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Cytoprotective Effects of Dihydrolipoic Acid and Lipoic Acid on the Oxidative Stress in Cultured Rat Cortical Neurons

Won-Ki Kim

Department of Pharmacology, College of Medicine; Division of Neuroscience, Medical Research Center, Ewha Womans University, Seoul 158–056, Korea

In brain hypoxic-ischemia, an excess release of glutamate and a marked production of reactive oxygen species (ROS) occur in neuronal and non-neuronal cells. The present study investigated the effect of the biological antioxidants dihydrolipoic acid (DHLA) and lipoic acid (LA) on *N*-methyl-D-aspartate (NMDA)- and ROS-induced neurotoxicity in cultured rat cortical neurons. DHLA enhanced NMDA-evoked rises in intracellular calcium concentration ($[Ca^{2+}]_i$). In contrast, LA did not alter the NMDA-evoked calcium responses but decreased after a brief treatment of dithiothreitol (DTT), which possesses a strong reducing potential. Despite the modulation of NMDA receptor-mediated rises in $[Ca^{2+}]_i$, neither DHLA nor LA altered the NMDA receptor-mediated neurotoxicity, as assessed by measuring the amount of lactate dehydrogenase released from dead or injured cells. DHLA, but not LA, prevented the neurotoxicity induced by xanthine/xanthine oxidase-generated superoxide radicals. Both DHLA and LA decreased the glutathione depletion-induced neurotoxicity. The present data may indicate that biological antioxidants DHLA and LA protect neurons from ischemic injuries via scavenging oxygen free radicals rather than modulating the redox modulatory site(s) of NMDA receptor.

Key Words: $[Ca^{2+}]_i$, Neurotoxicity, Dihydrolipoic acid (DHLA), Lipoic acid (LA), NMDA receptor, Glutathione, Reactive oxygen species (ROS)

INTRODUCTION

In brain hypoxic-ischemia, neurons may die from an extracellular overflow of excitatory amino acids such as glutamate. Excess increases in $[Ca^{2+}]_i$, mainly through the *N*-methyl-D-aspartate (NMDA) receptor subtype of glutamate receptors, have been associated with various neurodegenerative diseases including cerebral ischemia. During the ischemic insult, a continuous and marked production of reactive oxygen species (ROS) also occur in neuronal and non-neuronal cells. Thus, it may be plausible to protect neurons from ischemic insults via modulating the activity of NMDA receptor-ion channel complexes and scavenging reactive oxygen species.

Reduced (DHLA) and oxidized α-lipoic acid (LA) are endogenous compounds which act as cofactors in mitochondrial dehydrogenase complexes (Koike & Koike, 1975; Stryer, 1988). DHLA and LA help to maintain biological antioxidants such as glutathione and a-tocopherol in their reduced states (Scholich et al, 1989; Bast & Haenen, 1988). In animal models, ischemic injury was prevented by pretreatment of DHLA and LA (Serbinova et al, 1992; Prehn et al, 1992). These sulfur-containing cofactors can readily interconvert via thiol-disulfide exchange reactions and appear to be involved in the several redox processes (Spector et al, 1988).

NMDA-evoked calcium responses have been showed to be modulated by antioxidants via a redox-modulatory site(s) of the NMDA receptor, a subtype of the excitatory amino acid glutamate receptors (Aizenman et al, 1989; Lazarewicz et al, 1989; Levy

Corresponding to: Won-Ki Kim, Department of Pharmacology College of Medicine, Ewha Womans University, 911-1 Mok-6-dong, Yangchun-ku, Seoul 158-056, Korea. (Tel) 650-5745, 653-8891, E-mail: won_k@mm.ewha.ac.kr

et al, 1990; Reynolds et al, 1990). Thus, DHLA and LA are expected to alter the activity of NMDA receptor. Also, these endogenous biological antioxidants will also buffer the oxidative stress owing to the redox potentials. The present study was, therefore, aimed to investigate whether DHLA and LA could alter the activity of NMDA receptors through the redox modulatory site(s) and decrease the oxidative stress-induced neuronal death.

METHODS

Culture of cerebral cortical neurons

Cerebral cortical neurons were prepared from the prefrontal cerebra from embryonic day 16~17 rat embryos, as previously described (Lipton et al, 1997). In brief, the cerebra were separated from brain stem, and cerebellum in Eagle's minimum essential medium (EMEM). Then, hippocampus and meninges were completely removed. The cerebra were transferred to 0.03% trypsin-containing, serum-lacking EMEM supplemented with 11 mM glucose, 2 mM glutamine, and 60 U/ml penicilline and streptomycin, minced and incubated at 35°C for 2 h. The tissue was washed three times with growth media (Dulbecco's minimum essential media (DMEM)/F12/FCS (8 : 1 : 1) supplemented with 2 mM glutamine, 60 U/ml penicilline and streptomycin) and incubated in the same buffer without trypsin for 20 additional minutes. The tissue suspension was then diluted with several volumes of the growth medium with 5% fetal serum, triturated, and filtered through a 135 μm nylon mesh, and then a 35 μm. Cells (4.5×10^5 viable cells/35 mm dish) were plated onto the 35-mm dishes or coverslips precoated with poly-D-lysine and maintained in 95% air/5% CO_2 incubator. 14 days after culturing, in order to inhibit the proliferative cells, cells were treated with 10 mg/ml cytosine arabinoside for 48 h. All experiments (for both cell death and $[Ca^{2+}]_i$ measurements) were performed 7 to 10 days after the second treatment of cytosine arabinoside.

Determination of cell death

Neuronal cell death was determined by measuring the amount of LDH released to the bathing medium 12~24 hours after NMDA treatments, as previously described (Lipton et al, 1997). The LDH amount corresponding to complete neuronal death without glial damage (defined as total LDH) was measured in sister cultures exposed 24 hours to 2 mM NMDA in the presence of 1 μM glycine. Cell viability was expressed as % of total LDH. Basal LDH levels (generally <15% of total LDH) were determined in sister cultures subjected to sham wash and subtracted from the levels in experimental conditions to yield the LDH signal specific to experimenal injury.

Determination of intracellular calcium concentration ($[Ca^{2+}]_i$)

Intracellular calcium concentration was analyzed with fura-2 acetoxymethyl ester (fura-2/AM; Molecular Probes) using a protocol for digital Ca^{2+} imaging that has been detailed previously (Kim et al, 1994). In brief, cells on the 12-mm glass coverslips were twice rinsed with Krebs-Ringer-Hanselite (KRH) buffer (125 mM NaCl, 1.2 mM KH_2PO_4, 6 mM glucose, and 1 mM $CaCl_2$, 25 mM HEPES, pH 7.4) and were loaded with 10 μM fura-2/AM for 30 min at 37°C. Cells were washed thrice with KRH buffer and were left in KRH for 20 min at room temperature before measuring $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured every 10 seconds before and during the drug treatment. NMDA were always coapplied with glycine (1 μM) and tetrodotoxin (TTX; 0.5 μM). All redox reagents including dithiothreitol (DTT; 2 mM), 5,5'-dithio-bis-(2nitrobenzoic acid) (DTNB; 0.5 mM), DHLA (2 mM) and LA (2 mM) were treated for 2 min, washed away with Mg^{2+} -free KRH buffer for 3~4 min. The NMDA (35 μM)-elicited calcium responses were then assessed from at least 3 separate cortical neurons for each experiment. Changes in fluorescence intensity of fura-2 at excitation wavelengths of 340 and 380 nm were determined at room temperature using an image processing system (Quantex QX7-210, Sunnyvale, CA) interfaced to an IBM personal computer. $[Ca^{2+}]_i$ was determined as described by Grynkiewicz et al (1985).

Materials

NMDA, tetrodotoxin (TTX), glycine, poly-D-lysine, para-aminobenzoic acid, glutamate, glutamine, DMEM, EMEM, F12, lactate dehydrogenase diagnostic kit, L-buthionine-(S,R)-sulfoximine (BSO), lipoic acid (LA) and dihydrolipoic acid (DHLA) were purchased from Sigma Chemical Company (St. Louis,

MO, USA). Trypsin, DNAase, fetal calf serum, penicilline, streptomycin were obtained from Gibco (BRL Life Technologies, Inc. NY, USA). Fura-2/AM and fura-2 pentaphosphate were purchased from Molecular Probes (Eugene, OR, USA). All other chemicals were obtained from standard commercial sources.

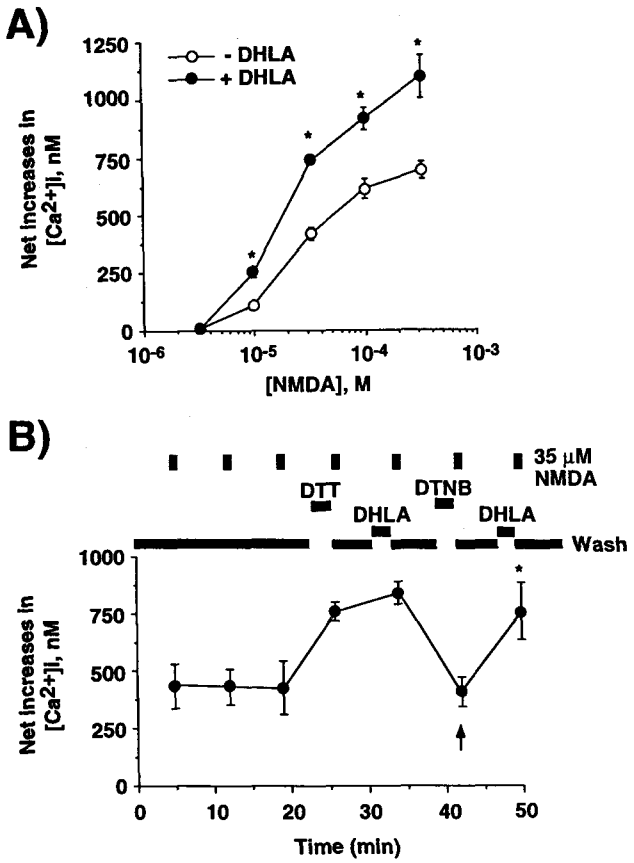

Fig. 1. Dihydrolipoic acid potentiates NMDA-evoked increases in $[Ca^{2+}]_i$. Cells were stimulated with NMDA for 8 seconds using a pneumatic puffer. $[Ca^{2+}]_i$ was measured at the end of the 8-second puffing. DTT, DTNB and DHLA were treated for 2 min and washed thoroughly with KRH buffer as depicted as solid bars. A) Concentration-response relationships. $[Ca^{2+}]_i$ rises were evoked by various concentrations of NMDA with or without a 2-min pre-exposure to 2 mM DHLA. B) DHLA increased the NMDA-mediated calcium responses. For both A and B, data are representative from 5 separate experiments. For both A and B, $*P<0.05$; analyzed with analysis of variance [ANOVA] followed by Scheffe's multiple comparison of means. A) Statistical comparison between the groups pre-treated for 2 min or untreated with 2 mM DHLA were made for calcium responses evoked at each concentration of NMDA. B) The $[Ca^{2+}]_i$ rise evoked by 35 μM NMDA after pre-exposure to DHLA was compared with that previously obtained after DTNB pre-treatment (indicated as an arrow).

Statistical analysis

Data are presented as mean standard error of the mean and were analyzed for statistical significance using a paired-*t* test or analysis of variance [ANOVA] and Scheffe's multiple comparison test.

RESULTS

Redox modulation of NMDA receptor activity

Basal $[Ca^{2+}]_i$ of cotical neurons were 47 ± 6.8 nM. All redox reagents used in the present study, including DTT (2 mM), DTNB (0.5 mM), DHLA (2 mM) and LA (2 mM), did not themselves alter $[Ca^{2+}]_i$ (data not shown). NMDA increased $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 1A). The reducing reagent DTT and the oxidizing reagent DTNB altered the NMDA-induced increases in $[Ca^{2+}]_i$ (Fig. 1B). Thus, DTT potentiated $73 \pm 13.2\%$ the NMDA-induced increases in $[Ca^{2+}]_i$ (Fig. 1B). The cells that did not respond DTT were not used for the studies on DHLA and LA. Similar to DTT, DHLA enhanced $63 \pm 11.9\%$ the NMDA (35 μM)-evoked calcium responses (Fig. 1, A and B) and the enhancing effect of DHLA was completely reversed by the oxidizing reagent DTNB (Fig. 1B). The DHLA effect was not additive to that of DTT, suggesting that the DHLA effect may be mediated via a redox-regulatory site(s) of the NMDA receptor (Fig. 1B).

Lipoic acid (2 mM) did not alter the NMDA-mediated calcium responses (Fig. 2A), but reversed the potentiating effect of DTT on the NMDA-mediated calcium responses (Fig. 2B). LA did not further inhibit the NMDA response of the neurons previously treated with DTNB (0.5 mM). Pretreatment of N-ethyl-maleimide, which has been shown to block the action of redox-modulatory reagents by alkylating the free sulfhydryl moiety of cysteins (Tang & Aizenman, 1993), also prevented the action of LA (Fig. 2B).

Effect on NMDA excitotoxicity

DHLA and LA themselves did not alter the

viability of cortical neurons (Fig. 3). A simultaneous and brief (10 min) treatment of DHLA, which enhanced the NMDA receptor-mediated increase in $[Ca^{2+}]_i$, did not influence the NMDA-evoked death of cortical neurons (Fig. 3). Simultaneous treatment of LA for 10 min also did not attenuate the NMDA-evoked death of cortical neurons (Fig. 3).

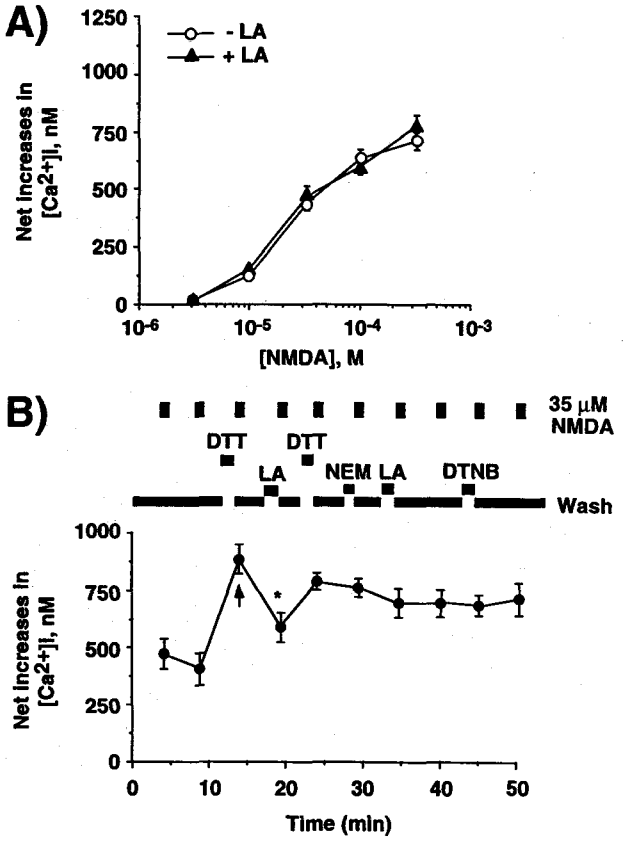

Fig. 2. Effects of Lipoic acid on NMDA-evoked increases in $[Ca^{2+}]_i$. DTT, DTNB, LA and N-ethyl maleimide (NEM, 1 mM) were treated for 2 min and washed thoroughly with KRH buffer as depicted as solid bars. A) Concentration-response relationships. $[Ca^{2+}]_i$ rises were evoked by various concentrations of NMDA with or without a 2-min pre-exposure to 2 mM LA. B) LA decreased NMDA-mediated calcium responses. For both A and B, data are representative from 5 separate experiments. For both A and B, *$P<0.05$; analyzed with analysis of variance [ANOVA] followed by Scheffe's multiple comparison of means. A) Statistical comparison between the groups pre-treated for 2 min or untreated with 2 mM LA were made for calcium responses evoked at each concentration of NMDA. B) The $[Ca^{2+}]_i$ rise evoked by 35 μM NMDA after pre-exposure to LA was compared with that previously obtained after DTT pre-treatment (indicated as an arrow).

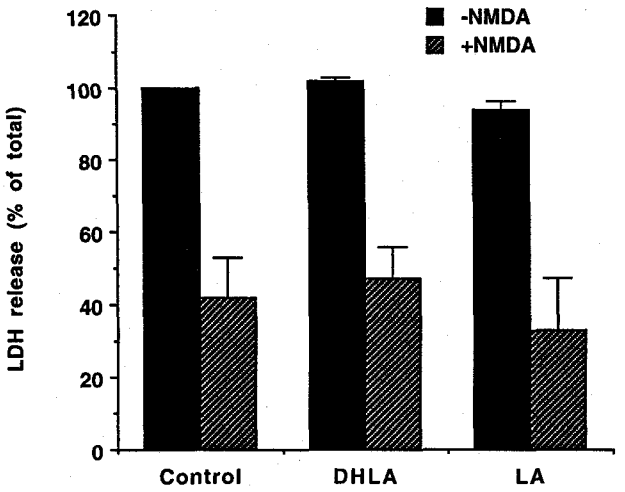

Fig. 3. Effects of DHLA and LA on NMDA-evoked neurotoxicity. Cells were treated for 10 min with 200 μM NMDA in the presence and absence of DHLA and LA. The LDH levels in the media were measured 12~18 h after NDMA treatment. Data are expressed as % change compared with the amount of LDH (total LDH) released by a 24 h incubation with DMEM containing 1 mM NMDA and 1 μM glycine. N=4.

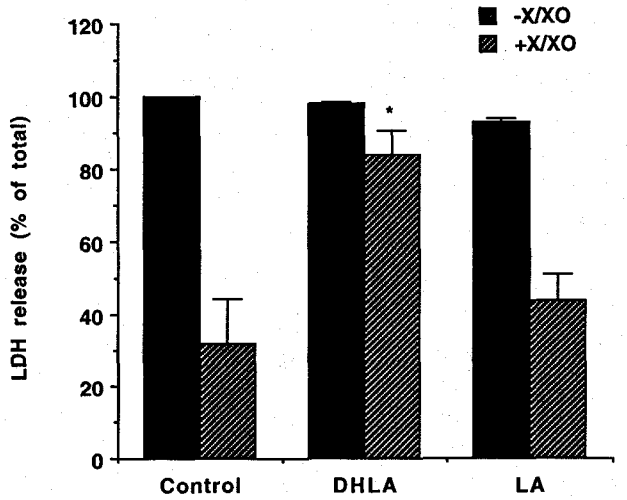

Fig. 4. Protective effects of DHLA on xanthine/xanthine oxidase-evoked neurotoxicity. Cells were treated for 18 h with DHLA or LA in the presence and absence of xanthine (X, 200 μM)/xanthine oxidase (XO, 20 mU/ml). Data are expressed as % change compared with the amount of LDH released by a 24 h incubation with DMEM containing 1 mM NMDA and 1 μM glycine. N=4. *$P<0.05$; compared with the LDH release from NMDA-treated control group, analyzed with analysis of variance [ANOVA] followed by Scheffe's multiple comparison of means.

DHLA and LA buffer oxidative stress

Superoxide has been shown to be generated in vitro by a mixture of xanthine and xanthine oxidase. DHLA prevented 77±7.3% neuronal death caused by xanthine (200 μM)/xanthine oxidase (20 mU/ml) (Fig. 4). In contrast, LA did not alter the superoxide-induced neuronal death (Fig. 4).

Glutathione is a key antioxidant combating oxidative stress (Meister, 1991; Shan et al, 1990; Sies, 1993). L-buthionine-(S,R)-sulfoximine, a γ-glutamylcysteine synthetase inhibitor, significantly induced the death of cortical neurons. Both DHLA and LA prevented the cortical neuronal death evoked by glutathione depletion (Fig. 5).

DISCUSSION

Excess increases in $[Ca^{2+}]_i$ have been associated with various neurodegenerative diseases including cerebral ischemia. NMDA receptors have many different allosteric modulatory sites including the sites modulated by the reagents having redox potentials

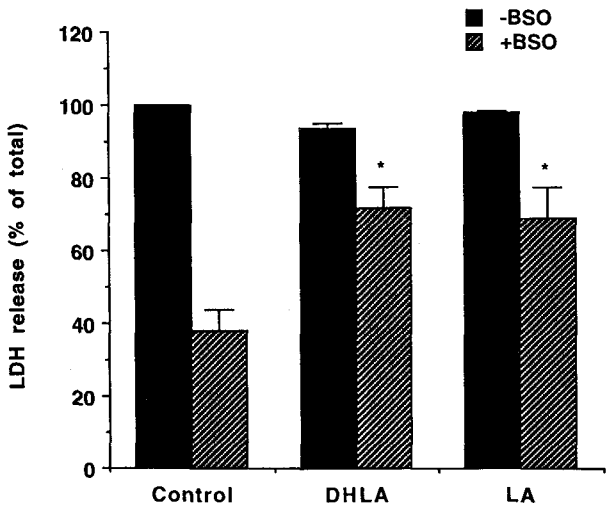

Fig. 5. Protective effects of DHLA and LA on glutathione depletion-induced neurotoxicity. Cells were treated for 18 h with DHLA or LA in the presence and absence of 1 mM BSO. Data are expressed as % change compared with the amount of LDH released by a 24 h incubation with DMEM containing 1 mM NMDA and 1 μM glycine. N=4. *P<0.05; compared with the LDH release from NMDA-treated control group, analyzed with analysis of variance [ANOVA] followed by Scheffe multiple comparison of means.

(Aizenman et al, 1989; Lazarewicz et al, 1989; Levy et al, 1990; Reynolds et al, 1990). A recent report identified the site of the redox-modulation of the NMDA receptor by showing two cysteine residues in the rat hippocampal NMDA R1 subunit (Cys-744 and Cys-798) mediating redox-modulation of the NMDA receptor-channel complex (Sullivan et al, 1994). Via this site, the reducing agent such as DTT potentiated NMDA-evoked calcium responses, whereas the oxidizing agent DTNB reversed the effect of DTT (Aizenman et al, 1989; Lazarewicz et al, 1989; Levy et al, 1990; Reynolds et al, 1990)

Endogenous antioxidants such as DHLA and LA may modulate the activity of NMDA receptor, possibly through the redox modulatory sites. Under the present experimental conditions, DHLA enhanced the NMDA-mediated rises in $[Ca^{2+}]_i$. However, DHLA did not alter the NMDA receptor-mediated neuronal death. The neurotoxic effect of DHLA by increasing NMDA receptor-ion channel activity could be concurrently cancelled out by its neuroprotective antioxidant effect.

In contrast to DHLA, the oxidizing agent LA did not alter the NMDA-evoked calcium responses. Similarly, LA did not alter the NMDA-evoked neurotoxicity. After a brief and prior exposure to DTT or DHLA, however, LA decreased the NMDA calcium responses. These data may indicate that the redox modulatory sites of NMDA receptors possess fully oxidized free sulfhydryl groups. In fact, the well-known strong oxidizing agent DTNB also did not decrease the NMDA calcium responses below the control NMDA responses (Fig. 1A). Furthermore, in pathophysiological conditions redox-modulatory sites of NMDA receptors are thought to be fully oxidized due to environmental oxidative stress.

In the present study, DHLA, but not LA, protected neurons from the superoxide generated by xanthine/xanthine oxidase. This result may be consistent with previous reports on the scavenging effects of DHLA on superoxide ($O_2^{\cdot -}$) (Suzuki et al, 1991): DHLA was reported to be very effective for elimination of both superoxide ($O_2^{\cdot -}$)and hydroxyl radical ($OH^{\cdot}$). In contrast, LA has been shown to effectively eliminate $OH^{\cdot}$, but not $O_2^{\cdot -}$ in the milieu.

Glutathione (GSH) plays a critical role in buffering the cellular oxidative stress. Inhibition of glutathione synthesis in rats has been shown to enlarge mitochondria in the brain (Jain et al, 1991). Inherited deficiencies of GSH synthesis in man causes severe

brain damage (Meister & Larsson, 1989). Glutathione scavenges oxygen free radicals and is a key factor in recycling other antioxidants (Meister, 1991; Shan et al, 1990; Sies, 1993). Therefore, glutathione depletion may allow the oxidative species normally produced by mitochondria to damage cellular constituents. DHLA is a strong reducing agent with a standard reduction potential of 320 mV and can chemically reduce extracellular cystine to cysteine (Han et al, 1997; Sen et al, 1997). As a result, DHLA may prevent the oxidative stress via supplying cysteine for restoring GSH synthesis inside the cell. LA is likely to be reduced into DHLA both outside and inside the cell after it is favorably taken up (Packer, 1992; Handelman et al, 1994). In this pattern, the oxidizing effect of LA could be cancelled out by the reducing one of DHLA converted metabolically from LA. This may explain the previously-reported neuroprotective effect of LA on striatal lesion caused by stereotaxic injection of NMDA (Greenamyre et al, 1994).

In summury, DHLA and LA can alter the activity of NMDA receptor-ion channel complex via its redox-modulatory site(s). However, the alteration of NMDA calcium responses alone appear not to be much responsible for the NMDA receptor-mediated neurotoxicity. Both oxygen free radical formation and $[Ca^{2+}]_i$ rises must be taken into consideration to prevent the hypoxia/ischemia-induced neurotoxicity. The effects of LA and DHLA on NMDA receptor activity and oxidative stress are complicated by the metabolic interconversion between the two thiol reagents. In toto, both thiol antioxidants used in the present study appear to have therapeutic potential in protecting against neurological injuries involving glutamate and oxidative stress.

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