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Anti-apoptotic Effect of Acetyl-*l*-carnitine and *l*-Carnitine in Primary Cultured Neurons

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ABSTRACT—Although exogenously administered acetyl-*l*-carnitine (ALCAR, (2-acetoxy-3-carboxypropyl)-trimethylammonium) and *l*-carnitine (LC, (3-carboxy-2-hydroxypropyl)-trimethylammonium) prevent brain damage in several ischemic models, the protective mechanism of these compounds remains unclear. Here, we evaluated the effect of ALCAR and LC in primary cultured neurons from the cerebral cortex, striatum and thalamus of 18-day-old rat embryos. Deprivation of the serum from cultured medium for 3 days reduced the number of viable cells and mitochondrial activity and induced cell death with characteristics of apoptosis such as DNA fragmentation, nuclear condensation and histone-DNA release into the cytoplasm. ALCAR (1 – 100 μM) and LC (1 – 100 μM) promoted neuronal survival and mitochondrial activity in a concentration-dependent manner. Moreover, these compounds attenuated DNA fragmentation and nuclear condensation in cultured neurons and significantly decreased histone-DNA release into the cytoplasm. These results indicate that anti-apoptotic actions of ALCAR and LC contribute to their neuroprotective effect.

Keywords: Primary cultured neuron, Apoptosis, Neuroprotective, Acetyl-*l*-carnitine, *l*-Carnitine

Primary cultured neurons are frequently used for elucidating cellular and molecular mechanisms involved in several diseases including ischemia. Cultured cerebral neurons are damaged following the application of various ischemic stresses, such as hypoxia (1), excitatory amino acid exposure (2) and serum deprivation from cultured medium (3). It is documented that the ischemic condition causes energy loss (4) and loss of mitochondrial membrane potential (5), and finally terminates the cell survival (1). Furthermore, reports have accumulated that neuronal cell death by ischemic stress is closely related to apoptosis in both in vivo and in vitro studies (1, 6, 7).

Acetyl-*l*-carnitine (ALCAR) and *l*-carnitine (LC) are widely distributed in the tissues including the central nervous system (8). Carnitine acetyltransferase converts ALCAR and LC to each other (9), and LC is known to be an essential cofactor for the transport of long-chain acyl CoA through the inner mitochondrial membrane for β-oxidation (9). Recently, in vivo pharmacological studies have revealed that ALCAR and LC prevent the brain damages in several ischemic models (4, 10, 11). However, it is not well known whether apoptotic events are prevented by ALCAR and LC in the central nervous system.

In the present study, we examined the neuroprotective effect of ALCAR and LC on primary cultured neurons from rat embryo focusing on apoptotic cell death.

MATERIALS AND METHODS

Chemicals

ALCAR ((2-acetoxy-3-carboxypropyl)-trimethylammonium chloride) was supplied by Sigma Tau Co., Ltd., (Rome, Italy). LC ((3-carboxy-2-hydroxypropyl)-trimethylammonium hydrochloride), MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide), cresyl violet, DNase I and Hoechst 33258 were obtained from Sigma (St. Louis, MO, USA). Recombinant human basic fibroblast growth factor (b-FGF) was obtained from Funakoshi (Tokyo). Glial fibrillary acidic protein (GFAP) was obtained from Immunon (Pittsburgh, PA, USA). All other chemicals used in this study were obtained commercially and of the highest purity available.

Cell culture and experimental schedule

Cell culture is performed according to the method of Abe et al. (12). Briefly, whole brains were isolated from embryonic day 18 Wistar rats (Nippon SLC, Hamamatsu), and the desired regions were further dissected out. The brain regions tested were the cerebral cortex, striatum and thalamus for the cell survival assay and thalamus for apop-

tosis, respectively. Because more neuroprotective effects of ALCAR and LC were recognized in thalamic neurons, the anti-apoptotic assay was performed using the thalamic neurons. Each tissue was cut into pieces with a razor blade knife, incubated with 0.25% trypsin (1:250; Difco, Detroit, MI, USA) and 0.01% DNase I at 37°C for 30 min, and added with heat inactivated horse serum (Gibco BRL, Gland Island, NY, USA). The pellet was pipetted and suspended in 7.42 mg/ml modified Eagle's medium (MEM) (Nissui Pharmaceutical, Tokyo) supplemented with 8.2 mg/ml glucose (Nacalai Tesque, Kyoto), 0.294 mg/ml *l*-glutamine (Sigma), 1.7 mg/ml sodium bicarbonate (Nacalai Tesque), 99 μg/ml sodium pyruvate (Wako, Osaka), 54 μg/ml penicillin G potassium (Meiji Seika, Tokyo), 90 μg/ml streptomycin sulfate (Meiji Seika) and 10% (v/v) fetal bovine serum (Nichirei, Tokyo). The cell suspension was passed through a cell strainer filter (40-μm mesh; Falcon, Franklin Lakes, NJ, USA) to remove cell lumps.

All multi plates and plastic chamber slides were precoated by 0.01% poly-*l*-lysine (Sigma) for 6 h and washed three times with distilled water. The suspended cells with the above medium were plated with 5×10^4 cells/cm^2 on 48-well multi plates (Nunc, Naperville, IL, USA) for the cell survival assay (12) and on 96-well multi plates (Nunc) for the MTT assay (13) and detection of histone-DNA release (14).

The suspended cells were plated on 2-well plastic chamber slides (Nunc) for detecting chromatin condensation (15) and on 6-well multi plates (Nunc) for the DNA laddering detection test (16) at a density of 1.5×10^5 cells/cm^2. They were all cultured at 37°C in a humidified 5% CO_2 – 95% air atmosphere.

Twenty-four hours after plating, the medium was changed to serum-free Dulbecco's modified Eagle's medium /Ham's nutrient mixture F12 (DMEM/F12) medium (Gibco BRL) supplemented with 100 μg/ml transferrin (Sigma), 5 μg/ml insulin (Sigma), 20 nM progesterone (Sigma), 100 μM putrescine (Sigma) and 30 nM selenium. ALCAR and LC were first dissolved in distilled water, and human b-FGF, used as a positive control, was first dissolved in distilled water supplemented with 1 mg/ml bovine serum albumin (Sigma) to prevent non-specific binding of the protein. They were further diluted to the desired concentration by serum-free DMEM/F12 medium. Three days after drug treatment, the following assays were performed. In the present culture condition, the percentages of astroglial cells (immunostained with a GFAP antibody) in the cerebral cortex, striatum and thalamus were <5%, <5% and <10% of the total cells, respectively.

Survival cell counting

Neurons were fixed with 4% paraformaldehyde (Katayama Chemical, Osaka) for 30 min and rinsed twice with PBS. Then they were stained with 0.1% cresyl violet in PBS for 10 min. The bottom of the well was divided into 16 checkers with lines. The number of survival neurons in five out of nine crossing points were counted and finally expressed as cells/cm^2 (each point, 0.25 mm^2).

MTT assay

The MTT assay was performed according to the method of Hansen et al. (13). Briefly, MTT was added to cultures (final concentration of 1 mg/ml). After 2-h incubation at 37°C, the reaction was stopped by adding a lysis buffer (20% sodium lauryl sulfate in 50% *N,N*-dimethyl formamide, pH 4.7). The absorbance was measured using a spectrophotometer (M-SP max 250, Wako) at 570 nm (reference wavelength of 700 nm) after an overnight incubation at 37°C.

DNA Laddering

Total genomic DNA was extracted from neuronal cells as described by D'Mello et al. (16) and electrophoresed on a 2.0% agarose gel. PHY marker (Takara, Kyoto) was used for the standard. DNA samples were visualized by ethidium bromide staining of the gel.

Hoechst 33258 staining

Neurons were fixed with 4% paraformaldehyde for 20 min, and rinsed twice with PBS. Then, they were stained with Hoechst 33258 (1 μg/ml) in PBS for 15 min and examined under UV illumination using a fluorescence microscope (PM-30; Olympus, Tokyo). Apoptotic nuclei with condensation were measured randomly in at least 5 different areas from 5 – 8 photographs and expressed as % of apoptotic nuclei.

Histone-DNA complex release

In addition to qualitative detection of DNA laddering and nuclear condensation, we quantitatively measured release of the histone-DNA complex into the cytoplasm by the ELISA method. The neuronal apoptosis was assessed by a cell death detection ELISA kit (Roche Diagnostics, Tokyo) for histone-associated DNA fragments.

Neuronal cells in 96-well multi plates were centrifuged at $200 \times g$ for 10 min, and the supernatant was removed. The cell pellets were resuspended with 200 μl lysis buffer provided by the kit and incubated for 30 min at a room temperature. Following the centrifugation of the plate, 20 μl of the supernatant was applied on a streptavidin-coated plate, and incubated for 2 h with 80 μl of incubation buffer containing monoclonal antibodies directed against DNA and histone (provided by the kit). After wash-out of unbound antibodies, substrate buffer was applied and the amount of immunocomplex of histone-DNA-antibodies was measured by a spectrophotometer (M-SP max 250,

Wako) at 405 nm (reference wavelength of 492 nm).

Statistics

Results were expressed as the mean ± S.E.M. The data were analyzed by one-way ANOVA followed by the Bonferroni/Dunn test. In the surviving cell counting study, ANOVA with randomized block design followed by Dunnett's test was performed.

RESULTS

Effect of ALCAR and LC on cell survival

Cell count (Fig. 1): Numbers of surviving cells in the cultured neurons from the cerebral cortex, striatum and thalamus decreased to less than 20% of the initial numbers (5×10^4 cells/cm^2) in this model. ALCAR and LC inhibited the cell death in all regions in a concentration-dependent manner ($1-100\,\mu$M). b-FGF (3 ng/ml) also inhibited the cell death in this assay. The minimum effective concentrations of ALCAR and LC were 10 and 100 μM, respectively.

MTT assay (Fig. 2): ALCAR ($1-100\,\mu$M) and LC ($1-100\,\mu$M) concentration-dependently inhibited mitochondrial activity in all regions. b-FGF (3 ng/ml) also inhibited the decrease of mitochondrial activity in all regions.

Effect of ALCAR and LC on apoptotic neuronal cell death

DNA laddering (Fig. 3): In the control group, extensive DNA laddering as a sign of broken DNA was confirmed in this assay. ALCAR (100 μM), LC (100 μM) and b-FGF (3 ng/ml) obviously decreased the appearance of DNA laddering.

DNA condensation (Fig. 4): In the control group, there was DNA condensation (Fig. 4A) in this assay. ALCAR (100 μM), LC (100 μM) and b-FGF (3 ng/ml) profoundly diminished the appearance of DNA condensation (Fig. 4: B–D). The percentage of apoptotic nuclei in the control was 35.1% and those in the groups treated with ALCAR (100 μM), LC (100 μM) and b-FGF (3 ng/ml) were 7.0%, 9.5% and 5.8%, respectively (Fig. 4E).

Histone-DNA complex release (Fig. 5): The release of histone-DNA complex into the cytoplasm was greatly enhanced in control cells. ALCAR ($1-100\,\mu$M) and LC ($1-100\,\mu$M) concentration-dependently inhibited the release of histone-DNA complex into the cytoplasm. The minimum effective concentration of ALCAR and LC was 10 μM. b-FGF (3 ng/ml) also inhibited the release of histone-DNA. ALCAR (100 μM), LC (100 μM) and b-FGF (3 ng/ml) inhibited the release of histone-DNA complex by 28.4%, 34.6% and 34.3% of the control level, respectively.

DISCUSSION

ALCAR and LC protect neuronal cells in both central and peripheral nervous systems of in vivo neurodegenerative models (11, 17, 18). In the present study, these compounds promoted the survival of neuronal cells from various parts of the brain. These observations suggested that ALCAR and LC exert neurotrophic factor-like activities. The promoting effect of ALCAR and LC on neuronal sur-

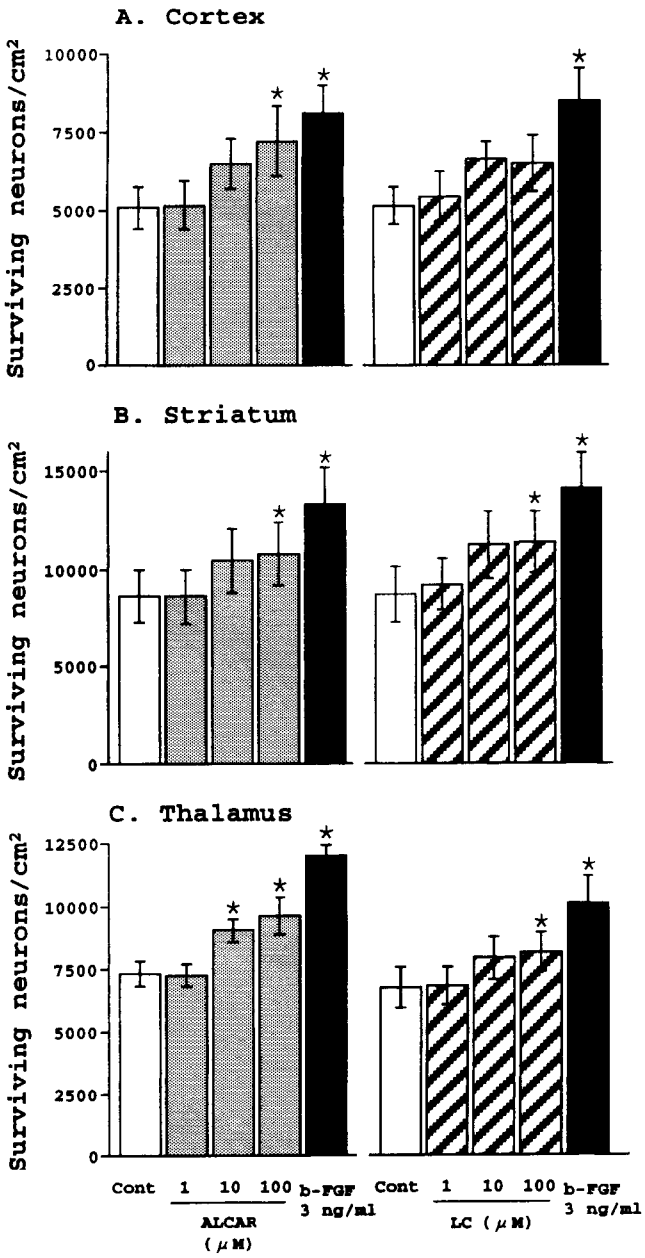

Fig. 1. Concentration-dependent effects of ALCAR and LC on cell death of primary cultured neurons from the cerebral cortex (A), striatum (B) and thalamus (C). Cells were plated at an initial density of 5×10^4 cells/cm^2. Viable cells were stained with cresyl violet and counted microscopically. The data were expressed as the mean ± S.E.M. (n = 8). *$P<0.05$ vs control group (ANOVA with randomized block design following by Dunnett's test).

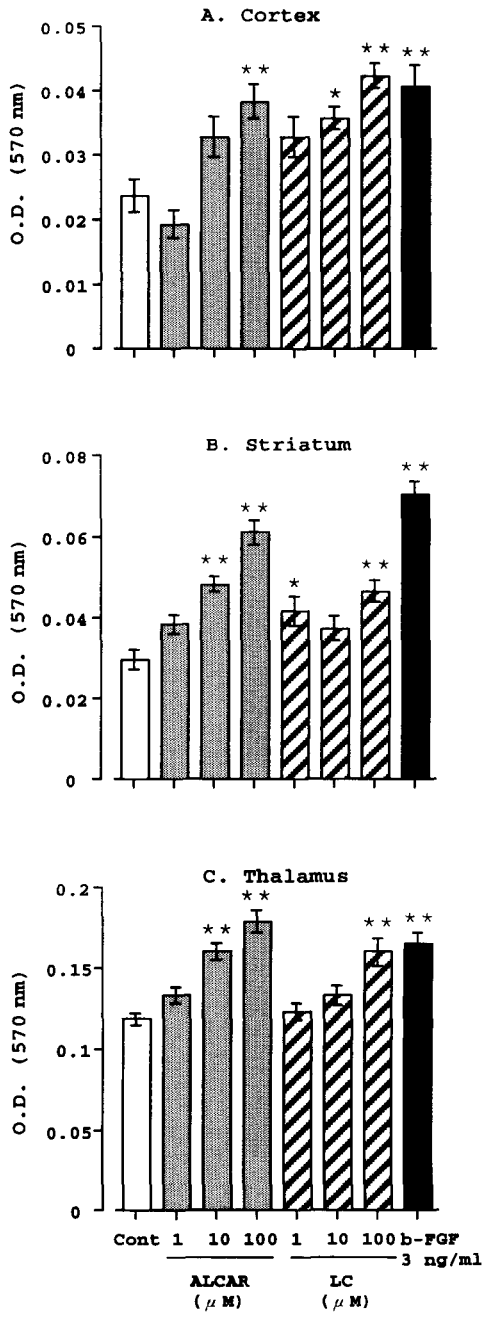

Fig. 2. Concentration-dependent neuroprotective effects of ALCAR and LC on mitochondrial dysfunction in primary cultured neurons from the cerebral cortex (A), striatum (B) and thalamus (C). The neuroprotective effect was determined by an MTT assay. The ordinate indicates the optical density (570 nm) value in the MTT assay. The data were expressed as the mean ± S.E.M. (n = 6). *$P<0.05$, **$P<0.01$ vs control group (Bonferroni/Dunn test).

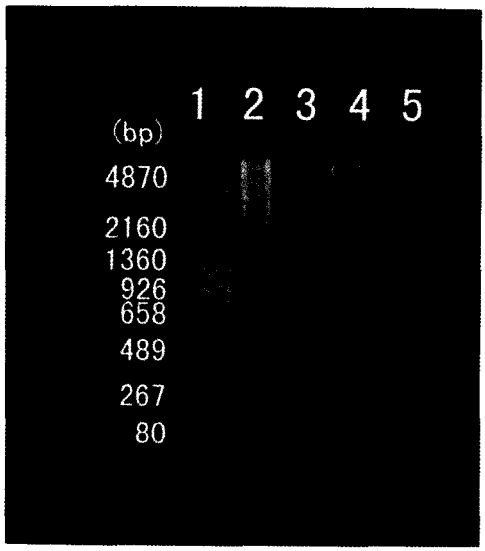

Fig. 3. Agarose gel electrophoresis of DNA extracted from the cultured neurons of the thalamus. DNA standard (lane 1), control (lane 2), ALCAR (100 μM) (lane 3), LC (100 μM) (lane 4) and b-FGF (3 ng/ml) (lane 5).

vival may underlie their in vivo neuroprotective actions.

Serum deprivation from the medium causes cell death of primary cultured neurons accompanied by both necrotic and apoptotic events (19). In the present study, we demonstrated that the neuronal death in this model was accompanied with the nuclear condensation, DNA laddering and histone-DNA release into the cytoplasm. These distinct characteristics of apoptosis (14, 20) were prevented by ALCAR, LC and b-FGF. Inhibition of apoptosis by ALCAR or LC has been reported in non-neuronal cell lines like P 19 teratoma cells (20) and C2.8 hepatocyte (21). To our knowledge, this is the first report demonstrating the anti-apoptotic effects of ALCAR and LC in primary cultured neuronal cells. According to our results from the apoptotic nuclei staining study, the anti-apoptotic action of these compounds may profoundly contribute to their improvement of survival of the cultured neurons.

ALCAR and LC are endogenous substrates that are widely distributed among tissues including the central nervous system (8). These compounds readily penetrate across the cell membrane and act as an essential co-factor for the transport of long-chain Acyl CoA through the inner mitochondrial membrane (9). ALCAR and LC restore and/or accelerate mitochondrial transport functions such as energy metabolism (22) and pyruvate dehydrogenase activity (23). It is well known that one of the initial events of apoptosis is cytochrome c release from mitochondria to the cytoplasm (24) due to mitochondrial dysfunction. The anti-apoptotic action of ALCAR and LC, therefore, is likely to be related to recovery from mitochondrial dysfunction.

It has been demonstrated that ALCAR enhances the NGF response in PC12 cells (25). Since NGF inhibits apoptosis induced by serum deprivation (26), the neurotrophic effect of these compounds may also contribute to the sup-

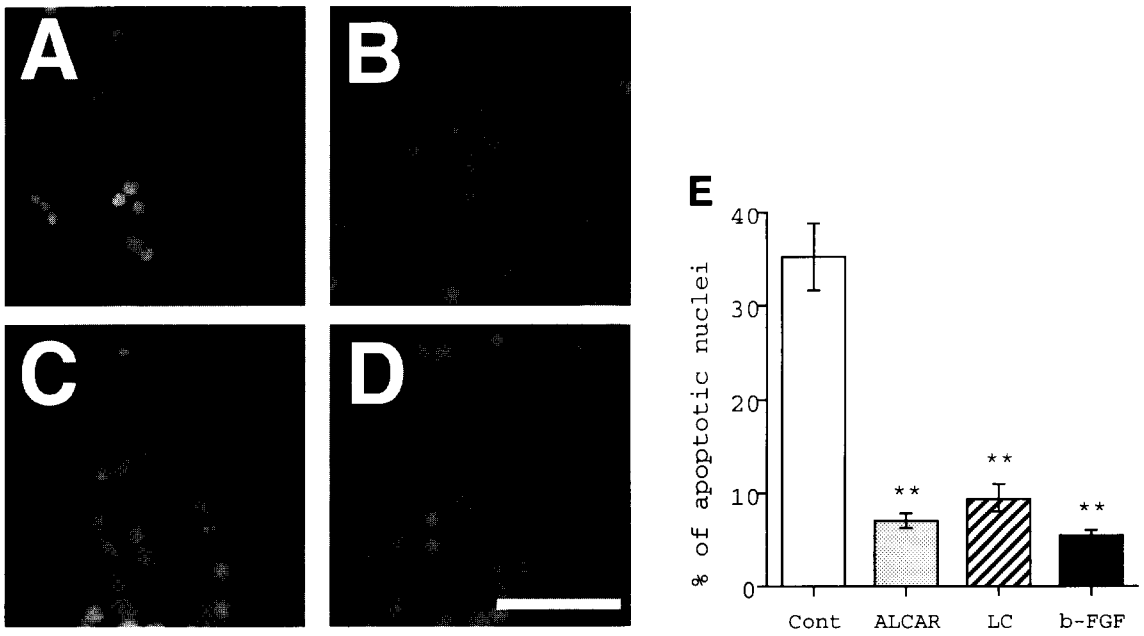

Fig. 4. Effects on nuclei of cultured neurons from the thalamus. Morphological changes of the nuclei with no drug (A), or in the presence of 100 μM ALCAR (B), 100 μM LC (C) or 3 ng/ml b-FGF (D). The nuclei were stained with Hoechst 33258. Scale bar represents 20 μm. Effect of ALCAR (100 μM), LC (100 μM) or b-FGF (3 ng/ml) on nulear condensation (E). Ordinate indicates the percentage of apoptotic nuclei, obtained by counting randomly from at least five determinations. The data were expressed as the mean ± S.E.M. (n = 5 – 8). $^{**}P<0.01$ vs control group (Bonferroni/Dunn test).

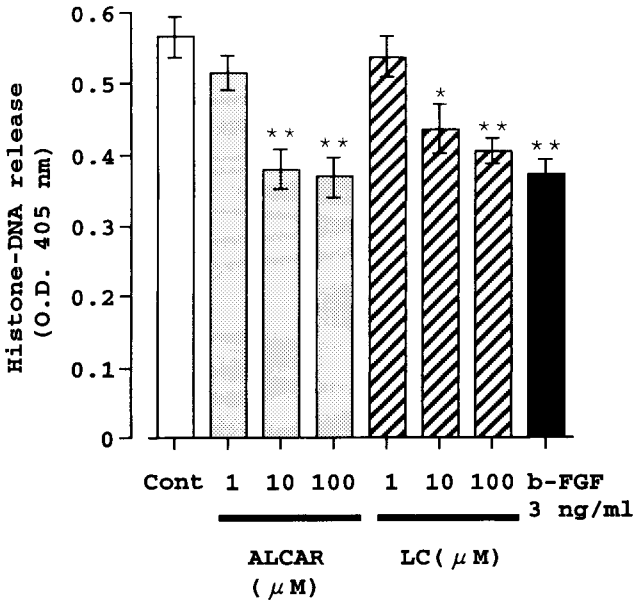

Fig. 5. Histone-DNA release from the cultured neurons. Anti-apoptotic activity was determined by an ELISA of histone-DNA. Ordinate indicates optical density (405 nm). The data were expressed as the mean ± S.E.M. (n = 5). $^{*}P<0.05$, $^{**}P<0.01$ vs control group (Bonferroni/Dunn test).

pression of apoptotic events. Although ALCAR and LC easily pass through the cell membrane, these compounds are also reported to act outside of the membrane. ALCAR prevents neuronal cell death induced by NMDA (27) and restored the glutamate uptake through the NMDA binding site (28). It is possible that ALCAR has a neuroprotective effect via the NMDA receptor. Further work will be needed to prove these speculations.

b-FGF inhibits the activation of caspase-3 and exerts an anti-apoptotic action in several cultured neuron models (1, 29). The neuroprotective effect of b-FGF in the present model may also be through the anti-apoptotic mechanism. To understand the mechanism of the anti-apoptotic effects by ALCAR or LC, future studies will be focused on the b-FGF-like activities of these drugs.

There is now substantial evidence that the process of apoptotic cell death underlies neuronal death and/or degeneration after ischemic brain damage (6, 7, 30, 31), particularly in the penumbra region, which is a possible therapeutic area. Therefore, agents with an anti-apoptotic action have been long awaited for the therapy of patients with brain damage. The present results, therefore, indicate that ALCAR and LC may be useful as agents for the treatment of brain damage and/or neurodegeneration associated with the apoptotic process.

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