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Effects of Ascorbic and Dehydroascorbic Acids on Apoptotic Cell Death in Hippocampal Slice Culture

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ABSTRACT

Ascorbic acid (AA) and dehydroascorbic acid (DHA) are known to have protective effects in experimental central nerve system disorder models such as stroke, ischemia, and epileptic seizures. The present study was conducted to examine the protective effect of AA and DHA on kainic acid (KA) neurotoxicity using organotypic hippocampal slice cultures (OHSC). Protective effects of AA and DHA on KA-induced cell death were evaluated by analyzing caspase-3. In addition, to determine if the prooxidant effect of AA is related to iron, the effect of AA on cell death was examined using desferrioxamine (DFO), an iron chelator. After 12h-KA treatment, significant delayed neuronal death was detected in CA3 region, but not in CA1. The AA (500 μM) and DHA (100 and 500 μM) pretreatments significantly prevented cell death by inhibiting caspase-3 activation in CA3 region. In the concentration of 1,000 μM, however, AA pretreatment might have prooxidant effect, but AA-induced oxidative reaction is mainly not related to transition metal ions. These data showed that the pretreatments of intermediate-dose AA and DHA protected KA-induced neuronal damage in OHSCs and co-pretreatment of AA and DFO did not affect cell death except for a few cases. These data suggest that both AA and DHA pretreatment have antioxidant or prooxidant effect depending on doses treated on KA-induced neuronal injury and the possible prooxidant effect of AA may not depend on the Fenton reaction.

Key words: ascorbic acid, dehydroascorbic acid, kainic acid, organotypic hippocampal slice culture, caspase-3

INTRODUCTION

It has been shown that excitotoxicity may contribute to neuronal death associated with a number of central nervous system (CNS) insults or disorders (Choi, 1987; Mattson et al., 2000). Kainic acid (KA), an agonist for kainate and α-amino-3-hydroxy-5-methyl-4-isoxasole-proprionic acid (AMPA) receptors, is an excitotoxin in the hippocampus (Monaghan and Cotman, 1987). Seizure activity is induced by KA results in the selective degeneration

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of vulnerable neuronal populations in limbic system including hippocampal formation and the pyriform cortex (Tanaka et al., 1992; Bruce and Baudry, 1995).

Ascorbic acid (AA) is a potent antioxidant and accumulates in the brain at high concentration (Rice, 2000). It is well known that AA protects the brain against injuries resulting from ischemia and excitotoxicity (Majewska et al., 1990; MacGregor et al., 1996; Stamford et al., 1999). However, the role of AA in protecting against oxidative stress is controversial because AA also has prooxidant natures (Carr and Frei, 1999). Dehydroascorbic acid (DHA) is an oxidized form of AA. It has been shown that DHA treatment circumvents the prooxidant effects of AA (Puskas et al., 2000).

Recently, we observed that pretreatment with intermediate doses of AA and DHA have dose-dependent neuroprotective effects on KA-induced neuronal injury by inhibiting reactive oxygen species (ROS) generation and mitochondrial dysfunction (Kim et al., 2008). The present study was conducted to assess the possible involvement of apoptosis in these processes, using organotypic hippocampal slice cultures (OHSC). In addition, the effect of AA on cell death was examined using an iron chelator in order to determine if the prooxidant effect of AA is related to iron.

MATERIALS AND METHODS

Preparation and maintenance of OHSC

All animal experiments were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine. OHSC were prepared by the method of Stoppini et al (1991). Briefly, 7-day-old Sprague-Dawley rat pups were killed by instant decapitation, without anesthesia, and the two hippocampi were rapidly dissected out in an ice cold dissection medium consisting of minimum essential medium (MEM, Gibco, Grand Island, NY, USA) with 25 mM HEPES (Sigma, Saint Louis, Missouri, USA) and 4 mM L-gultamine (Gibco, Grand Island, NY, USA). Transverse section 350 μm thick was cut on a McIlwain tissue chopper (Mickle Laboratory Engineering Ltd, Surrey, UK). Slices were placed on top of Millicell- CM Tissue Culture Inserts (0.4 μm pore, Millipore, Billerica, MA, USA) in six-well plate and maintained at 35°C in a humidified incubator with 5% CO_2 for 10～14 days in a culture medium composed of 50% MEM, 25% heat-inactivated horse serum (Gibco, Grand Island, NY, USA), 25% Hanks's balanced salt solution (HBSS, Gibco, Grand Island, NY, USA), and 25 mM HEPES, supplemented with penicillin-streptomycin (50 mg/ml, Gibco, Grand Island, NY, USA) and D-glucose (5.5 g/L, Sigma, Saint Louis, MO, USA). pH was adjusted to 7.3 by using 5 mM Tris and 4 mM $NaHCO_3$. The medium was changed on the first day after culture and every 3～4 days subsequently. Before study, slices were examined under the light microscope and ones showing degeneration were excluded from experiments.

Drug treatment

KA (5 μM) was applied for 12 h after mature cultures were incubated in serum-free culture medium overnight because serum may protect against excitotoxicity in cortical neuron (Dux et al., 1992). After KA treatment, cultures were allowed to recover for 48 h in fresh serum-free medium. AA (Sigma, Saint Louis, MO, USA) and DHA (Sigma, Saint Louis, MO, USA) were dissolved in 0.1 M phosphate buffered saline (PBS). Cultures were pretreated with AA or DHA at different concentrations for 1 h before KA treatment (Fig. 1). According to our previous study (Kim et al., 2008), 500 μM AA and 100 and 500 μM DHA were chosen to be used

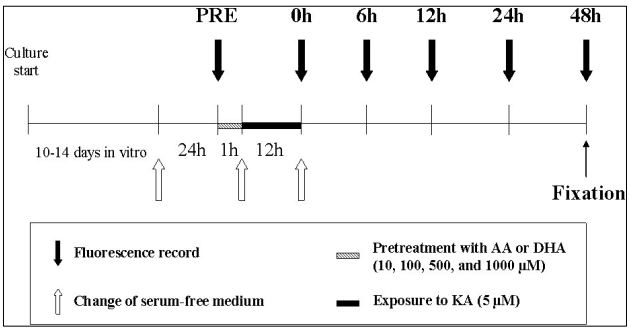

Fig. 1. Experimental paradigm. OHSC were incubated for 10～14 days in vitro. Culture were then transferred to wells containing serum-free medium before study and pretreated with ascorbic acid (AA) or dehydroascorbic acid (DHA) at different concentrations for 1 h before KA (kainic acid, 5 μM) treatment. Slices were exposed to KA for 12 h. Fluorescence images were recorded before AA and DHA treatment (PRE) and at 0, 6, 12, 24 and 48 h of recovery time after withdrawal of KA. Cultures were fixed in 4% paraformaldehyde for 6 h after 48 h of recovery time.

as intermediate doses. To determine if the prooxidant effect of AA was related to iron, cells were co-pretreated with desferrioxamine (DFO), an iron chelator, and AA for 1 h before KA treatment.

Assessment of neuronal injury

Neuronal injury was assessed by using a fluorescence cell death marker propidium iodide (PI, Sigma, Saint Louis, MO, USA) that is a stable dye. Normally, PI is a polar compound which only enters dead or dying cells with a damaged or leaky cell membrane. Inside the cell it binds to nucleic acids and produces a bright red fluorescence. PI was added to the medium 24 h prior to the experiments. Images of PI-labeled cells were captured with a digital camera under a fluorescence microscope (Model BX-51, Olympus, Tokyo, Japan) and quantified by using the MetaMorph Imaging system (Universal Image Co., Downingtown, PA, USA).

Immunohistochemistry

Immunocytochemical expression of active caspase-3 was used as a marker for caspase-3 dependent apoptosis. Common pathway of apoptosis which is mediated through oxidative stress is the activation of caspases. Capase-3 is potent effecter of apoptosis, and cleaves specific aspartate residues in a variety of structural, housekeeping, and regulatory proteins (Thornberry and Lazebnik, 1998). Culture medium was sucked off after 48 h of recovery. Inserts were briefly washed three times with cold PBS followed by fixation with 4% cold paraformaldehyde in PBS for 6 h at 4°C and if not immediately processed, placed in 25% sucrose solution for 72 h. After rinsing three times with cold PBS, the slices were carefully detached from inserts and transferred to 24 well plates containing permeabilization buffer (0.5% Triton X-100 in PBS) for 1 h, and then washed with PBS. Non-specific binding sites were blocked by treatment with Blotto solution (5% skim milk, 1% horse serum, 0.02% Azide in 0.05 M Tris-saline) for 1 h, and slices were incubated with primary antibody, monoclonal mouse anti-caspase-3 (1 : 50, Santa Cruz Biotechnology, Santa Cruz, CA, USA), at 4°C overnight. Primary antibodies were then removed, and slices were washed two times for 10 min in PBS before being incubated for 1 h in secondary antibody, FITC donkey anti-mouse IgG (1 : 250, Jackson, West Grove, PA, USA). They were washed two times for 10 min with PBS, and then transferred to glass slides and cover-slipped using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Images were acquired with a fluorescence microscope (Model BX-51, Olympus, Tokyo, Japan).

Statistical analysis

Data were presented as the mean±standard error of the mean (S.E.M.). Differences among groups were assessed by one-way ANOVA followed by Dunnett's post-hoc multiple comparisons or paired two-tailed Student's t-test as appropriate. In all cases, a p value less than 0.05 was considered significant.

RESULTS

KA neurotoxicity in OHSC

An initial experiment was to determine relationship of KA and neuronal death in OHSC. To exhibit the temporal development of cell death following 12 h-exposure to KA (5 μM), representative PI fluorescence images of dead cells captured at 0 and 48 h recovery times are shown in Fig. 2. In untreated slices, no noticeable PI fluorescence was observed. The treatments of slices with KA resulted in neuronal death which showed a selective uptake of PI fluorescence in CA3 region, while faint PI staining

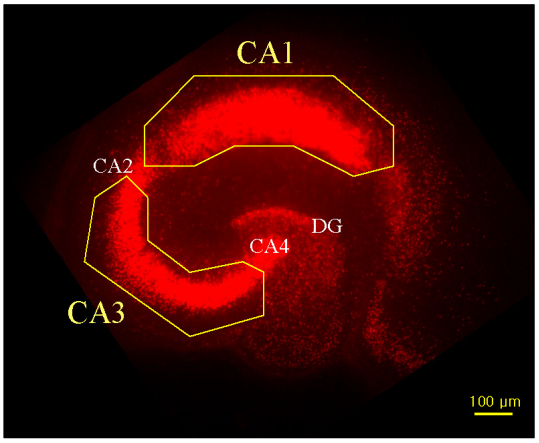

Fig. 2. A fluorescence image showing the standardized areas for measuring propidium iodide (PI) -incorporated area in CA1 and CA3 regions. CA1: cornu ammonis 1 region. CA2: cornu ammonis 2 region. CA3: cornu ammonis 3 region. CA4: cornu ammonis 4 region. DG: dentate gyrus.

was observed in CA1 region after 0 and 48 h of recovery times. The PI-staining area significantly continued to increase until 48 h of recovery time in CA3 region rather than CA1.

Attenuation of KA-induced caspase-3 activation by AA and DHA

Caspase-3 activation following AA and DHA pretreatments and KA exposure was studied using caspase-3 antibody (Fig. 3). In the control, there was a weak and diffuse background immunostaining over the hippocampus (Fig. 3A). In addition, cultures stained at 48 h of recovery time showed immunostaining in pyramidal neurons of CA3 region in every group (Fig. 3A). Caspase-3 fluorescence signals in CA3 region was, however, widely and remarkably seen only in KA -treated group compared with the other groups (Fig. 3A). In the case of 500 μM AA, and 100 and 500 μM DHA pretreatment groups, caspase-3 fluorescence was a few and condensed in CA3 region than KA -treated group (Fig. 3A). Furthermore, KA -treated group increased caspase-3 levels by almost 10-fold, and 500 μM AA, and 100 and 500 μM DHA pretreatment groups reduced the KA-induced caspase-3 activation by almost 4-fold compared to control. Moreover, caspase-3 activation which induced by KA exposure were significantly inhibited by 500 μM AA, and 100 and 500 μM DHA pretreatment. In summary, intermediated-dose AA and DHA prevented KA-induced apoptosis (Fig. 3B).

Effects of co-pretreatment with AA and DFO on KA-induced cell death

Hyrogen peroxide (H_2O_2) and lipid hydroperoxide (LOOH) can be changed to the hydroxyl radical

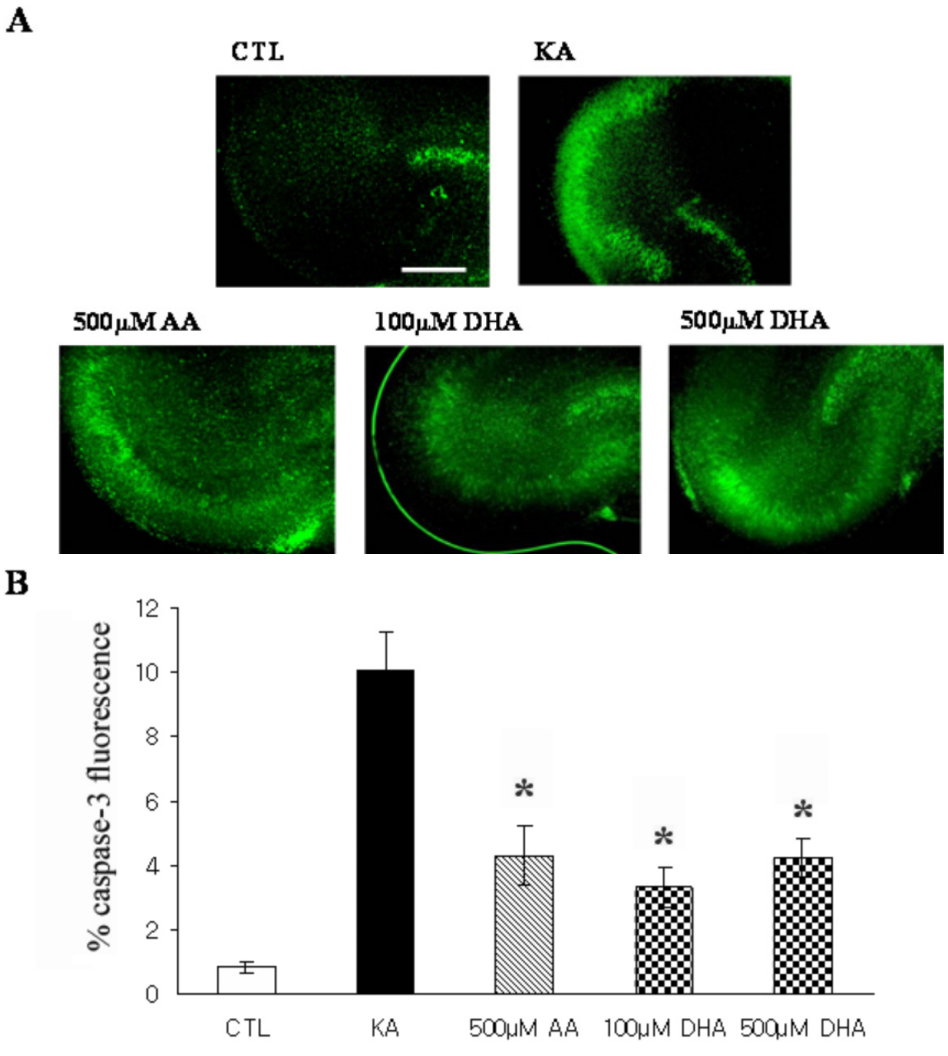

Fig. 3. The effect of AA and DHA on KA-induced change in level caspase-3. (A) Photographs of immunoreactivity of capase-3 at 48 h of recovery following pretreatment with AA or DHA and withdrawal of exposure to KA in CA3 region. (B) Quantification of caspase-3-fluorescence images in CA3 region. Data are shown as mean±S.E.M., with n=10 for CTL, n=13 for KA and 500 μM DHA, n=9 for 500 μM AA, and n=12 for 100 μM DHA. *$p<$ 0.001 vs. KA. Scale bar, 200 μm.

(HO・) and alkoxyl radical in the presence of Fe^{2+} by Fenton reaction. AA is a physiologic reductant that reduces Fe^{3+} to Fe^{2+}. If the presence of chelator can prevent Fe^{3+} reduction by AA, available Fe^{2+} for peroxide reduction would be diminished. It is well known that DFO, a hydroxamate chelator that forms a 1 : 1 complex with Fe^{3+}, prevents AA-mediated reduction of Fe^{3+} (Dean and Nicholson, 1994). To determine if the aggravating effects of AA on KA-induced cell death were caused by the Fenton reaction, slices were pretreated with 1,000 μM AA and 300 μM DFO, an iron-chelator, before KA exposure (Fig. 4). Co-pretreatment with AA and DFO reduced the area of PI uptake in pyramidal cells at 0 h of recovery time in CA3 region than KA only and AA pretreament, but did not at 48 h of recovery time (Fig. 4A). In this study, co-pretreatment with AA and DFO significantly inhibited the aggravating effects of AA on KA-induced cell death at 0 and 6 h after KA-treatment, but it did not affect cell death at 12, 24 and 48 h of recovery time (Fig. 4B). Therefore, high-concentration of AA may have a prooxidant rather than an antioxidant effect, because it could not prevent from cell death after KA exposure. This prooxidant effect of AA may not depend on the Fenton reaction, because co-pretreatment of AA and DFO did not affect cell death except for a few cases.

DISCUSSION

In the present study, the neuronal damage in CA3 was prominent during recovery time in fresh medium after 12 h of KA exposure. The mechanisms by which the cell death is augmented after the withdrawal of KA are not known, but Lahtinen et al. (2001) recently reported that excitotoxicity exerts its effects on vulnerable neurons in a direct manner as well as an enhancement of indirect network excitability and finally leads to progressive cell death. Therefore, this delayed neuronal loss after KA withdrawal may be caused by pathological activation of the intrahippocampal network.

The following hypothesis is proposed to explain the excitotoxicity of KA. KA neurotoxicity results in the activation of presynaptic kainate receptors and the release of endogenous glutamate (Ferany et al., 1982; Coyle and Puttfarcken, 1993). The released glutamate then acts postsynaptically on N-methyl-D-aspartate (NMDA) receptors which can contribute to the neuronal damage (Lafon-Cazal et al., 1993; Vajda, 2002). There is excessive calcium entry which

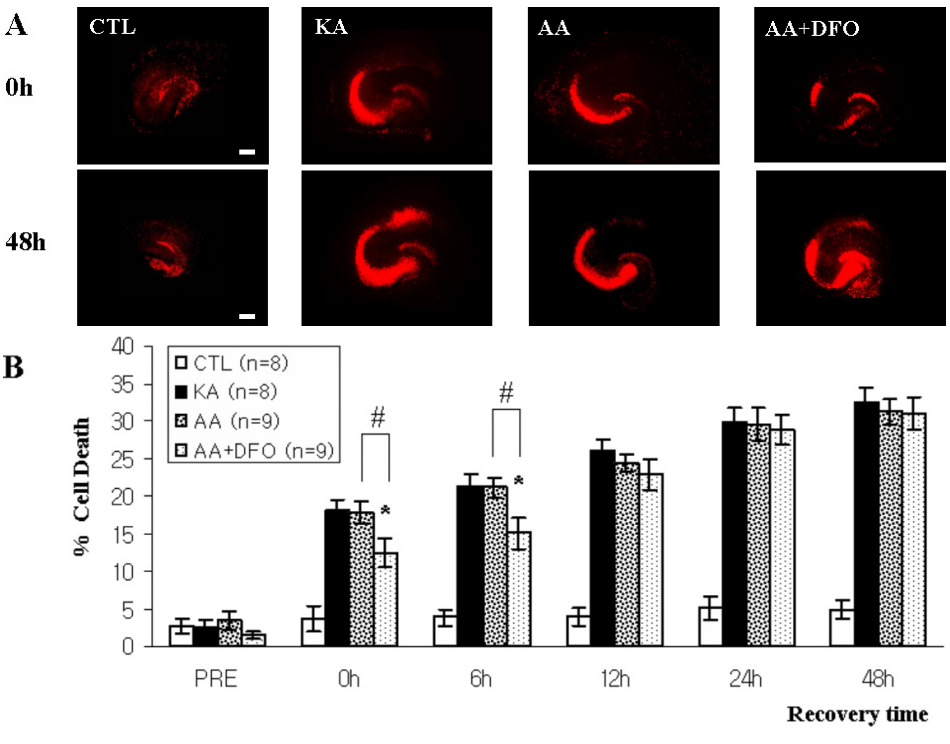

Fig. 4. Effects of co-pretreatment with AA and desferrioxamine (DFO) on KA-induced cell death. (A) Representative PI fluorescence images of OHSC at 0 h and 48 h of recovery following AA or DFO pretreatment and withdrawal of KA exposure. (B) Quantification of PI-incorporated area in CA3 region. Date are show as mean±S.E.M., with n=8 for CTL and KA, and n=9 for 1,000 μM AA, and 1,000 μM AA+DFO. *$p<0.05$ vs. KA, #$p<0.05$ vs. AA. Scale bar, 200 μm.

is accumulated by intracellular mitochondria, and causes mitochondrial membrane potentials (MMPs) collapse and reactive oxygen species (ROS) generation, resulting in cytochrome C release that may induce apoptosis by activating caspase (Nichols and Budd 1998; Liu et al., 2001). Several lines of recent evidence suggest that ROS plays a cardinal role in the pathogenesis of excitoxic cell death (Coyle and Puttfarcken, 1993; Nichols and Budd 1998). Liang et al. (2000) reported that systemic kainate administration specifically increases mitochondrial superoxide (O_2^-) radical production. Another in vitro study has been shown to cause the formation of free radical generation in cultured retinal neurons injured by kainite (Dutrait et al., 1995). Furthermore, KA-induced neuronal damage can be prevented by certain antioxidants (Puttfarcken et al., 1993; MacGregor et al., 1996).

Our data demonstrated that AA and DHA pretreatment can afford protection against KA exposure in OHSC. To determine the protective effects of AA and DHA on cell death, slices were measured caspase-3 activation. Activation of caspase-3 is an important event during apoptosis. Apoptotic cell death following exposure to KA was significantly prevented by 500 μM AA and 100 and 500 μM DHA pretreatment in OHSC. AA and DHA pretreatment with intermediated-dose inhibited caspase-3 activation.

AA is a well-known antioxidant with reducing activity and is involved in several types of protective mechanisms. AA is also known to act as prooxidant, but the mechanism of AA-induced oxidative action and apoptosis is not established. A biphasic effect of AA, anti- oxidant or pro-oxidant has been shown by Carr and Frei (1999). It was believed that AA-induced oxidative reaction is due to interaction between ferrous ion and AA. AA can reduce Fe^{3+} to Fe^{2+} and can produce free HO· by Fenton reaction, which has a very high oxidative potential (Carr and Frei, 1999). According to other reports, however, AA-induced oxidative stress is not caused by interaction between AA and transition metal ions (Chen et al., 2000). It demonstrated that AA acts as an antioxidant even in presence of ion overload. Kim et al. (2005) reported that AA and DFO co-pretreatments could inhibit the aggravating effect of AA on H_2O_2-induced oxidative cell death in the early stage, but not in the later phase. In the present study, although co-pretreatment with 1,000 μM AA and DFO in slices could prevent KA-induced cell death at 0 and 6 h of recovery, it could not prevent at 12, 24 and 48 h of recovery. Therefore, AA-induced oxidative action is mainly not related to transition metal ions.

DHA has been used to circumvent the prooxidant effects of AA with Fenton reaction. In the present study, 100 and 500 μM DHA inhibited KA-induced caspase-3 activation. Intermediated-dose of DHA, therefore, has protective effect against KA-induced cell death in OHSC. On the other hands, 1,000 μM DHA pretreatment did not prevent KA-induced neuronal damage. Song and coworkers demonstrated that AA and DHA induced lipid peroxide production and cell death at high concentration in PC12 cell and liver slices (Song et al., 2001a; Song et al., 2001b; Song et al., 2003). The paradoxical oxidative action of AA is due to a serial redox reaction (Song et al., 2001a; Song et al., 2001b). AA is oxidized to DHA and rapidly carried into cells by facilitative glucose transporter in the form of DHA, and then the cytosolic DHA is reduced back to AA by the action of DHA reductase (Vera et al., 1993; Meister, 1994; Wilson, 2002). The reduction process generates oxidative stress, which decreases quantities of cellular reducing agents such as GSH, and increases quantities of oxidized cellular components such as lipid peroxide (Song et al., 2001a; Song et al., 2001b). Rapid and high uptake of DHA is a critical factor to generate oxidative stress and to damage on vital cellular compound (Song et al., 2001a).

In the present study, intermediate-dose AA and DHA pretreatment prevent KA-induced apoptotic cell death. These data suggest that both AA and DHA pretreatment have antioxidant or prooxidant effect depending on the doses treated on KA-induced neuronal injury. In addition, co-pretreatment of AA and an iron chelator did not affect cell death except for a few cases. This leads to indicate the possible prooxidant effect of AA may not depend on the Fenton reaction.

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First two authors equally contributed to this work.

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