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

メタデータ	言語: English 出版者: 公開日: 2017-10-03 キーワード (Ja): キーワード (En): 作成者: Yamada, Takashi, Hashida, Koji, Takarada-Iemata, Mika, Matsugo, Seiichi, Hori, Osamu メールアドレス: 所属:
URL	http://hdl.handle.net/2297/29609

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

Takashi Yamada[1], Koji Hashida[2,3], Mika Takarada-Iemata[2,3], Seiichi Matsugo[1,4], and Osamu Hori[2,3,*]

[1] Graduate school of Natural Science and Technology, Division of Material Engineering, Kanazawa University, Kanazawa, Ishikawa, Japan
[2] Department of Neuroanatomy, Kanazawa University Graduate School of Medical Science, Kanazawa, Ishikawa, Japan,
[3] CREST, JST (Japan Science and Technology), Tokyo, Japan
[4] College of Science and Engineering, School of Natural System Bioengineering Course, Kanazawa University, Kanazawa, Ishikawa, Japan

* Address correspondence to:
Dr. Osamu Hori,
Department of Neuroanatomy,
Kanazawa University Graduate School of Medical Science,
13-1 Takara-Machi, Kanazawa, Ishikawa 920-8640, Japan,
TEL: +81-76-265-2162,
FAX: +81-76-234-4222,
e-mail; osamuh3@staff.kanazawa-u.ac.jp

Running title: α-lipoic acid and neuroprotection

Keywords: neuronal cell death, glutathione, kinase

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.

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., 2004). 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., 2004, 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).

Alpha-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 et al., 1990), scavenging reactive oxygen species (ROS) (Matsugo et al, 1995), recycling / inducing endogenous anti-oxidants (Konishi et al., 1996), anti-inflammation (Fuchs et al., 1994), activation of cell signaling (Packer et al., 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 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.

Materials and Methods

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

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) (1mM: 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 (1μM: Nacalai Tesque), which is an inhibitor of proteasome, thapsigargin (Tg) (0.2μM: Sigma), which is an inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), and tunicamycin (Tm) (1μg/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.

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.

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 1mM BSO for the indicated times. Cells were then harvested, and lysed in 0.2ml 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 5μM 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.

Cell lysis, Western blotting and immunostaining. Cells were lysed in RIPA buffer containing 10mM Tris, 1mM EDTA, 150mM 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).

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.

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 sulphonate] to $ABTS.^{+}$

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

Results

Protection of SH-SY5Y cells by LA against glutathione depletion.

To analyze the neuroprotective effects of LA, human neuroblastoma SH-SY5Y cells were treated with different types of stressors such as BSO, H_2O_2, MG132, tunicamycin (Tm) and thapsigargin (Tg). After 24-36 h incubation, cell viability and cell death were measured using MTT assay (Fig.1A, C, D, Fig.S1) and LIVE/DEAD assay (Fig.1 B), respectively. Each stressor decreased cell viability to 40-60% of control cells (Fig. 1 A, Fig.S1 A). Co-treatment of the cells with 100-400 μM of LA (racemate) significantly improved cell viability against the toxicities induced by BSO (Fig.1 A) and, to a lesser extent, by diethyl maleate (DEM) (Fig.S1 B), but not by other stressors (Fig. S1 A), suggesting that LA may protect cells most effectively against glutathione depletion. Consistent with these results, LIVE/DEAD assay revealed that BSO-induced cell death was attenuated by co-treatment with LA (Fig. 1B). Treatment of the cells with LA alone did not affect cell viability (data not shown). Next, we compared the neuroprotective properties of each LA-enantiomer (racemate, R-LA or S-LA) at lower concentrations (5-50 μM) to estimate the dose dependency in BSO-treated SH-SY5Y cells. The results revealed that every type of LA significantly improved cell viability in a similar manner (Fig. 1 C). As previous reports demonstrated that pre-treatment of the cells with LA ameliorated neuronal injury (Zhang et al., 2001, Jia et al., 2008), we studied the effect of LA pre-treatment in our systems. Cells were incubated under three different conditions including treatment with LA in the presence of BSO (co-treatment) for 24 h, pre-treatment with LA alone for 48 h followed by BSO treatment for 24 h, and pre-treatment with LA alone for 48 h followed by treatment with LA in the presence of BSO for 24 h (pre- + co- treatments). In all conditions, LA improved cell viability, but the most effective condition was the combination of pre- and co-treatment of LA (Fig. 1D). This combined treatment of LA also improved cell viability against the toxicity of H_2O_2 (Fig. S1 C), as described previously (Zhang et al., 2001), but not those of other stressors such as

MG132, Tm and Tg (data not shown). As IC_{50} of LA against BSO-induced cell death was calculated to be 18.4μM in our system, two different concentrations, 20 and 200 μM, of LA were used for further experiments.

Effects of LA on the amount of intracellular glutathione.

As it is known that LA increases intracellular glutathione levels (Shay et al., 2009), we hypothesized that LA may protect SH-SY5Y cells against BSO and DEM by modulating glutathione content as previously described (Bharath S et al., 2002). The levels of total glutathione were measured after LA (racemate) treatment in the presence or absence of BSO. Treatment of the cells with LA alone enhanced glutathione levels to 150 % of those in control cells both at 16 h and 72 h (48 h+24 h) (Fig. 2 B, C). In contrast, Treatment of the cells with BSO alone reduced glutathione levels to 45 %, 20 % and 8 % of those in control cells at 8 h, 16 h and 24 h, respectively. Treatment of the cells with LA in the presence of BSO for 16 h did not restore glutathione levels (Fig. 2 B), and, importantly, pre-treatment of the cells with LA for 48 h followed by further treatment with LA in the presence of BSO for 24 h only mildly restored the glutathione levels to 15 % of those in control cells (Fig. 2 C), suggesting that the protective effect of LA in our system may be different from its glutathione-modulating property.

Effects of LA on ROS and oxidative stress response.

As LA can reduce oxidative stress directly by attenuating ROS and indirectly by enhancing the oxidative stress response, a fluorescent dye DCFH-DA was employed to measure the level of ROS. Treatment of SH-SY5Y cells with LA reduced the basal level of ROS to 31 % of that in control cells, while treatment of the cells with BSO enhanced it to 218 % of that in control cells (Fig 3A). Further analysis revealed that treatment of the cells with LA in the presence of BSO only mildly reduced ROS levels of the cells treated with

BSO alone (189 %) (Fig. 3 A). These results suggested that the direct quenching effect of LA toward ROS was not strong enough to scavenge ROS produced in the reaction system.

The oxidative stress response was next estimated by analyzing expressions of antioxidant genes and phase II detoxification genes at mRNA levels. qRT-PCR analysis revealed that downstream genes of Nrf2 such as HO-1 and NQO1 were strikingly upregulated by LA in the presence or absence of BSO (Fig. 3 B I, II), as described previously (Jia et al., 2008, Shay et al., 2009). Similar tendency was obtained when cells were treated with R- or S-LA (Fig. S2 A). Immunocytochemical analysis confirmed translocation of Nrf2 from the cytosol to the nucleus upon LA treatment (Fig.S2 C). In our system, LA also upregulated other anti-oxidant genes such as MnSOD and Trx to lesser degrees (Fig. S2 B I, II). Consistent with these results, reduction of anti-oxidant capacity in the cells after BSO treatment was restored by co-treatment with LA (Fig. 3 C).

To analyze the effect of upregulation of Nrf2-dependent anti-oxidant genes and phase II detoxification enzymes (HO-1 and NQO1) on the LA-mediated cell protection, cells were treated with Sn-mesoporphyrin IX (Sn-MP), an inhibitor of HO-1, or dicoumarol, an inhibitor of NQO1, in the presence or absence of BSO and LA. Dicoumarol (Fig. 3 D), but not Sn-MP (data not shown), reduced the protective effect of LA at the doses which did not affect cell viability.

Activation of the PI3-kinase/Akt pathway by LA.

As it was reported that LA promoted neuronal cell survival through the activation of signaling pathways including PI3K / Akt (Zhang et al., 2001, Wang et al., 2009), we examined the effect of LA on the phosphorylation of several signaling molecules. Western Blot analysis revealed that all LA enantiomers activated Akt (Fig. 4 A I, II), but not ERK (Fig. 4 A I), JNK (Fig. 4 A I), or p38 (data not shown), at 1-2h after LA treatment. Consistently, the protective effect of LA against BSO was reduced in the cells treated with

the PI3K inhibitor (LY294002), but not with the JNK inhibitor (SP600125), the p38 inhibitor (SB202190) (Fig. 4 B), or the ERK inhibitor (PD98059) (Fig. S3).

To elucidate the effect of Akt activation on the upregulation of antioxidant genes and phase II detoxification genes, qRT-PCR was performed using samples derived from cells treated with LY294002 in the presence of LA. LY294002 significantly reduced the levels of expressions of HO-1 (data not shown) and NQO1 (Fig. 4 C) in the presence of LA treatment.

Discussion

Glutathione is the most abundant antioxidant in the brain which serves to detoxify H_2O_2 by decomposing it into water and oxygen, and keeps the thiol groups of proteins in the reduced state (Mytilineou et al., 2002). Numerous studies have demonstrated decreased levels of glutathione in pathological conditions including brain ischemia and neurodegenerations. Postmortem studies have revealed that glutathione levels in the substantia nigra (SN) are decreased with age, and dramatically reduced in the brain of PD patients in concert with the death of dopaminergic neurons in the SN (Perry et al., 1982, Perry and Yong, 1986, Riederer et al., 1989). In experimental animal studies, acute depletion of glutathione caused extensive carbonylation of brain proteins (Bizzozero et al., 2006) and morphological changes of dopaminergic neurons in the nigrostriatal pathway that mimic alterations observed in the brains of aged animals (Andersen et al., 1996). It is, therefore, critical to find pathways which can improve neuronal survival / activity in the glutathione-depleted condition.

In this paper, we demonstrated that treatment of the cells with LA most effectively prevented cell death induced by drugs which deplete intracellular glutathione in SH-SY5Y cells (Fig. 1, Fig. S1). Accumulating evidence has revealed that LA-mediated neuroprotection requires relatively long periods of pre-treatment (hours to days) (Zhang et al., 2001, Blarath et al., 2002, Wang et al., 2010), raising a hypothesis that it may play more critical roles in enhancing the defense system against oxidative stress which includes upregulation of anti-oxidants such as glutathione and HO-1, and phase II enzymes such as NQO1, than directly chelating metals or scavenging reactive oxygen species (ROS) (Shay et al., 2009). Consistent with this hypothesis, LA-mediated cell protection against BSO was enhanced in the combination of pre-treatment for 48h and co-treatment for 24h in our system (Fig. 1 D). H_2O_2-induced toxicity was also attenuated in the combination of pre- and co-treatment of LA (Fig. S1 A II). Furthermore, expressions of endogenous anti-oxidants such as HO-1 and phase II enzymes such as NQO1 were strikingly upregulated by LA treatment in the presence of BSO (approximately 20-25 fold increase, Fig. 3 B I, II). The reduction of anti-oxidant

capacity upon BSO treatment was also restored by treatment with LA (Fig. 3 C). Furthermore, an inhibitor of NQO1, but not HO-1, significantly reduced the protective effect of LA against BSO (Fig. 3 D). In contrast, LA had only mild effects on BSO-mediated glutathione depletion (Fig. 2) or ROS generation (Fig. 3 A).

How does LA enhance the defense system against oxidative stress? One possibility is activation of signaling pathways such as PI3K/Akt, p38 MAPK, ERK1/2 and JNK (Shay et al., 2009). Consistent with previous reports (Zhang et al., 2001, Abdul et al., 2007), LA activated Akt (Fig. 4 A), and an inhibitor of PI3K, LY294002, suppressed the effect of LA on NQO1 expression (Fig. 4 C) and cell viability upon BSO treatment (Fig. 4 B). Another possibility is that LA, as a pro-oxidant, increases both Nrf2-dependent and independent transcriptional activity (Shay et al., 2009). Mild upregulation of MnSOD and Trx expressions (approximately 3-3.5 fold increase, Fig. S2 B, I, II) upon LA treatment in the presence of BSO may support this, but this is not so likely because the level of ROS was not enhanced either in the cells (Fig. 3 A) or in the media (unpublished observation) after LA treatment.

Regarding LA enantiomers, every LA (racemate, R-LA or S-LA) protected cells against BSO toxicity (Fig. 1 C) and upregulated endogenous anti-oxidants (Fig. 3 B, Fig. S 2 A) in a similar manner. Previous reports demonstrated that R- and S-LA are differentially metabolized, and R-LA may exert more rapid and clearer bioactivities (e.g. glucose uptake and glycogen synthesis) than S-LA (Shay et al., 2009). Further studies are required, especially *in vivo,* to determine the differences between LA enantiomers for their neuroprotective properties.

In conclusion, we demonstrated that all LA enantiomers most effectively prevented cell death induced by drugs which deplete glutathione through several mechanisms including PI3K /Akt-mediated upregulation of phase II enzymes NQO1.

Acknowledgments

We thank Dr. Tetsuya Konishi (Niigata University of Pharmacy and applied life sciences) for providing the LAs (racemate, R-LA and S-LA). We are also grateful to Mr. Kei Gunjima for technical assistance. This work was supported partly by Grant-in Aid for Scientific Research (19500319, 20059014) from the Ministry of Education, Science, Technology, Sports and Culture of Japan.

References

Andersen JK, Mo JQ, Hom DG, Lee FY, Harnish P, Hamill RW, McNeill TH. Effect of buthionine sulfoximine, a synthesis inhibitor of the antioxidant glutathione, on the murine nigrostriatal neurons. *J Neurochem.* 1996 Nov;67(5):2164-71.

Bharath S, Cochran BC, Hsu M, Liu J, Ames BN, Andersen JK. Pre-treatment with R-Lipoic acid alleviates the effects of GSH depletion in PC12 Cells: Implications for Parkinson's Disease Therapy. *Neurotoxicology.* 2002, 23 479-486.

Bizzozero OA, Ziegler JL, De Jesus G, Bolognani F. Acute depletion of reduced glutathione causes extensive carbonylation of rat brain proteins. *J Neurosci Res*. 2006, 83(4):656-67.

Bossy-Wetzel, E., Schwarzenbacher, R. and Lipton, S.A. (2006). Molecular pathways to neurodegeneration. *Nat Med*. 10 Suppl., S2-9.

Cakatay U. Pro-oxidant actions of alpha-lipoic acid and dihydrolipoic acid. *Med Hypotheses*. 2006;66(1):110-7. Epub 2005 Sep 13.

Clark WM, Rinker LG, Lessov NS, Lowery SL, Cipolla MJ. Efficacy of antioxidant therapies in transient focal ischemia in mice. *Stroke.* 2001 Apr;32(4):1000-4.

Fuchs J, Milbradt R, Antioxidant inhibition of skin inflammation induced by reactive oxidants: evaluation of the redox couple of dihydrolipoate / lipoate. *Skin Pharamcol* 1994, 7: 278-284.

Hori, O., Ichinoda, F., Yamaguchi, A., Tamatani, T., Taniguchi, M., Koyama, Y., Katayama, T., Tohyama, M., Stern, D.M., Ozawa, K., Kitao, Y. and Ogawa, S. Role of Herp in the endoplasmic reticulum stress response. *Genes Cells*. 2004, 9(5), 457-469.

Jia Z, Hallur S, Zhu H, Li Y, Misra HP. Potent upregulation of glutathione and NAD(P)H:quinone oxidoreductase 1 by alpha-lipoic acid in human neuroblastoma SH-SY5Y cells: protection against neurotoxicant-elicited cytotoxicity. *Neurochem Res*. 2008 May;33(5):790-800. Epub 2007 Oct 17

Karunakaran S, Diwakar L, Saeed U, Agarwal V, Ramakrishnan S, Iyengar S, Ravindranath V. Activation of apoptosis signal regulating kinase 1 (ASK1) and translocation of death-associated protein, Daxx, in substantia nigra pars compacta in a mouse model of Parkinson's disease: protection by alpha-lipoic acid. *FASEB J.* 2007, 21(9):2226-36.

Konrad D, Somawat K, Sweeney G, Yaworsky K, Hayashi M, Ramlal T, Klip A The antihyperglycimic drug a-lipoic acid stimulates glucose uptake via both GLUT4 translocation and GLUT4 activation: Potential role of p38 mitogen-activated protein kinase in GLUT4 activation *Diabetes* 2001. 50: 1464-1471.

Konishi T, Handelman G, Matsugo S, Mathur VV, Tritschler HJ, Packer L. Amplified determination of lipoyl groups by lipoamide dehydrogenase in the presence of oxidized glutathione. *Biochem Mol Biol Int* 1996 36 (6) 1155-1161.

Matsugo S, Yan LJ, Han D, Tritschler HJ, Packer, L. Elucidation of antioxidant activity of α-lipoic acid toward hydroxyl radical *Biochem. Biophys Res. Commun* 1995, 208(1);161-167

Mehta SL, Manhas N, Raghubir R. Molecular targets in cerebral ischemia for developing novel therapeutics. *Brain Res Rev.* 2007, 54(1):34-66. Epub 2007 Jan 12.

Muller L, MenzelH, Studies on the efficacy of lipoate and dihydrolipoate in the alternation of cadmium 2+ toxicity in isolated hepatocytes. *Biochim Biophys Acta* 1990, 1052: 386-391

Mytilineou C, Kramer BC, Yabut JA. Glutathione depletion and oxidative stress. *Parkinsonism Relat Disord*. 2002, 8(6):385-7.

Packer L, Cadenas E.. Lipoic acid: energy metabolism and redox regulation of transcription and cell signaling. *J Clin Biochem Nutr* 2011 48(1):26-32.

Perry TL, Yong VW. Idiopathic Parkinson's disease, progressive supranuclear palsy and glutathione metabolism in the substantia nigra of patients. *Neurosci Lett.* 1986, 67(3):269-74

Riederer P, Sofic E, Rausch WD, Schmidt B, Reynolds GP, Jellinger K, Youdim MB. Transition metals, ferritin, glutathione, and ascorbic acid in Parkinsonian brains. *J Neurochem*. 1989, 52(2):515-20

Shay KP, Moreau RF, Smith EJ, Smith AR, Hagen TM. Alpha-lipoic acid as a dietary supplement: molecular mechanisms and therapeutic potential. *Biochim Biophys Acta.* 2009, 1790(10):1149-60.

Siedlak SL, Casadesus G, Webber KM, Pappolla MA, Atwood CS, Smith MA, Perry G. Chronic antioxidant therapy reduces oxidative stress in a mouse model of Alzheimer's disease. *Free Radic Res.* 2009, 43(2):156-64

Smith, W.W., Jiang, H., Pei, Z., Tanaka, Y., Morita, H., Sawa, A., Dawson, V.L., Dawson, T.M. and Ross, C.A. (2005). Endoplasmic reticulum stress and mitochondrial cell death pathways mediate A53T mutant alpha-synuclein-induced toxicity. *Hum Mol Genet*. 14(24), 3801-3811.

Song, D.D., Shults, C.W., Sisk, A., Rockenstein, E. and Masliah, E. (2004). Enhanced substantia nigra mitochondrial pathology in human alpha-synuclein transgenic mice after treatment with MPTP. *Exp Neurol*. 186(2), 158-172.

Takano K, Kitao Y, Tabata Y, Miura H, Sato K, Takuma K, Yamada K, Hibino S, Choshi T, Iinuma M, Suzuki H, Murakami R, Yamada M, Ogawa S, Hori O. A DIBENZOYLMETHANE (DBM) DERIVATIVE PROTECTS DOPAMINERGIC NEURONS AGAINST BOTH OXIDATIVE STRESS AND ENDOPLAMIC RETICULUM (ER) STRESS. *Am J Physiol cell Physiol.* 2007, 293(6):C1884-94.

Wang X, Zhang X, Cheng Y, Li C, Zhang W, Liu L, Ding Z. Alpha-lipoic acid prevents bupivacaine-induced neuron injury in vitro through a PI3K/Akt-dependent mechanism. *Neurotoxicology.* 2010, 31(1):101-12. Epub 2009 Oct 29

Wolz P, Krieglstein J. Neuroprotective effects of alpha-lipoic acid and its enantiomers demonstrated in rodent models of focal cerebral ischemia. *Neuropharmacology*. 1996 Mar;35(3):369-75

Zhang L, Xing GQ, Barker JL, Chang Y, Maric D, Ma W, Li BS, Rubinow DR. Alpha-lipoic acid protects rat cortical neurons against cell death induced by amyloid and hydrogen peroxide through the Akt signaling pathway. *Neurosci Lett*. 2001, 312(3):125-8

Figure legends

Figure 1. Effects of LA on BSO toxicity. (A), (B) Improved viability and reduced cell death in LA-treated cells. SH-SY5Y cells were treated with BSO at the indicated concentrations for 36h in the presence or absence of LA (racemate). Cell viability and death were measured using MTT assay (A) and LIVE/DEAD assay (B), respectively. (C) Effects of LA-enantiomers on BSO toxicity. Cells were treated with 1mM of BSO in the presence or absence of LA enantiomers (racemate, R-LA or S-LA) at indicated concentrations. Cell viability was measured using MTT assay. (D) The effect of LA pretreatment on the BSO toxicity. Cells were treated with LA in the presence of BSO for 24 h, pre-treated with LA alone for 48 h followed by BSO treatment for 24 h, or pre-treated with LA alone for 48 h followed by treatment with LA in the presence of BSO for 24 h. Cell viability was measured using MTT assay. Values shown are the mean ± S.D. in three separate experiments. *$P<0.05$, **$P<0.01$, significantly different from values obtained under treatment with BSO alone. $^{\#}P<0.05$, significantly different between the two conditions.

Figure 2. Effects of LA on the amount of glutathione. Cells were treated with LA (racemate) for 8h (A) or 16h (B) in the presence or absence of BSO. Cells were also treated with LA (racemate) for 48h, followed by treatment with LA in the presence or absence of BSO for further 24h (C). The amount of intracellular glutathione was measured as described in the text. The values in the cells cultured in the medium alone were designated as 100. Values shown are the mean ± S.D. in three separate experiments. *$P<0.05$, **$P<0.01$, significantly different between the two conditions.

Figure 3. Effects of LA on ROS generation and oxidative stress response. (A) Measurement of ROS using a fluorescent dye DCFH-DA. Cells were treated with LA in the presence or absence of 1mM BSO for 16h, and then loaded with 5μM DCFH-DA for 20min. Typical

results were demonstrated (I). Quantification was performed using Image J as described in the text (II). (B) Upregulation of anti-oxidant genes. Cells were treated with LA in the presence or absence of 1mM BSO for 16h, and total RNA was extracted. qRT-PCR was performed with specific primers for HO-1 (I) and NQO1 (II). Values shown are the mean ± S.D. in four separate experiments. (C) Anti-oxidant capacity upon BSO treatment. Cells were treated with LA in the presence of BSO for 28h, and anti-oxidant assay was performed as described in the text. (D) Effect of dicoumarol on LA-mediated protection against BSO. Cells were treated with the indicated dose of dicoumarol in the presence and absence of BSO and LA for 36h. Cell viability was analyzed using MTT assay. Values shown are the mean ± S.D. in three separate experiments. *P<0.05, **P<0.01, significantly different from values obtained under incubation in the medium alone. $^{\#}$P<0.05 , significantly different between the two conditions.

Figure 4. Activation of PI3K / Akt pathway by LA. (A) Phosphorylation of stress-associated kinases after LA treatment. Cells were treated with 200μM LA (racemate, R-LA or S-LA) for the indicated times, and protein extracts were subjected to Western blotting using the indicated antibodies (I). Relative intensity was measured using Image J as described in the text (II). (B) Effects of kinase inhibitors on LA-mediated cell protection. Cells were treated with the indicated kinase inhibitors in the presence or absence of BSO and LA for 36h, and MTT assay was performed. Values shown are the mean ± S.D. in three separate experiments. (C) Suppression of LA-mediated NQO1 upregulation by PI3K inhibitor. Cells were treated with LY294002 in the presence of LA for 24h, and total RNA was extracted. qRT-PCR was performed with specific primers for NQO1. Values shown are the mean ± S.D. in four separate experiments. *P<0.05, **P<0.01, significantly different from values in the conditions incubated in the medium alone. $^{\#}$P<0.05 , significantly different between the two conditions.

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

Supplemental Figure Legends

Figure S1. (A) (B) Effects of LA on cell viability during different stresses. SH-SY5Y cells were treated with LA in the presence of various stressors such as H_2O_2 (24h), MG132 (24h), tunicamycin (Tm) (24h), thapsigargin (Tg) (24h) (A) or DEM (36h) (B), and MTT assay was performed. Values shown are the mean ± S.D. in three separate experiments. *P<0.05, significantly different from values obtained under treatment with BSO alone. (C) The effect of LA pretreatment on the H_2O_2 toxicity. Cells were pre-treated with LA alone for 48 h followed by treatment with LA in the presence of H_2O_2 for 24 h. Cell viability was measured using MTT assay. Values shown are the mean ± S.D. in three separate experiments. *P<0.05, **P<0.01, significantly different from values obtained under treatment with BSO or H_2O_2alone.

Figure S2. Upregulation of anti-oxidant genes and Nrf2 activation by LA. Cells were treated with LA (racemate, R-LA or S-LA) in the presence or absence of 1mM BSO for 16h. (A)(B) Expressions of anti-oxidant genes. Total RNA was subjected to qRT-PCR with specific primers for NQO1 (A), MnSOD (B I), or Trx (B II). Values shown are the mean ± S.D. in four separate experiments. *P<0.05, **P<0.01, significantly different from values obtained under incubation in the medium alone. (C) Cells were fixed and subjected to immunostaining with anti-Nrf2 antibody. Nucleus was visualized with DAPI.

Figure S3. Effect of an ERK inhibitor on LA-mediated cell protection. Cells were treated with PD98059 in the presence of BSO and LA for 36h, and MTT assay was performed.

Values shown are the mean ± S.D. in three separate experiments. **P<0.01, significantly different from values in the conditions incubated in the medium alone.

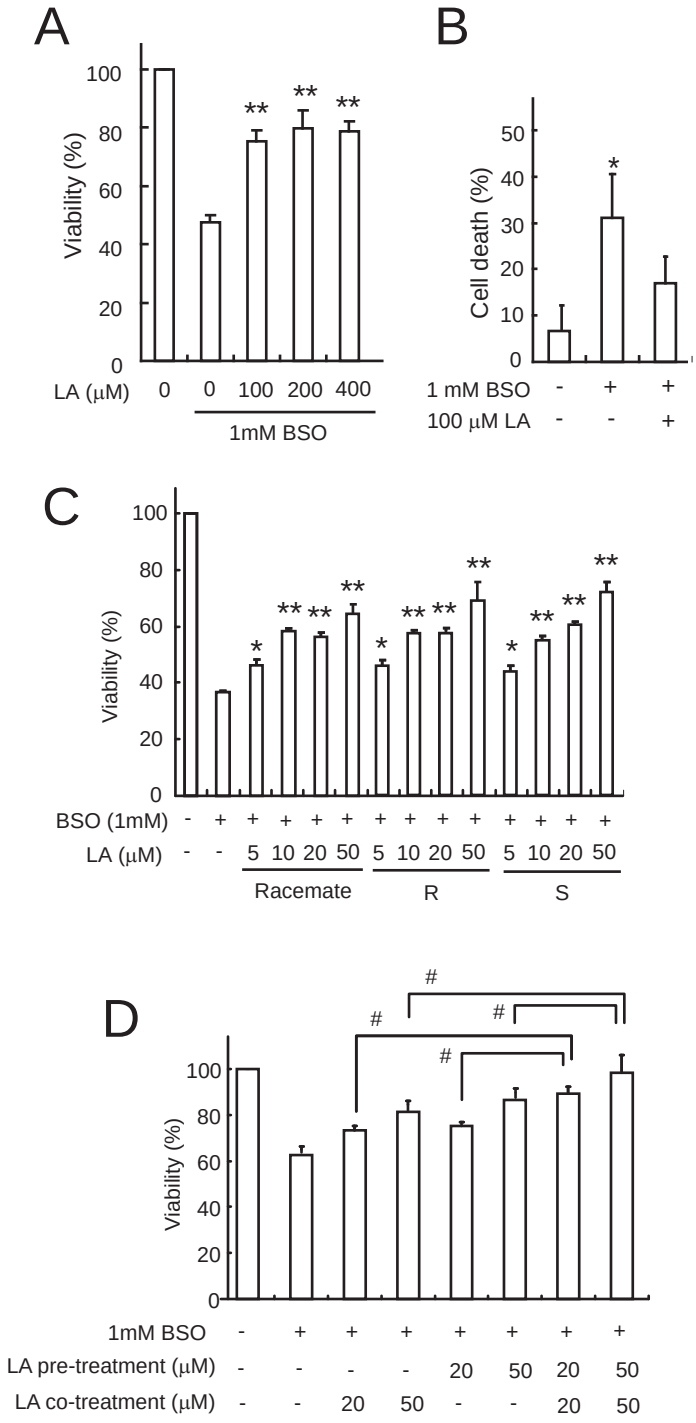


Fig.1 Yamada et al.

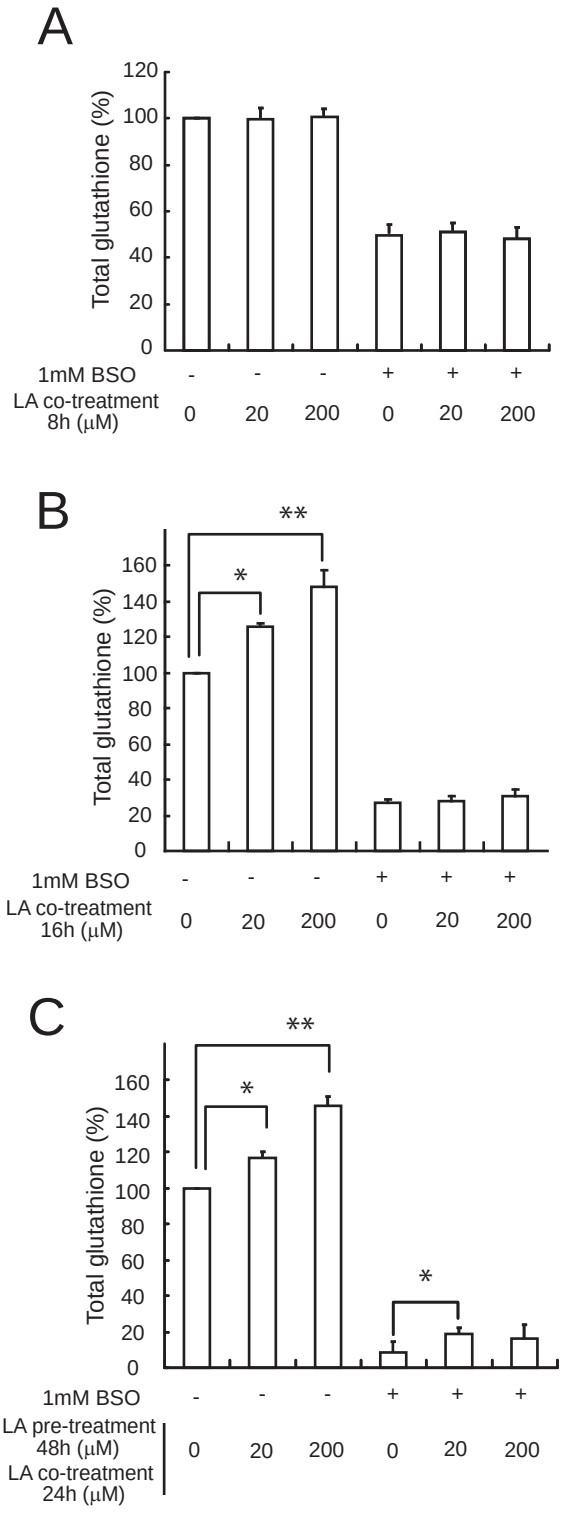


Fig.2 Yamada et al.

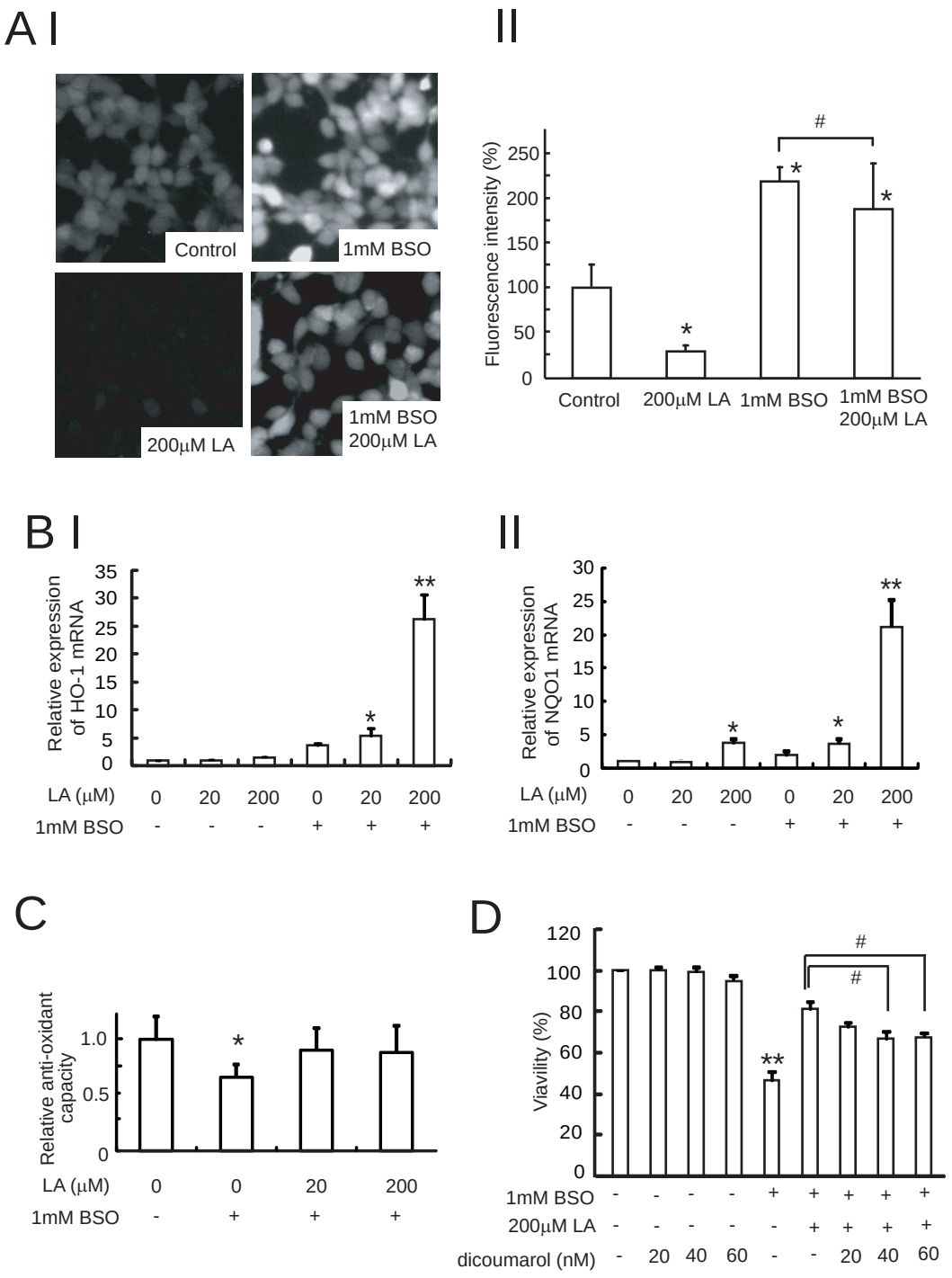


Fig.3 Yamada et al.

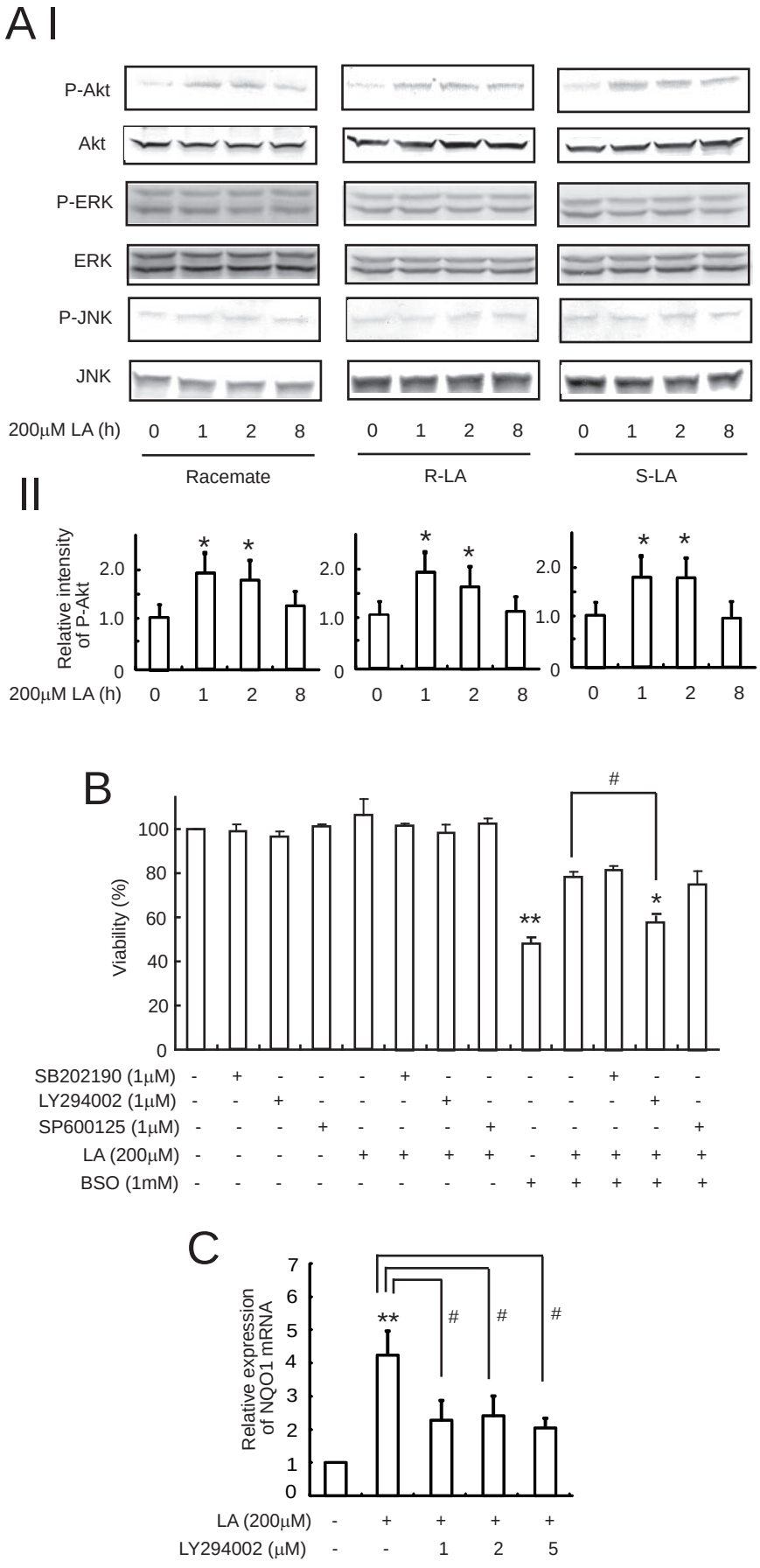


Fig.4 Yamada et al.

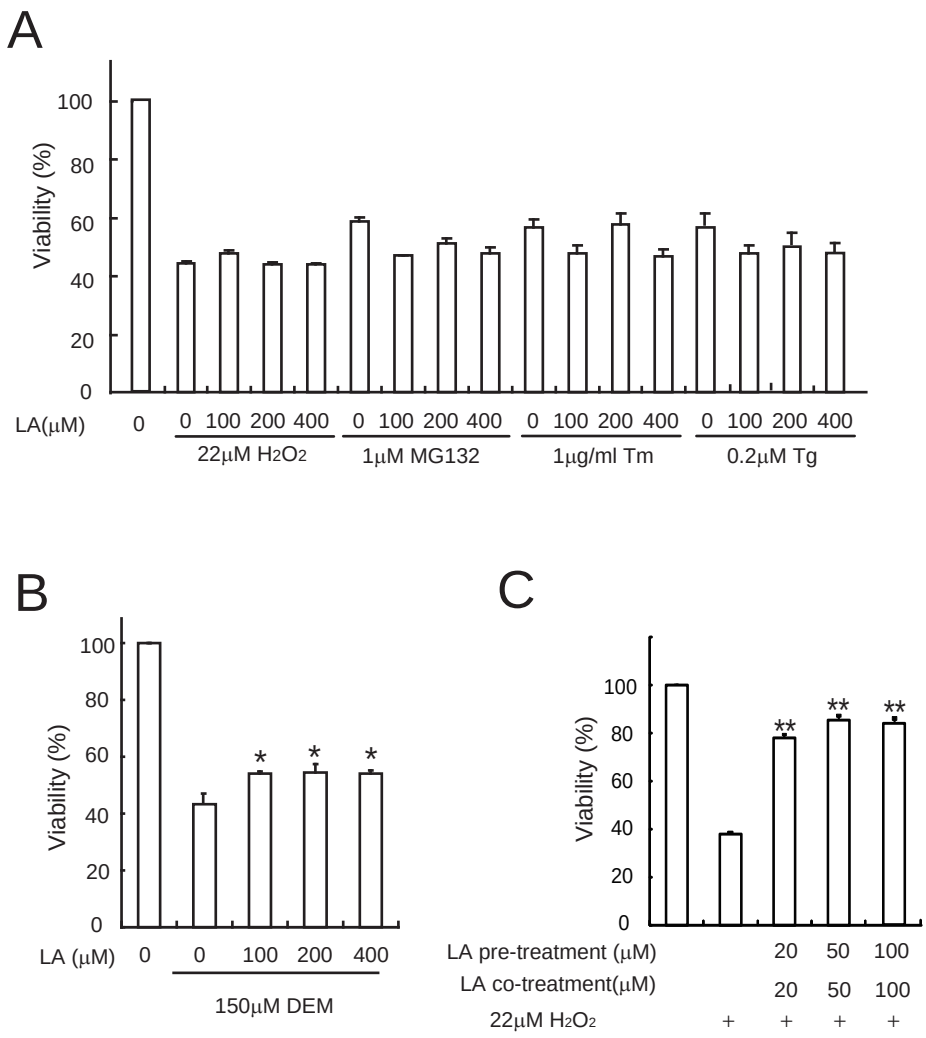
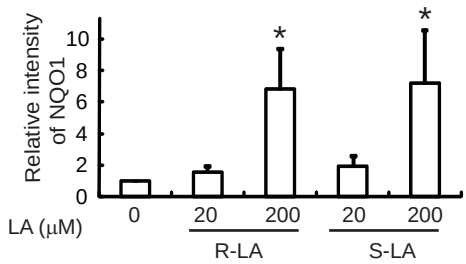
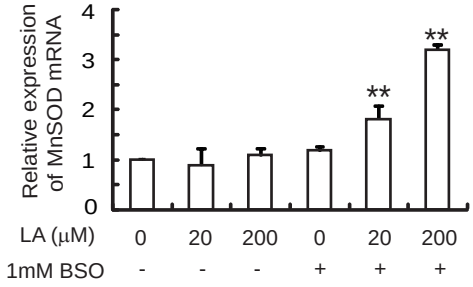


Fig.S1 Yamada et al.

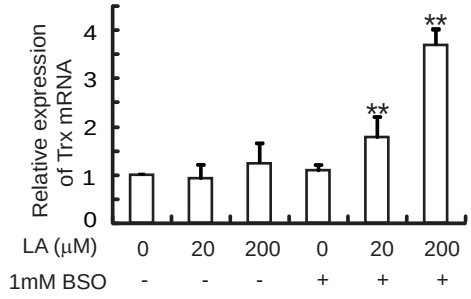
A



B I



II



C

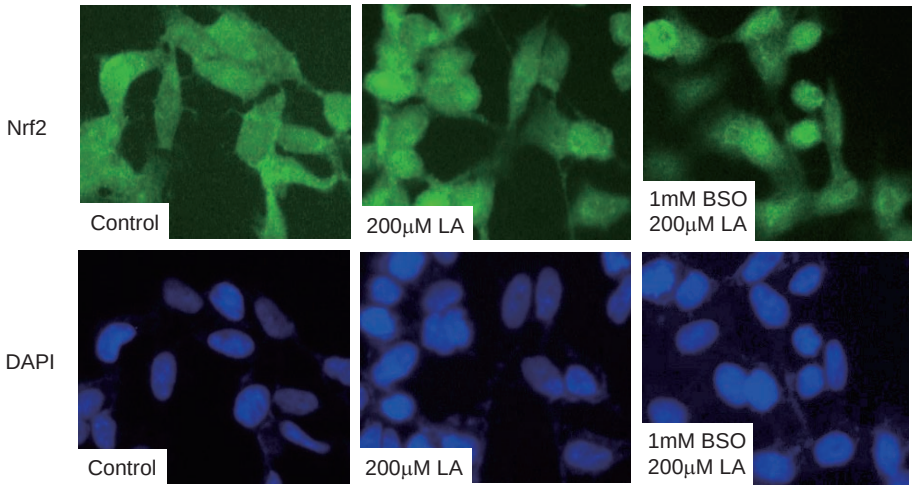


Fig.S2 Yamada et al.

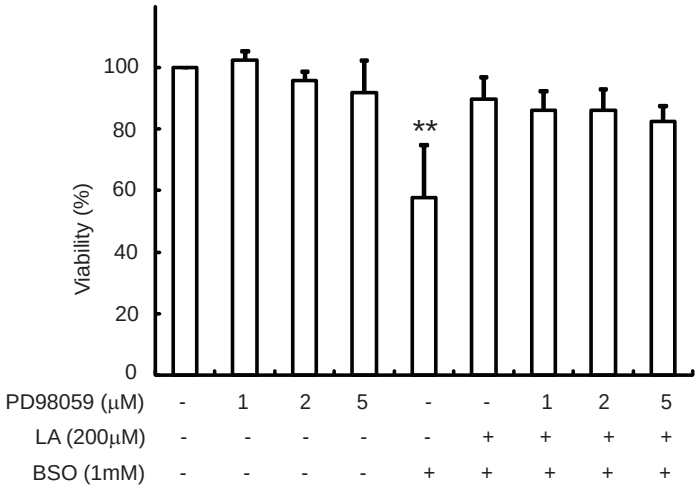


Fig.S3 Yamada et al.