Cell Viability

Mitochondrial function was evaluated by the 3-[4,5-dimethylthiazol-2-y]-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. Briefly, MTT was added to each well at a final concentration of 1.0 mg/ml and incubated for 1 hr in CO2 incubator. The dark blue formazan crystals formed in intact cells were extracted with 250 μl of dimethyl sulfoxide, and the absorbance was read at 595 nm with a microtiter plate reader (Bio-Tek Instruments, Winooski, VT). Results were expressed as the percentage MTT reduction (Liu et al., [1997]) vs. control cells (untreated cells).

Analysis of DNA Fragmentation

Cell death was measured by Hoescht 332584 (1 μg/ml) followed by propidium iodide (PI; 5 μg/ml) staining and detected by fluorescence microscopy (Darzynkiewicz et al., [1994]). Cortical neuronal cells were treated with Aβ42 (10 μg/ml)
of modified Laemmli buffer containing 0.125 M Tris base, pH 6.8, 4%
M dithiobis-2-nitrobenzoic acid (DTNB) was utilized to calculate the average
of the apoptotic cells in each of the groups was determined as a percentage of untreated control.

Measurement of Protein Carbonyls
Samples (5 μl) were incubated for 20 min at room temperature with 5 μl of 12% sodium dodecyl sulfate (SDS) and 10 μl of 2,4-dinitrophenylhydrazine (DNPH) that was diluted 10 times with PBS (pH 7.5) from a 200 mM stock. The samples were neutralized with 7.5 M of neutralization solution (2 M Tris in 30% glycerol). The resulting sample (250 ng) was loaded per well in the slot-blot apparatus; samples were loaded onto a nitrocellulose membrane under vacuum pressure. The membrane was blocked with 3% bovine serum albumin (BSA) in PBS containing 0.2% (v/v) Tween 20 (wash blot) for 1 hr and incubated with a 1:100 dilution of anti-DNP polyclonal antibody in wash blot for 1 hr. After completion of the primary antibody incubation, the membranes were washed three times in wash blot for 5 min each. An anti-rabbit IgG alkaline phosphatase secondary antibody was diluted 1:8,000 in wash blot and added to the membrane for 1 hr. The membrane was washed in wash blot three times for 5 min each and developed with Sigmafast tablets (BCIP/NBT substrate). Blots were dried, scanned with Adobe Photoshop (San Jose, CA), and quantitated in Scion Image.

Measurement of HNE
Levels of HNE were quantified by slot-blot analysis as described previously (Lauderback et al., 2001). Anti-HNE antibody raised in rabbit was used as the primary antibody (5:1:000 dilution). The membrane was developed with Sigmafast tablets (BCIP/NBT substrate). The blot was dried, scanned with Adobe Photoshop, and quantitated in Scion Image (PC version of Macintosh-compatible NIH Image) software.

Measurement of 3-Nitrotyrosine (3NT)
The sample (10 μl) was incubated with 10 μl of modified Laemmli buffer containing 0.125 M Tris base, pH 6.8, 4% (v/v) SDS, and 20% (v/v) glycerol. The resulting sample (250 ng) was loaded in the slot blot apparatus; samples were loaded onto a nitrocellulose membrane under vacuum pressure. The membrane was blocked with 3% (w/v) BSA in wash blot for 1 hr and incubated with a 1,200 dilution of 3-NT polyclonal antibody in wash blot for 90 min. After completion of the primary antibody incubation, the membranes were washed three times in wash blot for 5 min each. An anti-rabbit IgG alkaline phosphatase secondary antibody was diluted 1:8,000 in wash blot and added to the membrane for 120 min. The membrane was washed in wash blot three times for 5 min each and developed with Sigmafast tablets (BCIP/NBT substrate). Blots were dried, scanned with Adobe Photoshop, and quantitated in Scion Image (PC version of Macintosh-compatible NIH Image) software.

GSH Assay
Endogenous GSH was measured with a Glutathione Assay Kit (Cayman Chemical). Briefly, cells were deproteinated by treatment with 10% (w/v) metaphosphoric acid (Aldrich, Milwaukee, WI) and centrifuged at 2,000g for 2 min. The supernatant was collected and treated with 4 M triethanolamine. Standards of oxidized GSH (GSSG) were prepared varying from 0 to 8.0 μM. Fifty microliters of standard were aliquoted per well to establish a standard curve ranging from 0 to 16.0 μM GSH. Fifty microliters of sample was added per well. One hundred fifty microliters of assay cocktail (consisting of 2-(N-morpholino) ethanesulfonic acid (MES) buffer, cofactor mixture (NADP and glucose-6-phosphate), enzyme mixture (GSH reductase, and glucose-6-phosphate dehydrogenase) and 5,5-diethiobis-2-nitrobenzoic acid (DTNB) were added to each well, and the absorbance was followed at 405 nm for 30 min. All measurements were made in triplicate. The average absorbance at 25 min was calculated for each standard and sample. A plot of the corrected absorbance vs. the concentration of the GSH standards (in μM) was utilized to calculate the average concentration of GSH present in the samples.

Western Blot Analysis
Neuronal cultures were incubated with 10 μM Aβ1-42 alone or ALCAR alone for 24 hr or pretreated with ALCAR 2 hr prior to addition of Aβ1-42. Protein content of the cells was determined by using the Bio-Rad protein assay reagent (BCA). Equal amounts of proteins were separated on 4-15% SDS-polyacrylamide gels, transferred to nitrocellulose membrane, and then exposed to the appropriate antibodies. HO1, Hsp-72, iNOS, caspase-3, cytochrome C, and GAPDH were detected with specific primary antibodies. After incubation with the primary antibodies, the nitrocellulose membranes were incubated with a secondary alkaline phosphatase-conjugated antibody. Proteins were visualized by developing with Sigmafast tablets (BCIP/NBT substrate). Blots were dried, scanned with Adobe Photoshop, and quantitated in Scion Image (PC version of Macintosh-compatible NIH Image) software.

Statistical Analysis
Data were analyzed by one-way ANOVA, followed by inspection of all differences via Student-Newman-Keuls test.
RESULTS

ALCAR Inhibits Aβ_{1-42}-Induced Protein Oxidation and Lipid Peroxidation in a Dose-Dependent Manner

Protein carbonyls are elevated in vulnerable regions of AD brain (Hensley et al., 1995; Castegna et al., 2002a, b) and in brain treated in vivo with Aβ_{1-42} (Boyd-Kimbali et al., 2005). 3-NT is formed by the reaction of reactive nitrogen species (RNS) with proteins and is reported to be elevated in AD brain (Smith et al., 1997; Castegna et al., 2003). A dose-dependence study was carried out by adding ALCAR to the cortical neurons. A significant 38%, 30%, or 70% increase in protein carbonyls (P < 0.05), HNE (P < 0.05), or 3-NT (P < 0.01) levels, respectively, was induced in neurons following 24 hr of treatment with Aβ_{1-42} (Fig. 1a-c). Pretreatment of neurons with ALCAR was shown to inhibit Aβ_{1-42}-mediated protein oxidation and lipid peroxidation in a dose-dependent manner. At 100 μM ALCAR, there was a maximal significant decrease in protein oxidation (P < 0.05; Fig. 1a,c) and lipid peroxidation (P < 0.01; Fig. 1b). ZnPP IX, quercitin, and L-NMMA are the respective inhibitors of HO1 (Poon et al., 2004) Hsp72, and iNOS (Sultana et al., 2005). Pretreatment of neurons with ZnPP IX or quercitin or L-NMMA independently followed by 100 μM ALCAR produced less protection against Aβ_{1-42}-induced protein carbonyl (Fig. 1a), HNE (Fig. 1b), or 3-nitrotyrosine (Fig. 1c) formation than 100 μM ALCAR alone (P < 0.05), with elevated protein oxidation and lipid peroxidation compared with controls (P < 0.01).

![Figure 1](image1)

**Figure 1.** Protective effect of ALCAR against Aβ_{1-42}-induced oxidative stress. a: Protein oxidation (protein carbonyls). *P < 0.01, **P < 0.05 compared with the control. The treatment of cell cultures is as described in Materials and Methods. Hatched bars are presence of Aβ_{1-42}, and solid bars are without Aβ_{1-42}. Data are mean ± SEM expressed as percentage of control values. Statistical comparison was by ANOVA (n = 5). b: 4-Hydroxy-2-transnonenal (HNE) *P < 0.01, **P < 0.05, c: 3-Nitrotyrosine, *P < 0.01, **P < 0.05. Post hoc analysis was via Student-Newman-Keuls test, and the P values given are compared with the control.

ALCAR Protects Mitochondria From Aβ_{1-42}-Mediated Dysfunction in a Dose-Dependent Manner

Aβ_{1-42} was shown to decrease mitochondrial function assessed by MTT reduction by 50% compared with untreated control (Fig. 2). Treatment of neurons with ALCAR alone for 24 hr did not show any change in MTT reduction compared with the control. Pretreatment of neurons with 100 μM ALCAR (2 hr prior) followed by Aβ_{1-42} showed significant (P < 0.01) dose-dependent protection of mitochondrial function compared with that induced by Aβ_{1-42} alone. There is a maximal significant increase (P < 0.01) in cell viability with 100 μM ALCAR, followed by Aβ_{1-42}, but still a significant decrease in MTT reduction compared with untreated control. However, these results are consistent with the concentration of ALCAR that protected neurons against Aβ_{1-42}-mediated protein oxidation (Fig. 1a-c). The study on the degree of cytochrome C release suggests that there is an approximate 4-fold increase in cytochrome C levels in the cytosol of neurons treated with Aβ_{1-42}, which was gradually decreased in the presence of increasing concentration of ALCAR. However, the levels of cytochrome C released from mitochondria in neurons treated with 100 μM ALCAR alone were comparable to those of the control cells (untreated cells). Because 100 μM ALCAR protected neurons against protein oxidation and lipid peroxidation and prevented loss of mitochondrial function, this concentration was used for all subsequent experiments.

![Figure 2](image2)

**Figure 2.** a: Protective effect of ALCAR on cell viability against Aβ_{1-42}-induced cytotoxicity in primary cultured rat primary neurons. The dose-dependent effect of ALCAR on neuronal cell culture was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Hatched bars are presence of Aβ_{1-42}, and solid bars are without Aβ_{1-42}. Data are mean ± SEM expressed as percentage of control values. Statistical comparison was by ANOVA (n = 5). *P < 0.05, **P < 0.01, ***P < 0.02. Post hoc analysis was performed by Student-Newman-Keuls test, and the P values given are compared with the control. b: Levels of cytochrome C release. The treatments in b are the same as in a.

ALCAR Protects Against Aβ_{1-42}-Induced Loss of Neuronal Network and Neuronal Function

Differences were considered significant at P < 0.05.
Apoptosis
Phase-contrast microscopy was used to examine morphological changes in the neurons following treatment (Fig. 3). Neurons that were exposed to 10 μM Aβ1-42 for 24 hr (Fig. 3a, middle) showed loss of neuronal network, membrane blebbing, and cell shrinkage, processes that are normally associated with apoptotic cell death. Conversely, neurons pretreated with ALCAR 2 hr prior to addition of Aβ1-42 (Fig. 3a, right), showed intact networks and cell bodies similar to those of control neurons (Fig. 3a, left). To investigate further and to confirm the results obtained in the MTT assay, neuronal apoptosis studies were carried out with Hoechst and PI staining. Neurons treated with Aβ1-42 (Fig. 3b, c) showed extensive apoptotic bodies by both stains, from which we conclude that late apoptotic and necrotic cells are found under the conditions of this experiment. In contrast, pretreatment of neurons with 100 μM ALCAR followed by addition of Aβ1-42 (Fig. 3b, c) resulted in a significant (P < 0.01) reduction in apoptotic cells similar to the reductions in control (untreated) cells. The averages of late apoptotic cells were calculated and are reflected in the bar graph (Fig. 3d).

Figure 3. Protective effect of ALCAR on cortical neuronal cell viability. The cells were treated as described in Materials and Methods, after which cell morphology was visualized by phase-contrast microscopy (×100). The final concentration of Aβ1-42 or ALCAR is 10 μM or 100 μM, respectively. a: Images displayed here are those that exhibit the maximum morphological protection at the respective concentration specified. Arrowheads indicate apoptotic bodies. Hoechst staining (b) propidium iodide staining (c) for DNA fragmentation. Control neurons do not show any apoptotic neurons, but Aβ1-42-treated neurons show significant evidence of late-stage apoptotic and necrotic neurons. Conversely, 100 μM ALCAR-pretreated cells protect the neurons against Aβ1-42 apoptosis. d: Averages of late-stage apoptotic cells and necrotic cells were calculated and are reflected in this bar graph. Results are mean ± SEM expressed as percentage of control values. Each experiment was repeated three times with three independent samples. Statistical comparison was by ANOVA (n = 5), *P < 0.05, **P < 0.01 compared with the untreated control. Post hoc analysis was via Student-Newman-Keuls test, and the P values given are compared with the control.

ALCAR Increases Endogenous GSH Levels
To determine whether ALCAR protected neurons by up-regulation of GSH, an assay to measure the total GSH levels was conducted (Fig. 4). Aβ1-42 treatment led to a significant reduction (P < 0.01) in cellular GSH (50% compared with the untreated control cells). Treatment of cells with 100 μM ALCAR alone led to a mean 4% significant (P < 0.01) increase in GSH compared with control (untreated cells). However, ALCAR treatment followed by addition of Aβ1-42 raised total GSH levels significantly (P < 0.01) to 80% of control. That is, pretreatment of cortical neuronal cells with 100 μM ALCAR protected neurons against Aβ1-42-mediated loss of GSH.

Figure 4. GSH assay. Cell cultures were pretreated with ALCAR in a dose-dependent manner 2 hr prior to Aβ1-42, after which the cells were collected after 24 hr and assayed for total GSH. Hatched bars are presence of Aβ1-42, and solid bars are without Aβ1-42. Results are mean ± SD of three independent measurements. Treatment of neurons with Aβ1-42 led to a significant loss of GSH. No significant difference was noted in GSH levels between control and ALCAR alone (at lower concentrations)-treated neurons, but a significant increase was seen at 100 μM. ALCAR treatment followed by addition of Aβ1-42 raised total GSH levels to 80% of control cell levels. *P < 0.01, **P < 0.05; n = 4. The statistical comparison was performed between control and each treatment data set. Post hoc analysis was via Student-Newman-Keuls test, and the P values given are compared with the control.

ALCAR Protection of Neurons Against Aβ1-42-Induced Toxicity Is Associated With Up-Regulation of HO1 and Hsp72 and Down-Regulation of iNOS
Cortical neuronal cells treated with Aβ1-42 showed a significant 2.3-fold increase (P < 0.01) in HO1 protein levels, and pretreatment of cells with 100 μM ALCAR prior to Aβ1-42 induced HO1 by 4.3-fold (P < 0.05) compared with the control (Fig. 5a, c). A significant (P < 0.05) 2.5-fold increase in Hsp72 protein (Fig. 6a, c) levels was observed in Aβ1-42-treated cells compared with control, and pretreatment of cells with ALCAR before Aβ1-42 further significantly (P < 0.01) induced...
HSP 72 in a dose-dependent manner compared with the control. As shown in Figure 7, iNOS levels were significantly ($P < 0.01$) increased by 3.3-fold in Aβ$_{1-42}$-treated primary neuronal cells. Pretreatment with ALCAR significantly decreased ($P < 0.05$) the iNOS levels induced by Aβ$_{1-42}$, in a dose-dependent manner, compared with the control.

Figure 5. a: Representative Western immunoblot analysis of neuronal cells for heme oxygenase-1 (HO1) protein. One hundred micrograms of protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with a mouse monoclonal anti-HO1 antibody. Hatched bars are presence of Aβ$_{1-42}$, and solid bars are without Aβ$_{1-42}$. Lane 1: control (untreated cells); lane 2: Aβ$_{1-42}$; lane 3: 10 μM ALCAR plus Aβ$_{1-42}$; lane 4: 50 μM ALCAR plus Aβ$_{1-42}$; lane 5: 100 μM ALCAR plus Aβ$_{1-42}$; lane 6: 200 μM ALCAR plus Aβ$_{1-42}$; lane 7: 100 μM ALCAR alone (1-42). b: Anti-GAPDH blot. c: Densitometric analysis from five independent experiments (mean ± SEM of values expressed as relative units). Increased HO1 expression in a dose-dependent manner after ALCAR treatment. Significant differences were assessed by ANOVA. Each sample was compared with the control. *$P < 0.01$, **$P < 0.05$. Post hoc analysis was via Student-Newman-Keuls test, and the $P$ values given are compared with the control.

Figure 6. Western immunoblot analysis of neuronal cells for heat shock protein (HSP) 72 protein. One hundred micrograms of protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with a mouse monoclonal anti-Hsp72 antibody. The treatments of the samples are as described for Figure 5. a: Representative immunoblot of Hsp72 expression. b: Anti-GAPDH blot. c: Densitometric analysis from five independent experiments (mean ± SEM of values expressed as relative units). Increased Hsp72 expression in a dose-dependent manner after ALCAR treatment. Significant differences were assessed by ANOVA. Each sample was compared with the control. *$P < 0.01$, **$P < 0.05$. Post hoc analysis was via Student-Newman-Keuls test, and the $P$ values given are compared with the control.

Figure 7. Western immunoblot analysis of neuronal cells for inducible nitric oxide synthase (iNOS) protein. One hundred micrograms of protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with a mouse monoclonal anti-iNOS antibody. The treatments of the samples are as described for Figure 5. a: Representative immunoblot of iNOS expression. b: Anti-GAPDH blot. c: Densitometric analysis from five independent experiments (mean ± SEM of values expressed as relative units). Decreased iNOS expression in a dose-dependent manner after ALCAR treatment. Significant differences were assessed by ANOVA. Each sample was compared with the control. *$P < 0.05$, **$P < 0.01$. Post hoc analysis was via Student-Newman-Keuls test, and the $P$ values given are compared with the control.

**DISCUSSION**

Aβ is the principal component of senile plaques in the AD brain and plays a central role in the pathogenesis of AD (Selkoe [2001]; Butterfield et al., [2001]; Butterfield and Lauderback, [2002]). Aβ$_{1-42}$ is involved in free-radical formation that induces damage to neurons in vitro (Yatin et al., [1999]; Varadarajan et al., [2000], [2001]) and in vivo (Drake et al., [2003]; Boyd-Kimball et al., [2005]). The AD brain is under significant oxidative stress, evidenced by various markers of protein, lipid, DNA oxidation, and other aspects (Markesbery, [1997]; Butterfield et al., [2001]; Butterfield and Lauderback [2002]).

The AD brain also presents increased protein oxidation (Hensley et al., [1995]). Additionally, markers of oxidative stress include nitrotyrosine in AD (Calingasan et al., [1999]; Castegna et al., [2003]; Sultana et al., [2006]). Consonant with this notion, the current study showed a significant increase in protein oxidation (Fig. 1a), lipid peroxidation (Fig. 1b), and 3-NT formation (Fig. 1c) in primary neuronal cell cultures treated with Aβ$_{1-42}$. However, Aβ$_{1-42}$-induced oxidative stress was significantly decreased by pretreatment of neuronal cells with ALCAR.
ALCAR is the L-carnitine ester of acetic acid and is synthesized in the brain, kidney, and liver by the enzyme acetyl carnitine transferase. ALCAR reportedly enhances learning capacity in aging animals (Ando et al., [2001]), acts as a source of acetyl groups available for synthesis for acetylcholine, and is selectively taken up by the primate brain (Kuratsune et al., [1997]). ALCAR crosses the blood-brain barrier and improves neuronal energetics and repair mechanisms, while modifying acetylcholine production in the CNS (Carta et al., [1993]). The precise mechanism of action by which ALCAR is neuroprotective in aging and neurodegeneration is not known. However, ALCAR is involved in mitochondrial metabolism (Hagen et al., [1998]) and may have indirect antioxidant properties (Kaur et al., [2001]; Calabrese et al., [2006]).

Administration of ALCAR has been shown to decrease the level of lipid peroxidation in the aged rat brain (Kaur et al., [2001]) and to prevent free-radical-mediated protein oxidation in the frontal cortex of dogs following brain ischemia and reperfusion (Calvani and Arrigoni-Martelli, [1999]). Our results suggest that ALCAR protects cortical neurons against Aβ1-42-mediated oxidative stress. However, there is approximately a 10% increase in oxidative stress markers following treatment with ALCAR alone. We speculate that the increase in oxidative stress induced with ALCAR alone possibly is due to increased Hsp32 (HO1), which catalyzes a reaction with heme to produce Fe²⁺, and biliverdin. The latter is quickly converted to bilirubin, by biliverdin reductase. Conceivably, Fe²⁺ could react with endogenous H₂O₂ to produce hydroxyl radicals. Bilirubin, though an antioxidant at low levels, can be a prooxidant at high levels (Brito et al., [2004]). Thus, conceivably bilirubin could contribute to the slight increased oxidative stress markers observed in the absence of Aβ1-42.

Production of NO is catalyzed by NOS, which converts L-arginine to L-citrulline. The large amount of NO produced by iNOS has been closely correlated with the pathophysiology in a variety of diseases and inflammation (Bredt, [1999]). Increased protein nitration is found in AD brain tissue compared with the control (Smith et al., [1997]; Castegna et al., [2003]; Sultana et al., [2006]). In this study, we observed that both iNOS and 3-NT levels were increased in Aβ3-42-treated cells and that pretreatment with ALCAR showed a dose-dependent protection against this increase.

Studies in AD have established that high levels of Aβ3-42 result in neuronal cell death via apoptosis (Selkoe, [2001]). Additionally, an experiment with Aβ1-42 suggested the apoptotic nature of neurodegeneration (Morishima et al., [2001]). Aβ peptide also induces apoptosis in mouse neuron cultures (Loo et al., [1993]). Su et al. (1994) reported evidence for DNA fragmentation in neurons from subjects with AD, although evidence for apoptosis in AD remains controversial (Pollack and Leeuwenburgh, [2001]). Mitochondria are particularly vulnerable to oxidative damage, and mitochondrial dysfunction has been observed in AD brain (Hirai et al., [2001]). In the present study, we studied the cytotoxic effects (by MTT assay) and morphological changes by examining the dynamic neuronal-network processing in the Aβ1-42 or ALCAR-treated cortical neurons. The results suggest that there is an extensive loss of neuronal connections as well as the presence of dying cells in the Aβ1-42-treated cells. Pretreatment of neurons with ALCAR resulted in a morphology similar to that of control cells (without treatment), suggesting a protective role of this carnitine ester. To confirm our results, Hoechst or PI staining was used to detect the presence of apoptotic bodies as an indication of apoptotic cells; ALCAR demonstrated antia apoptotic properties on the neuronal cells by reducing the formation of apoptotic bodies. ALCAR (1-100 μM) promoted neuronal survival and mitochondrial activity in a concentration-dependent manner, attenuating DNA fragmentation and nuclear condensation in cultured neurons (Ishii et al., [2000]). To confirm these results obtained from the MTT assay, the level of cytochrome C release was measured in these treated samples, and our results are consistent with the above-mentioned studies in showing that the antiapoptotic action of ALCAR might contribute to its neuroprotective effect.

Aβ1-42 has been shown to deplete GSH levels in astrocytes, leading to a significant loss of neurons in vitro (Abramov et al., [2000]). GSH levels have been shown to decrease with aging, leaving neurons vulnerable to ROS attack (Lui and Choi, [2000]). In the present study, Aβ1-42 significantly depleted the cellular GSH level in cortical neuronal cells. Moreover, treatment of neurons with 100 μM ALCAR alone was shown to elevate GSH levels 24 hr after administration. Upon pretreatment with 100 μM ALCAR followed by 10 μM Aβ1-42, GSH levels almost returned to control level. Additionally, exogenous GSH has been shown to prevent Aβ1-42-induced apoptosis in human cortical neurons (Medina et al., [2002]). Pretreatment of neurons with 100 μM ALCAR protected these cells against Aβ3-42-mediated loss of mitochondrial function, protein oxidation, loss of neuronal network, and apoptotic cells. Taken together, these results suggest ALCAR induces up-regulation of GSH, which, in turn, protects neurons against protein oxidation and neurotoxicity.

Eukaryotic cells have evolved networks of responses to survive different forms of stress. One such kind of response is the heat shock response that has gained great attention in the recent years (Calabrese et al., [2003]; Poon et al., [2004]). The heat shock response plays a cytoprotective role in various neurodegenerative disease and aging (Poon et al., [2004]). This insight has opened new perspectives in medicine and pharmacology, as a possible candidate for novel cytoprotective strategies (Calabrese et al., [2003]). In this study, we tested the hypothesis that ALCAR protects primary neuronal cell cultures against Aβ3-42-mediated oxidative stress and neurotoxicity by up-regulating HSPs. HO is the rate-limiting enzyme in the degradation of heme, and the isoform HO1 is readily responsive to oxidative stress and heme.
We observed significantly increased expression of HO1 and Hsp72 proteins in Aβ42-treated neurons relative to controls. However, addition of ALCAR 2 hr prior to exposure of Aβ42 markedly increased (in dose-dependent manner) expression of HO1 and Hsp72 protein, which is consistent with a protective response. These data indicate that constitutive expression of these stress responses is very low in control cells compared with Aβ42-treated cells or ALCAR-treated cells. HO1 is markedly overexpressed in neurons and astrocytes of AD cerebral cortex and hippocampus relative to control (Premkumar et al., [1995]). Inhibition of HO1 or Hsp72 or iNOS proteins by inhibitors ZnPPIX or quercitin or L-NMMA, respectively, abrogated the protective effect of ALCAR, demonstrating that HO1, Hsp72, or iNOS was involved in the ALCAR-mediated cytoprotection (Fig. 1a-c) against the oxidative stress caused by Aβ42. Recent studies show that ALCAR is cytoprotective against inflammatory and oxidative insults in astrocytes in part by being able to up-regulate cytoprotective cellular stress responses, particularly induction of HO1 and Hsp60, while inhibiting induction of iNOS (Calabrese et al., [2005b]).

In summary, we have shown that ALCAR increased neuronal GSH levels and up-regulated HSPs. Moreover, this increase in GSH levels can protect neurons from alterations in mitochondrial function and increased protein oxidation, loss of neuronal network, and apoptosis induced by Aβ42. These findings are of importance in that Aβ42 may play a central role in the pathogenesis of AD, and GSH is a vital endogenous antioxidant found in millimolar concentrations in the brain (Cooper, [1997]), although GSH levels decrease with age (Liu and Choi, [2000]). Thus, agents such as ALCAR, by increasing the levels of GSH in the brain, may provide therapeutic benefit in oxidative stress-associated neurodegenerative diseases such as AD. HSPs are known to play a pivotal role in cytoprotection, and the molecules inducing the defense mechanism in them appear to be possible candidates for novel cytoprotective strategies. On these grounds, ALCAR may be used as a possible therapeutic strategy in AD patients early in the disease.

Acknowledgements

This work was supported in part by NIH grants AG-05119 and AG-10836 to D.A.B.

REFERENCES


