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α-Lipoic acid (LA) enantiomers protect SH-SY5Y cells against glutathione depletion

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ABSTRACT

Growing evidence suggests that α-lipoic acid (LA) has neuroprotective effects in various pathological conditions including brain ischemia and neurodegeneration. While anti-oxidative activity has been thought to play a central role in LA-mediated neuroprotection, the precise mechanism and the effect of LA enantiomers (R- and S-LA) are not fully clarified. We, therefore, estimated the neuroprotective effects of LA against different cellular stresses including oxidative stress, endoplasmic reticulum (ER) stress and proteolytic stress using human neuroblastoma SH-SY5Y cells. All types of LAs (racemate, R-LA and S-LA) most effectively prevented cell death induced by buthionine sulfoximine (BSO) which depletes intracellular glutathione. Although direct effects of LA on glutathione depletion or generation of the reactive oxygen species (ROS) were relatively small upon BSO treatment, LA enhanced expressions of anti-oxidative genes such as heme oxygenase-1 (HO-1) and phase II detoxification enzymes such as NAD(P)H:Quinone Oxidoreductase 1 (NQO1). An inhibitor of NQO1, but not that of HO-1, suppressed LA-mediated protection against BSO. Further experiments revealed that all types of LAs activated cell survival-associated kinase Akt, and an inhibitor of PI3K, LY294002, suppressed both LA-induced upregulation of NQO1 and cell protection against BSO. Our results suggest an important role of PI3K/Akt-mediated upregulation of genes including phase II enzymes such as NQO1 in LA-mediated neuroprotection.

1. Introduction

Recent studies have demonstrated that enhanced levels of oxidative stress contribute to neuronal death in various pathological conditions including brain ischemia and neurodegeneration (for review, Mehta et al., 2007; Bossy-Wetzel et al., 2006). Cerebral ischemia/reperfusion causes rapid production of reactive oxygen species (ROS) which easily overwhelms the anti-oxidative capacity of the brain. In those conditions, ROS can be generated through the activation of NMDA receptors, xanthine oxidation, NADPH oxidation and lipid peroxidation. Several stress-associated signaling cascades such as mitogen-activated protein kinases (MAP kinases) are activated by ROS, and cause neuronal death directly or indirectly via increased production of inflammatory cytokines. In neurodegenerative diseases, in contrast, ROS can be generated through the impairment of intracellular organelles such as mitochondria and endoplasmic reticulum (ER), or inflammatory response derived from glial cells. Accumulation of aggregation-prone proteins such as amyloid β and α-synuclein may also contribute to ROS generation (Bossy-Wetzel et al., 2006; Smith et al., 2005; Song et al., 2004). From the view of therapeutic potential, therefore, it is important to find small anti-oxidants which can cross the blood brain barrier (BBB).

α-Lipoic acid (LA), or 1,2-dithiolane-3-pentanoic acid is a small sterically hindered disulfide molecule that can dissolve in both water and lipid, and cross the BBB (for review, Shay et al., 2009). Although LA can exist in both R- and S-enantiomeric forms, naturally found α-lipoic acid is a R-isomer that can bind to the ε-amino moiety of lysine residues by an amide linkage, thus making this isomer essential as a co-factor in the mitochondrial energy metabolism. Chemically synthesized LA, in contrast, is a racemate (a mixture of equal amount of R- and S-LA), and is believed to exert additional beneficial bioactivities when administered exogenously. These include anti-oxidative activities such as chelating metals (Muller and Menzel, 1990), scavenging reactive oxygen species (ROS) (Matsugo et al., 1995), recycling/inducing endogenous anti-oxidants (Konishi et al., 1996), anti-inflammation (Fuchs and Milbradt, 1994), activation of cell signaling (Packer and Candenas, 2011), and enhancement of glucose uptake (Konrad et al., 2001). It was also reported that LA had a pro-oxidant activity which leads to cellular adaptation against oxidative stress (Cakatay, 2006).

In neurological fields, accumulating evidence suggests that LA (racemate or R-LA) has neuroprotective effects in the models of

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both brain ischemia (Wolz and Krieglstein, 1996; Clark et al., 2001) and neurodegeneration such as Alzheimer's disease (AD) (Siedlak et al., 2009) and Parkinson's disease (PD) (Karunakaran et al., 2007). Although the anti-oxidative property has been thought to play a pivotal role in the neuroprotective activity of LA, the precise reaction mechanism and the effect of LA enantiomers, especially that of S-LA, are not fully understood.

We report here that every LA enantiomer (racemate, R-LA or S-LA) most effectively prevented cell death induced by drugs which deplete intracellular glutathione in SH-SY5Y neuroblastoma cells. The underlying mechanism may include Phosphatidylinositol 3-kinases (PI3K) /Akt dependent upregulation of anti-oxidant and/or phase II detoxification genes.

2. Materials and methods

2.1. Cell cultures

Cells of the human neuroblastoma cell line, SH-SY5Y, were maintained in DMEM containing 15% FBS and 50 μg/ml of penicillin and 100 μg/ml of streptomycin. These cells were kept at 37 °C in humidified 5% CO_2/95% air.

2.2. Stress conditions

SH-SY5Y cells were cultured in 24-well plates up to 60–70% confluence, and treated with various stressors for 24–36 h to reduce the viability to 45–60% of the control cells (non-stressed cells). The stressors used in this study were buthionine sulfoximine (BSO) (1 mM: purchased from Wako chemicals, Osaka, Japan) which is an inhibitor of γ-glutamyl cysteine ligase (γ-GCL), the rate-limiting enzyme in glutathione synthesis, diethyl maleate (DEM) (150 μM: Wako chemicals), which is a chelator of glutathione, hydrogen peroxide (H_2O_2) (22 μM: Nacalai Tesque, Kyoto, Japan), MG132 (1 μM: Nacalai Tesque), which is an inhibitor of proteasome, thapsigargin (Tg) (0.2 μM: Sigma), which is an inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), and tunicamycin (Tm) (1 μg/ml: Sigma), which is an inhibitor of N-linked glycosylation in the ER. The latter two drugs were used to cause ER stress. α-lipoic acid (LA; racemate, R-LA or S-LA) was provided by Dr. Tetsuya Konishi (Niigata University of Pharmacy and Applied Life Sciences). LA was added to the cells in the presence or absence of stressors, and incubated for the indicated times. In some experiments, the cells were treated with LA for 48 h (pre-treatment) prior to the addition of stressors. Chemical inhibitors such as SB202190, a p38 Mitogen-activated protein kinase (MAPK) inhibitor (Sigma), LY294002, a Phosphatidylinositol 3-kinases (PI3K) inhibitor (Sigma), SP600125, a Jun amino-terminal kinase (JNK) inhibitor (Sigma), PD98059, Extracellular signal-regulated kinase (ERK) inhibitor (Sigma), Sn-mesoporphyrin IX (Sn-MP), a heme oxygenase-1 (HO-1) inhibitor (Frontier Scientific Inc., Logan, UT), and dicoumarol, a NAD(P)H: Quinone Oxidoreductase 1 (NQO1) inhibitor (Tokyo Chemical Industry Co., Tokyo, Japan) were added to the cells in the presence or absence of LA and BSO.

2.3. Cell viability and cell death assays

Cell viability and cell death were measured using MTT assay (Nacalai Tesque) and LIVE/DEAD assay (Invitrogen, Carlsbad, CA), respectively, as described previously (Hori et al., 2004). In the latter experiment, the number of dead cells out of the total cells is shown in the graph.

2.4. Measurement of total glutathione and ROS

Total glutathione (GSH/GSSG) content was measured using a Total Glutathione Quantification Kit (DOJINDO, Kumamoto, Japan). Briefly, SH-SY5Y cells were treated with LA in the presence or absence of 1 mM BSO for the indicated times. Cells were then harvested, and lysed in 0.2 ml of PBS using a sonicator (Braoson, Danbury, CT). The samples were deproteinized with 20 μl of 5% 5-sulfosalicylic acid (SSA: Sigma), and total glutathione was measured following the manufacture's instructions. Intracellular ROS levels were measured using a fluorescent probe, 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA: Invitrogen) as described previously (Takano et al., 2007). SH-SY5Y cells were loaded with 5 μM DCFH-DA for 20 min and the fluorescence measured by a microscope (Nikon TS100-F ECLIPSE) equipped with a CCD camera (Hamamatsu Photonics, Shizuoka, Japan). Quantification of the fluorescent intensity was performed using Image J (version 1.42, Wayne Rasband, National Institutes of health), and the results presented as a percentage to the control intensity.

2.5. Cell lysis, Western blotting and immunostaining

Cells were lysed in RIPA buffer containing 10 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.2% sodium deoxycholate and protease inhibitors. Samples were then subjected to Western blotting with anti-p-ERK antibody, anti-ERK antibody, anti-p-JNK antibody, anti-JNK antibody, anti-p-Akt antibody, and anti-Akt antibody (Cell Signaling technology, Beverly, MA). Sites of primary antibody binding were visualized using alkaline phosphatase-conjugated secondary antibodies. Immunostaining was performed with anti- NF-E2-related factor 2 (Nrf2) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) after fixing the cells. FITC-labled anti-rabbit IgG was used as secondary antibody. Nucleus was visualized with DAPI (Sigma).

2.6. Quantitative real time RT-PCR (qRT-PCR)

Total RNA was extracted from the SH-SY5Y cells using TRIzol (Invitrogen). RT reactions containing 1 μg of total RNA were performed using PrimeScript (Takara, Shiga, Japan). The individual cDNA species were amplified in a reaction mixture containing THUNDERBIRD™ SYBR qPCR® Mix (TOYOBO Co., Ltd., Osaka, Japan) and specific primers for HO-1, NQO1, manganese superoxide dismutase (MnSOD), thioredoxin (Trx), and β-actin. Comparative Ct method was employed for data analysis using MxPro 4.10 (Agilent technologies, Santa Clara, CA). Values for each gene were normalized to expression levels of β-actin.

2.7. Measurement of anti-oxidant capacity

The anti-oxidant capacity was measured using Antioxidant Assay Kit (Cayman Chemical Company), which measures the inhibitory effect against metmyoglobin-mediated oxidative reaction from ABTS(2,2′-azino-di-[3-ethylbenzthiazoline sulfonate] to ABTS.$^+$

2.8. Statistical analysis

Statistical analysis was performed using Bonferroni/Dunn test following a one-way ANOVA. Differences were considered statistically significant when p values were less than 0.05.