Antioxidant effect of ascorbic acid and dehydroascorbic acid on kainate-induced neural injury in organotypic hippocampal slice cultures

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Antioxidant effect of ascorbic acid and dehydroascorbic acid on kainate-induced neural injury in organotypic hippocampal slice cultures

Directed by Professor Bae Hwan Lee

The Master's Thesis submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Medical Science

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December 2006

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저자 씀

TABLE OF CONTENTS

ABSTRACT---------- 1

I. INTRODUCTION---------- 3

II. MATERIALS AND METHODS---------- 6

1. Preparation and maintenance of OHSC---------- 6
2. Drug treatment---------- 7
3. Assessment of neuronal injury---------- 8
4. Evaluation of intracellular ROS formation---------- 9
5. Assay of mitochondrial dysfunction---------- 10
6. Immunohistochemistry---------- 10
7. Statistical analysis---------- 11

III. RESULTS---------- 12

1. KA neurotoxicity in OHSC---------- 12
2. Effects of AA and DHA on cell death induced by KA---------- 14
3. Effects of AA and DHA on ROS generation induced by KA---------- 17

4. Effects of AA and DHA on mitochondrial dysfunction induced by KA---------- 19
5. Attenuation of KA-induced caspase-3 activation by AA and DHA---------- 21
6. Effects of co-pretreatment with AA and DFO on KA-induced cell death---------- 23

IV. DISCUSSION---------- 25

REFERENCES---------- 29

국문요약---------- 34

LIST OF FIGURES

Fig. 1. Photographs of organotypic hippocampal slice cultures (OHSC)---------- 7
Fig. 2. Experimental paradigm---------- 8
Fig. 3. A fluorescence image showing the standardized areas for measuring propidium iodide (PI) -incorporated area in CA1 and CA3 regions--------- 9
Fig. 4. KA-induced neuronal death in OHSC---------- 13
Fig. 5. Fluorescence images of the effects of AA and DHA on KA-induced PI uptake in OHSC---------- 15
Fig. 6. Quantification of the effects of AA and DHA on KA-induced PI uptake in OHSC---------- 16
Fig. 7. Effects of AA and DHA on reactive oxygen species (ROS) production---------- 18
Fig. 8. Effects of AA and DHA on mitochondrial membrane potentials---------- 20
Fig. 9. Effect of AA and DHA on KA-induced change in level caspase-3---------- 22
Fig. 10. Effects of co-pretreatment with AA and desferrioxamine (DFO) on KA-induced cell death---------- 24

ABSTRACT

Antioxidant effect of ascorbic acid and dehydroascorbic acid on kainate-induced neural injury in organotypic hippocampal slice cultures

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(Directed by Professor Bae Hwan Lee)

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. Initially it was thought that binding KA to KA receptor would spur an intracellular influx of calcium ions and increase mitochondrial free radical generation. It was believed that large amounts of reactive oxygen species (ROS) would be produced and may trigger apoptotic cell death. Ascorbic acid (AA) generally functions as an antioxidant known to quench ROS and accumulates in the brain at a much higher concentration than it does in any other tissues. It also has prooxidant properties, however, in the presence of free transition metals. Dehydroascorbic acid (DHA), an oxidized form of AA, has been used as a substitute for AA because of the prooxidant effects of AA. AA and DHA have been shown recently to have protective effects in experimental central nerve system (CNS) disorder models such as stroke, ischemia, and epileptic seizures. The present study examined the protective effect of AA and DHA in KA neurotoxicity using organotypic hippocampal slice cultures

(OHSC). To determine the protective effects of AA and DHA on KA-induced cell death, we measured ROS level, mitochondrial dysfunction, caspase-3 activation. In addition, to determine if the prooxidant effect of AA is related to iron, the effect of AA on cell death was examined using an iron chelator. After 12h KA treatment, significant delayed neuronal death was detected in CA3 region, but not in CA1. AA (500 μM) and DHA (100 and 500 μM) pretreatment significantly prevented cell death and inhibit ROS level, mitochondrial dysfunction and capase-3 activation in CA3 region, but the rest of groups did not. In the case of 1000 μM, however, AA pretreatment might be thought to have prooxidant effect, but AA-induced oxidative action is mainly not related transition metal ions. These data showed that intermediate-dose AA and DHA pretreatment protect KA-induced neuronal damage in OHSCs, but high-dose AA and DHA pretreatment did not prevent KA-induced cell death, because they have prooxidant effects. These data suggest that both AA and DHA pretreatment have antioxidant or prooxidant effects according to level of dose on KA-induced neuronal injury.

Key Words: antioxidants, ascorbic acid, dehydroascorbic acid, kainic acid, reactive oxygen species, organotypic hippocampal slice culture

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I. INTRODUCTION

Excitotoxicity is widely considered to be a contributing factor in neuronal death associated with a number of central nervous system (CNS) insults or disorders, including hypoxia, ischemia, and epileptic seizures.[1,2] 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.[3] KA-induced seizure activity results in the selective degeneration of vulnerable neuronal populations in limbic structures, including the CA3 and CA4 areas in hippocampal formation and the pyriform cortex.[4,5]

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.[6,7] The released glutamate then acts postsynaptically on N-methyl-D-aspartate (NMDA) receptors which can contribute to the neuronal damage.[8,9] There is excessive calcium entry which 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.[10,11] Several lines of recent evidence suggest that ROS plays a cardinal role in the pathogenesis of excitoxic cell death.[6,10] Liang et al.[12] 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 kainate.[13] Furthermore, KA-induced neuronal damage can be prevented by certain antioxidants.[14,15]

Ascorbic acid (AA) is a well known a potent antioxidant and accumulates in the brain at a much higher concentration than it dose in any other organs.[16] However, within the brain, AA levels are not homogeneous, with the highest levels being found in the amygdala, hippocampus and hypothalamus.[14] Neuroprotection by AA has been demonstrated in several recent studies, both in vitro and in vivo. AA protects the brain against injury resulting from ischemia and excitatory amino acid toxicity.[14,17,18] The role of AA in protecting against oxidative stress is controversial because AA also has prooxidant natures in the presence of free transition metals in vitro.[19] Several studies have shown that AA induced lipid peroxide production and cell death in cortical slices[20] or PC12 cells.[21] Therefore, the antioxidant or prooxidant properties of AA are different according to its concentration, experimental condition and cell types.

Dehydroascorbic acid (DHA) is an oxidized form of AA. It has been shown that DHA treatment circumvents the prooxidant effects of AA.[22] DHA are taken up by glucose transporter[23] and is regenerated into AA at the expense of the glutathione.[24] Pathological conditions that inhibit DHA recycling may decrease AA concentrations and thereby impair AA-dependent enzymatic and antioxidant activities.[25] DHA administration has resulted in normalization of oxidative stress markers and inflammation in hyperglycemic stroke models.[26] In vitro, DHA inhibited mitochondrial damage and cell death against oxidative injury.[22,27]

Organotypic hippocampal slice cultures (OHSC) have desirable advantages for examining hippocampal function by maturation of synapses, receptors, and intrinsic fiber pathways for a number of weeks in a well-controlled in vitro environment.[28,29] These cultures can apply experimental manipulation that is not possible in vivo such as allowing a precise concentration and time of drugs or factors and visualizing cell morphology and function, using fluorescent markers/probes within the same cultures for long-term periods.[28] OHSC were prepared from postnatal day 5-10 rats and have mature period in vitro for 2 weeks because it was shown the response of adult hippocampus by excitotoxic damage.[30]

The aim of the present investigation was firstly to assess the protective effect of AA and DHA according to different concentration on KA-induced neuronal death via production of ROS using OHSC and secondly to assess the possible involvement of a loss of MMPs and apoptosis in these processes.

II. MATERIALS AND METHODS

1. 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.[31] In briefly, 7-day-old Sprague-Dawley rat pups were killed by instant decapitation, without anesthesia, and the two hippocampi 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℃ 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 addition of 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 (Fig. 1).

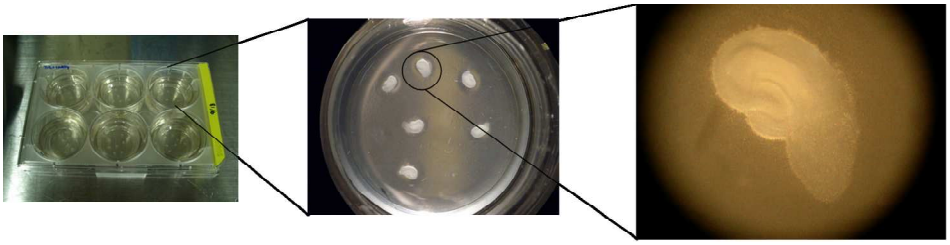

Fig. 1. Photographs of organotypic hippocampal slice cultures (OHSC).

2. Drug treatment

KA (5 μM) was applied for 12 h after mature cultures were incubated in serum-free culture medium overnight because Dux et al.[32] reported that serum may protect against excitotoxicity in cortical neuron. 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. 2). 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.

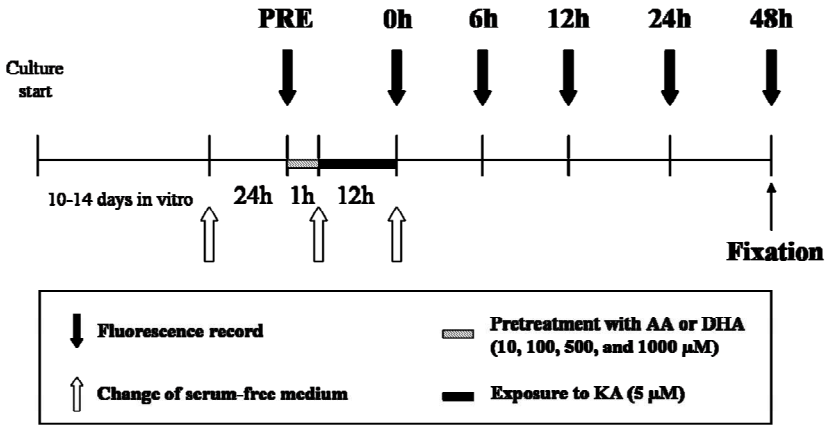

Fig. 2. Experimental paradigm. OHSC were incubated for 10-14 days in vitro. Culture were then transferred to wells containing serum-free medium before study and pretreated with ascorbic acid (AA) or dehydroascorbic acid (DHA) at different concentrations for 1 h before KA (kainic acid, 5 μM) treatment. 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.

3. Assessment of neuronal injury

Neuronal injury was assessed by using the fluorescence cell death marker propidium iodide (PI, Sigma, Saint Louis, MO, USA) that is a very 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 present in the medium from 24 h prior to the experiments and throughout the recovery period. Images of PI-labeled cells (Fig. 3) 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).

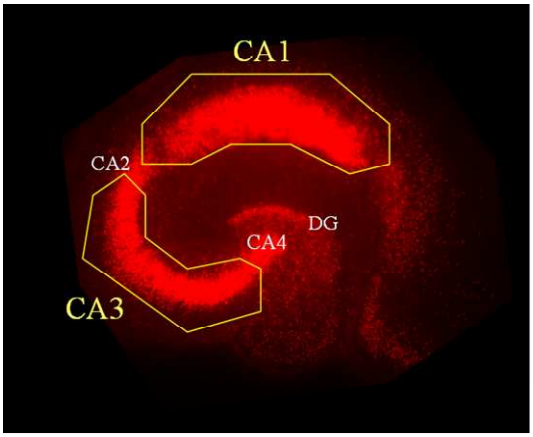

Fig. 3. A fluorescence image showing the standardized areas for measuring propidium iodide (PI) -incorporated area in CA1 and CA3 regions. CA1: cornu ammonis 1 region. CA2: cornu ammonis 2 region. CA3: cornu ammonis 3 region. CA4: cornu ammonis 4 region. DG: dentate gyrus.

4. Evaluation of intracellular ROS formation

Formation of intracellular peroxides was detected using an oxidant-sensing fluorescence probe, 2',7'-dichlorofluorescin diacetate (DCFH-DA, Sigma, Saint Louis, MO, USA). When the DCFH-DA added to cells, it diffuses across the cell membrane and hydrolyzed by intracellular esterases to liberate 2',7'-dichlorofluorescin (DCFH) which, upon reaction with oxidizing species, form its 2-electron oxidation product, the highly fluorescence compound 2',7'-dichlorofluorescein (DCF).[33] Cultures were incubated with 5 μM DCFH-DA at 35℃ for 30 min, and then washed with fresh serum-free medium. The fluorescence DCF was measured using a fluorescence microscope (Model BX-51, Olympus, Tokyo, Japan), and captured by a digital camera. The DCF fluorescence signals were analyzed using the MetaMorph Imaging system (Universal Image Co., Downingtown, PA, USA).

5. Assay of mitochondrial dysfunction

Mitochondrial dysfunction was assayed by measuring MMPs. For examining of MMPs, slice cultures were loaded with the cationic and voltage-sensitive fluorescent dye, rhodamine 123 (R-123, 5 μM, Molecular Probes, Eugene, OR, USA), for 2 min and washed three times with fresh serum-free medium before KA application. Under control conditions, R-123 is retained in mitochondrial matrix according to the Nernst equation.[34] Collapse of MMPs following depolarization of mitochondrial membranes releases R-123. The fluorescence signals were excited at 480 nm, and fluorescence images were recorded at 590 nm using a fluorescence microscope (Model BX-51, Olympus, Tokyo, Japan), and captured by a digital camera. The R-123 fluorescence signals were analyzed using the MetaMorph Imaging system (Universal Image Co., Downingtown, PA, USA).

6. 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.[35] 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℃ and if not immediately processed, placed in 25% sucrose solution for 72 h. After rinsing three times with cold PBS, the slices were carefully mechanically 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℃ 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).

7. Statistical analysis

Data are 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.

III. RESULTS

1. KA neurotoxicity in OHSC

An initial experiment was conducted 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 time are shown in Fig. 4A. In untreated slices, no noticeable PI fluorescence was observed (Fig. 4A). The treatment of slices with KA resulted in neuronal death which showed a selective uptake of PI fluorescence in CA3 region, while faint PI staining was observed in CA1 region after 0 and 48 h of recovery time (Fig. 4A). The PI-staining area significantly continued to increase until 48 h of recovery time in CA3 region (Fig. 4B). However, in the case of CA1 region did not show significant cell death at every recovery time (Fig. 4B).

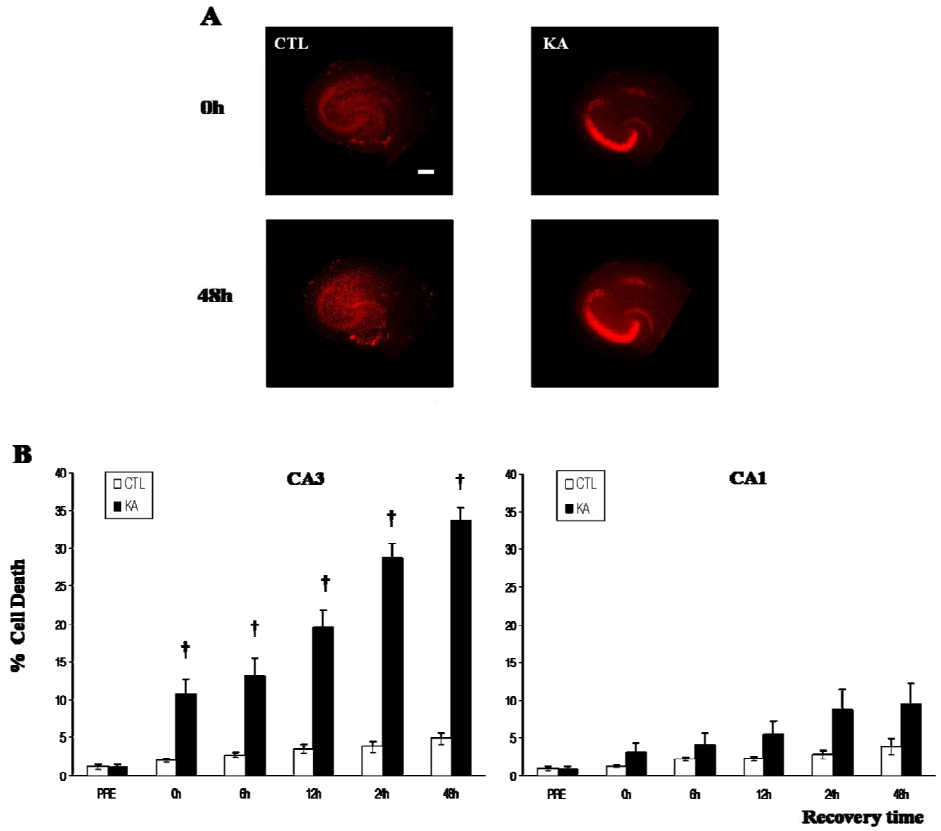

Fig. 4. KA-induced neuronal death in OHSC. A: Representative PI fluorescence images of OHSC at 0 and 48 h of recovery time in fresh medium after 12 h of KA exposure. B: Quantification of PI-incorporated area following withdrawal of exposure to KA in the CA3 and CA1 regions at each recovery time. Data are shown as mean ± S.E.M., with n = 13 in each group. †$p < 0.001$ vs. control. Scale bar, 200 μm.

2. Effects of AA and DHA on cell death induced by KA

The effects of AA and DHA on KA-induced cell death were observed by fluorescence microscopy: representative PI-stained images of increasing dose of AA and DHA (0, 10, 100, 500 and 1000 μM) are shown in Fig. 5. In control cultures, the PI uptake was very low, while in KA only cultures, area of PI-incorporated cell death was broad and deep in CA3 region as compared with the rest of groups (Fig. 5). Pretreatment with AA reduced the area of PI uptake in pyramidal cells at 500 μM in CA3 region (Fig. 5A). The neuroprotective effect of DHA pretreatment appeared at 100 and 500 μM in CA3 region (Fig. 5B). In addition, pretreatment with 500 μM AA significantly prevented cell death after 24 and 48 h recovery in CA3 region, but the rest of groups did not prevent it in same region (Fig. 6A). Furthermore, pretreatment with 100 μM DHA significantly prevented cell death at 12, 24 and 48 h of recovery in CA3 region (Fig. 6B). Moreover, pretreatment with 500 μM DHA significantly reduced cell death at 24 and 48 h of recovery in CA3 region (Fig. 6B). However, low- and high-dose of AA and DHA pretreatment did not prevent cell death at every recovery time. In the case of 1000 μM, therefore, AA and DHA pretreatment might be thought to have prooxidant effect.

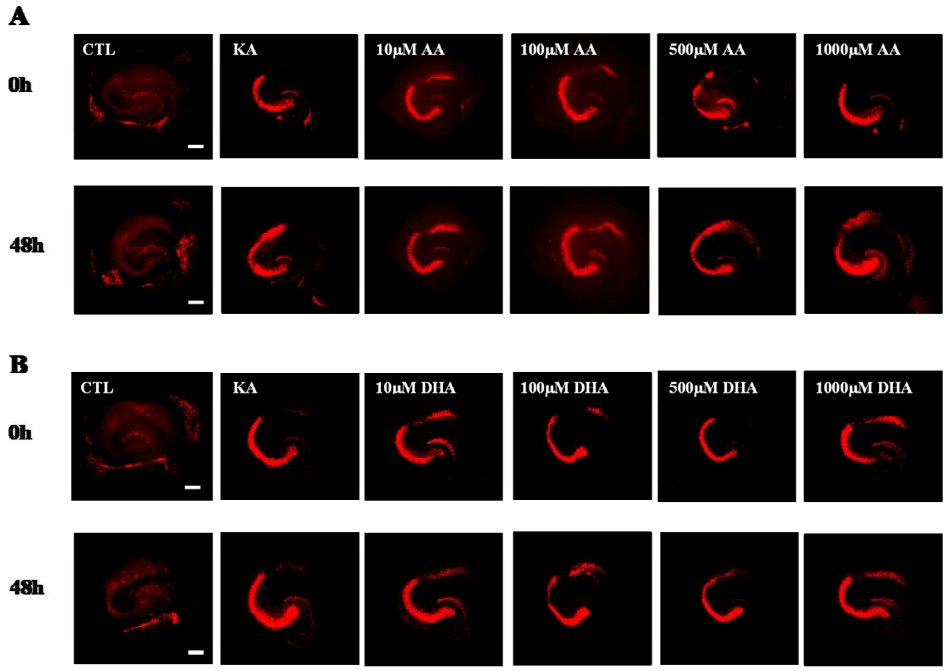

Fig. 5. Fluorescence images of the effects of AA and DHA on KA-induced PI uptake in OHSC. Slices were captured following different concentration of AA (A) or DHA (B) pretreatment and withdrawal of KA exposure at 0 and 48 h recovery time. Scale bar, 200 μm.

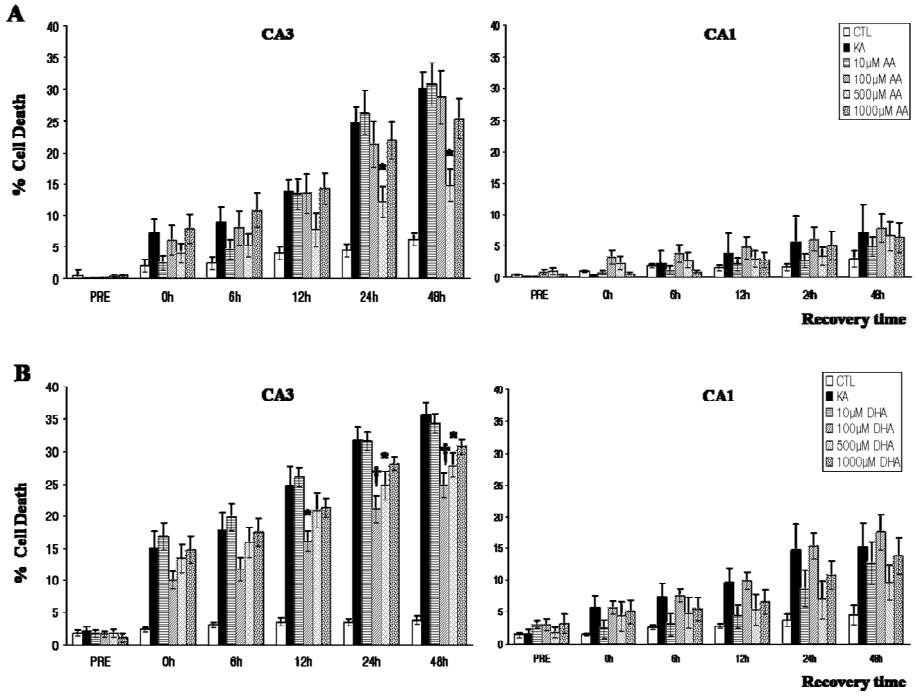

Fig. 6. Quantification of the effects of AA and DHA on KA-induced PI uptake in OHSC. A: Quantification of PI-incorporated cell death following pretreatment with AA and withdrawal of KA exposure in the CA3 and CA1 regions. Data are shown as mean ± S.E.M., with n = 7 for CTL, KA, and 10 μM AA, n = 9 for 100 and 1000 μM AA, and n = 10 for 500 μM AA. B: Quantification of PI-incorporated cell death following pretreatment with DHA and withdrawal of KA exposure in CA3 and CA1 regions. Data are shown as mean ± S.E.M., with n = 7 for CTL, KA, 100 and 1000 μM DHA, and n = 8 for 100 and 500 μM DHA. *$p < 0.05$ and †$p < 0.001$ vs. KA.

3. Effects of AA and DHA on ROS generation induced by KA

To evidence the generation of ROS in KA-induced cell death in OHSC, we measured using the DCFH-DA probe. As shown in Fig. 8A, KA-generated ROS reached a peak at 0 h of recovery time, and reduced after that time. To determine if AA and DHA have antioxidant effects, cultures were pretreated with increasing dose of AA and DAH for 1 h before exposure to KA and ROS levels were measured right after withdrawal of KA, which is the time of the highest level of ROS production after withdrawal of KA In KA only group, DCF staining was widely distributed throughout the slices and more intense in CA3 and CA1 regions as compared with untreated control (Fig. 7B and C). Furthermore, in 500 μM AA and 100 and 500 μM DHA pretreatment, intense DCF fluorescence was decreased throughout the whole hippocampus (Fig. 7B and C). In addition, pretreatment with 500 μM AA significantly quenched ROS, but the rest of groups did not (Fig. 7D). Moreover, DCF fluorescence was significantly decreased in pretreatment of 100 and 500 μM DHA (Fig. 7E). When 100 μM DHA was pretreated, it was more efficient than 500 μM DHA pretreatment (Fig. 7E). Pretreatment of 1000 μM AA and DHA, however, might be thought to have prooxidant effect, as the generation of ROS was also increased (Fig. 7D and E).

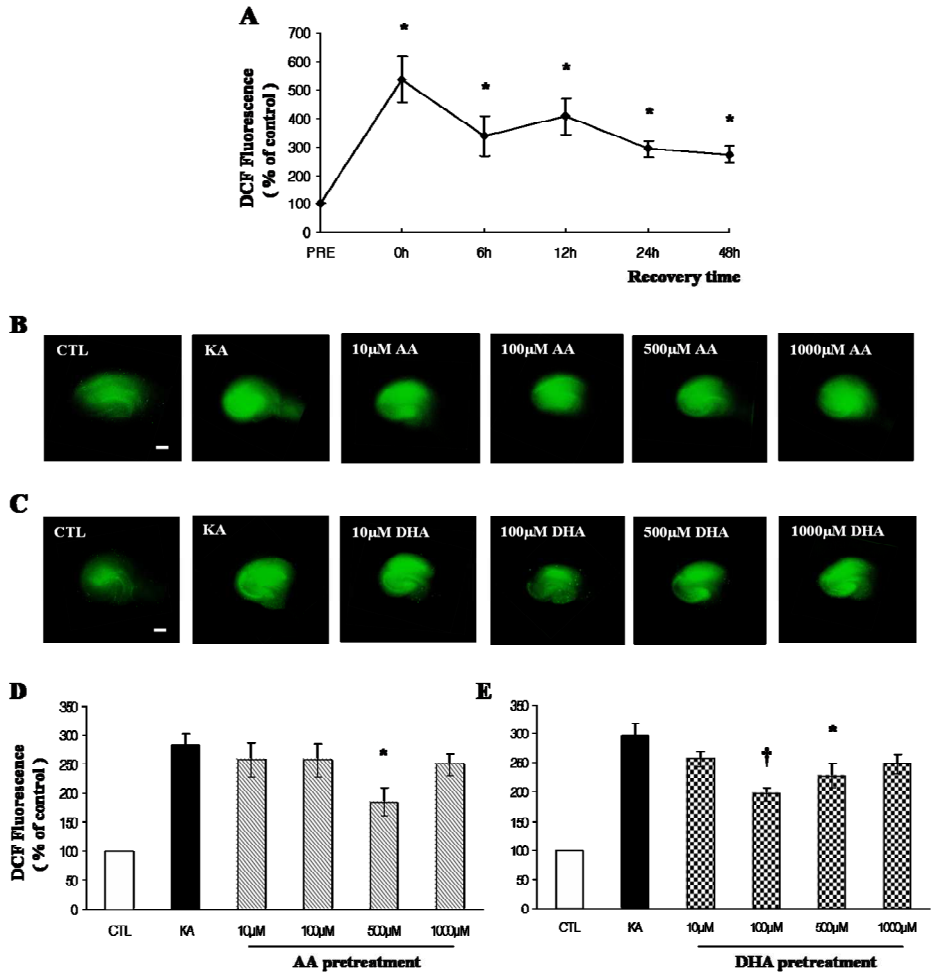

Fig. 7. Effects of AA and DHA on reactive oxygen species (ROS) production. A: The time course of ROS generation after KA exposure for 12 h. Data are shown as mean ± S.E.M., with n = 5 for KA. Representative 2',7'-duchlorofluorescein (DCF) fluorescence images and quantification of DCF fluorescence images of OHSC at 0 h of recovery time following pretreatment with either AA (B and D) or DHA (C and E) and withdrawal of KA exposure. Data are shown as mean ± S.E.M., with n = 8 in each group. *$p < 0.05$, †$p < 0.001$ vs. KA. Scale bar, 200 μm.

4. Effects of AA and DHA on mitochondrial dysfunction induced by KA

To determine if AA or DHA could prevent KA-induced mitochondrial dysfunction, we measured MMPs using fluorescence dye R-123. In control cultures, the R-123 was retained inside mitochondria as indicated stable fluorescence images in all regions (Fig. 8A). In contrast, in KA only cultures, area of R-123-incorporation staining was very small in CA3 region as compared with other groups because KA led to mitochondrial dysfunction (Fig. 8A). 500 μM AA and 100 and 500 μM DHA pretreatment was shown to intercept collapse of MMPs in CA3 region (Fig. 8A). Furthermore, KA suddenly lost MMPs by 25-16% with respect to control (100%) from 0 h to 48 h of recovery time in CA3 region (Fig. 8B). Interestingly, pretreatment with 500 μM AA and 100 and 500 μM DHA significantly prevented mitochondrial dysfunction at every recovery time in CA3 region (Fig. 8B). In addition, 100 μM DHA pretreatment lost MMPs by 74-42% with respect to control from 0 h to 48 h of recovery time in CA3 region, so it was more effective against depolarization of mitochondrial membrane than others (Fig. 8B).

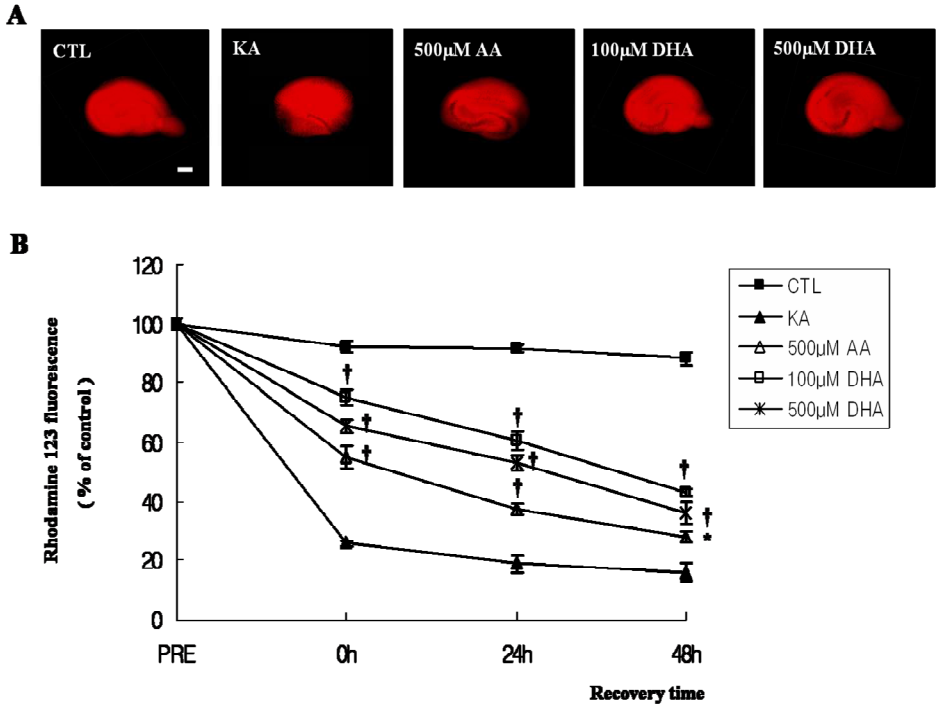

Fig. 8. Effects of AA and DHA on mitochondrial membrane potentials. A: Representative rhodamine 123 (R-123) fluorescence images of OHSC at 48 h of recovery following pretreatment with AA or DHA and withdrawal of KA exposure. B: Quantification of R-123 fluorescence images in CA 3 region. Data are shown as mean ± S.E.M., with n = 5 in each group. *$p < 0.05$, †$p < 0.001$ vs. KA. Scale bar, 200 μm.

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

Caspase-3 activation following AA and DHA pretreatment and KA exposure was studied using an antibody of caspase-3. In control cultures, there was a weak and diffuse background immunostaining in the whole hippocampus (Fig. 9A). In addition, cultures stained at 48 h of recovery time showed immunostaining in pyramidal neurons of CA3 region in every group (Fig. 9A). Caspase-3 fluorescence signals in CA3 region was, however, widely and remarkably seen in KA only group compared with the other groups (Fig. 9A). 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 only group (Fig. 9A). Furthermore, KA only group increased caspase-3 levels by almost 10-fold of control, and 500 μM AA, and 100 and 500 μM DHA pretreatment groups reduced the KA-induced caspase-3 activation by almost 4-fold of 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 other words, intermediated-dose AA and DHA prevented KA-induced apoptosis (Fig. 9B).

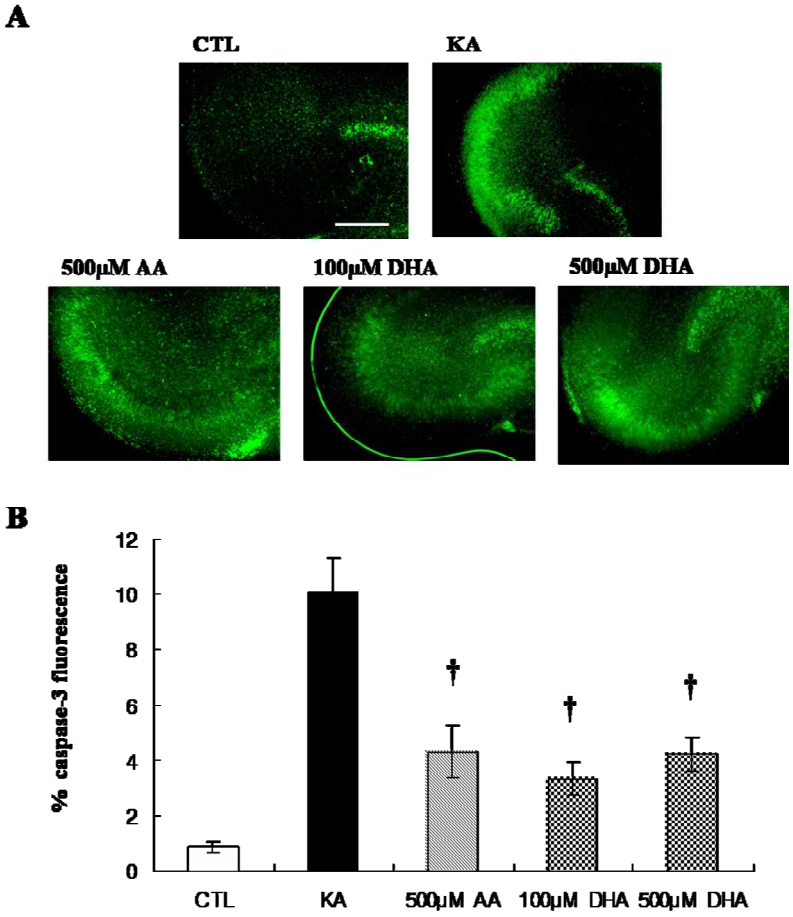

Fig. 9. Effect of AA and DHA on KA-induced change in level caspase-3. A: Photographs of immunoreactivity of capase-3 at 48 h of recovery following pretreatment with AA or DHA and withdrawal of exposure to KA in CA3 region. B: Quantification of caspase-3-fluorescence images in CA3 region. Data are shown 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.

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

Hyrogen peroxide (H_2O_2) and lipid hydroperoxide (LOOH) can be reduced to the hydroxyl radical (HO·) and alkoxyl radical in the present 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, the Fe^{2+} pool available 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+}.[36] To determine if the aggravating effects of AA on KA-induced cell death were caused by the Fenton reaction, slices were pretreated with 1000 μM AA and 300 μM DFO, the iron-chelating, before KA exposure. 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. 10A). 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 of recovery, but it increased cell death at 12, 24 and 48 h of recovery time (Fig. 10B). Therefore, high-concentration of AA could not prevent cell death after KA exposure without the Fenton reaction.

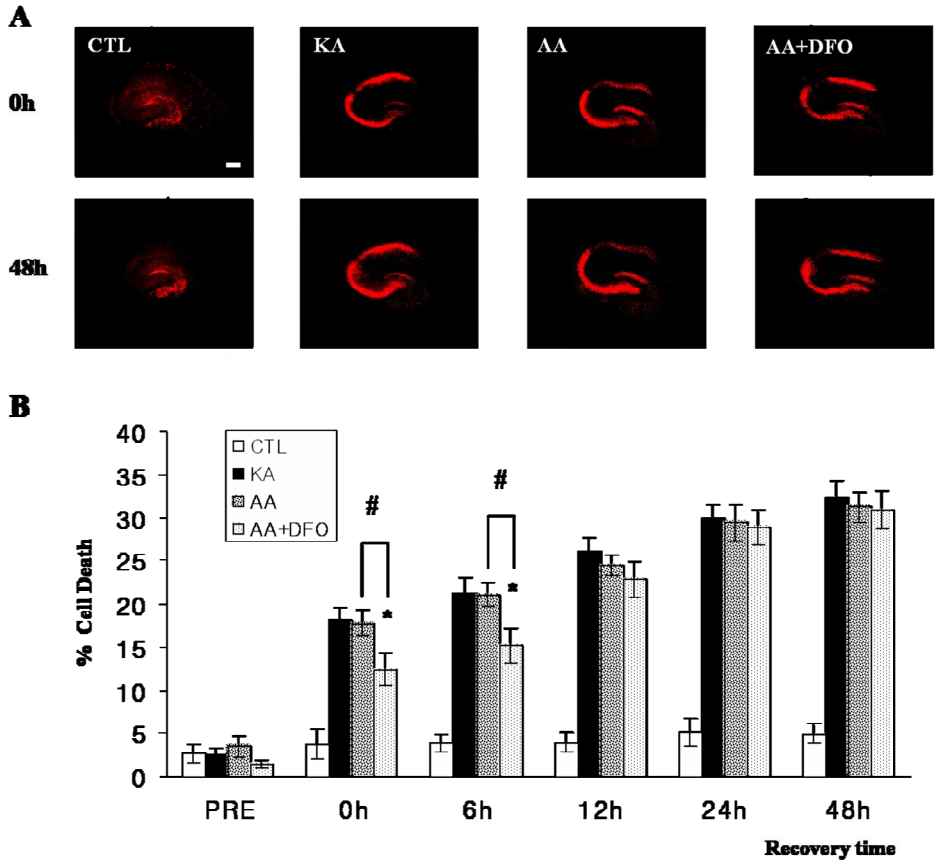

Fig. 10. Effects of co-pretreatment with AA and desferrioxamine (DFO) on KA-induced cell death. A: Representative PI fluorescence images of OHSC at 0 h and 48 h of recovery following AA or DFO pretreatment and withdrawal of KA exposure. B: Quantification of PI-incorporated area in CA3 region. Date are show as mean ± S.E.M., with n = 8 for CTL and KA, and n = 9 for 1000 μM AA, and 1000 μM AA+DFO. *$p < 0.05$ vs. KA, #$p<0.05$ vs. AA. Scale bar, 200 μm.

IV. DISCUSSION

The present study indicates that KA specifically causes damage in CA3 pyramidal neurons, whereas few CA1 pyramidal neurons are injured by KA. Low concentrations of KA result in a specific loss of CA3 neurons while high concentrations result in complete loss of neurons.[37] These results are consistent with earlier observations of a specific susceptibility of this region after KA treatment.[4,5,30,37] Specific neuronal death induced by KA has been shown to be related to the high density of KA-binding sites in CA3 region than other regions.[3,30,38] KA-binding sites have been localized on mossy fiber terminals and on the soma of CA3 pyramidal cells.[3] When the mossy fibers are immature or absent, KA acts only on the soma of CA3 pyramidal cells, so this depolarization is not sufficient to induced toxicity.[37] Therefore, mossy fiber synapses are necessary for CA3 toxicity induced by KA.[3,30,37,38] The mechanisms of KA-induced neuronal injury are not yet well-understood. In addition, KA-induced pyramidal cell death observed in OHSC is decisively mediated by generation of ROS via mitochondrial dysfunction following excessive calcium entry.[4,5,10,12,13] An essential factor in cell death resulting from apoptosis is ROS, so KA-induced cell death exhibits several apoptosis characteristics.[11] Furthermore, KA-induced neuronal injury can be prevented by certain free radical scavengers.[14,15] The damage of CA3 neurons showed the temporal development of cell death during 48 h 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 recently Lahtinen et al.[39] 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.

In the present study, it was 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 ROS level, mitochondrial dysfunction, and caspase-3 activation. Loss of MMPs and activation of caspase-3 are important events 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, whereas low- and high-dose of AA and DHA pretreatment did not prevent it. Intermediated-dose of AA and DHA pretreatment diminished intracellular DCF fluorescence and inhibited collapse of MMPs and caspase-3 activation.

AA diffuses from cerebrospinal fluid (CSF) to the brain extracellular fluid and is taken up to brain cells. In rat brain, the concentrations of AA in CSF are approximately 200-500 μM, but those in neuron and glia are approximately 10 mM and 1 mM.[16,40] However, anterior regions such as cerebral cortex and hippocampus consistently show higher AA levels compared to other brain structures.[14] Resent studies have shown that AA and DHA are transported into neurons and astrocytes by vitamin C recycling.[41,42] Continuous vitamin C recycling clearly helps protect neuronal cell types from oxidative damage.[25,41,42] AA and DHA are transported into the brain by two distinct mechanisms. The transport of AA into neurons is mediated mainly by Na^+-dependent transport.[43] Neuronal intracellular AA is oxidized in generating DHA by ROS, which may exit neurons through facilitated transport and enter astrocytes, which help protect neurons, through facilitative glucose transporter and is reduced subsequently to AA.[23,25,41] Rice and Russo-Menna[44] reported that glutathione (GSH) which is DHA reductase is more concentrated in glia than in neurons. AA efflux from astrocytes may occur through glutamate-ascorbate heteroexchagers and extracullar AA is again transported back into neurons.[42] In astrocyte, extracelluar glutamate uptake should be accelerated by exchange for intracellular AA.[42] Drugs which block glutamate uptake prevent the release of AA.[45,46] Conversely, if the intracellular AA concentration determined the capacity of glutamate uptake, one might expect elevated intracellular AA to enhance glutamate uptake.[17,47] Such action would be neuroprotective and might

explain the need for pretreatment of the animals or slices with AA in all possibility.[17] On the other hand, one might anticipate such an action to protect in the hippocampus where glutamate contributes significantly to injury.[6,7,17] In this study, intermediated-dose of AA and DHA pretreatment was significantly protective against KA neurotoxicity which results in the release of endogenous glutamate.

AA is a well-known antioxidant that is important reducing agent 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.[19] It has been generally believed that AA-induced oxidative action 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.[19] According to other reports, however, AA-induced oxidative stress is not caused by interaction between AA and transition metal ions. Chen et al.[48] demonstrated that AA act as an antioxidant even in presence of ion overload. Kim et al.[49] reported that AA and DFO co-pretreatment could inhibit the aggravating effect of AA on H_2O_2-induced oxidative cell death at early stage, but it could not inhibit at later stage. In the present study, although co-pretreatment with 1000 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. Puskas et al.[22] reported that DHA can elevate GSH levels through stimulation of the pentose phosphate pathway. Sagun and coworks[27] indicated that DHA enters mitochondria via facilitative glucose transporter and prevents mitochondrial membrane depolarization. In the present study, 100 and 500 μM DHA prevented KA-induced cell death in OHSC. In addition, those diminished ROS level, mitochondrial dysfunction, caspase-3 activation. Intermediated-dose

of DHA, therefore, has protective effect against KA-induced oxidative stress in OHSC. On the other hands, 1000 μM DHA pretreatment did not prevent KA-induced neuronal damage. Song and coworkers[20,21] demonstrated that AA and DHA induced lipid peroxide production and cell death at high concentration in PC12 cell and liver slices.[21,51] The paradoxical oxidative action of AA is due to a serial redox reaction.[20,21] 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.[23-25] 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.[20,21] Rapid and high uptake of DHA is a critical factor to generate oxidative stress and to damage on vital cellular compound.[20] In the present study, therefore, it was shown that high-dose AA and DHA has prooxidant effects on KA-induced cell death in OHSC. The uptake rate of DHA by facilitative glucose transporter is rapid, whereas that of AA via Na^+-dependent transport is slow.[52] Therefore, these results showed that when AA and DHA was pretreated, 100 μM DHA group was more efficient antioxidant than 500 μM AA and DHA, because it could be rapidly transported into cells via facilitative glucose transporters.

In conclusion, these data showed that KA-induced neuronal death in OHSC is region-specific in CA3 region. Furthermore, KA-induced neurotoxicity involves oxidative stress that triggers mitochondrial dysfunction and caspase-dependent apoptotic pathway. Moreover, Intermediate-dose AA and DHA pretreatment prevent KA-induced apoptotic cell death. In contrast, high-dose of AA and DHA pretreatment did not prevent KA-induced neuronal injury, because they have prooxidant effects. These results suggest that both AA and DHA pretreatment have antioxidant or prooxidant effects in accordance with level of dose on KA-induced neuronal damage.

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ABSTRACT (IN KOREAN)

Organotypic hippocampal slice culture 에서 kainate 에 의해 유발된 신경세포의 손상에 대한 ascorbic acid 와 dehydroascorbic acid 의 항산화 효과

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김 은 진

Kainic acid(KA)는 kainate와 α-amino-3-hydroxy-5-methyl-4-isoxasole-proprionic acid(AMPA) receptor의 흥분제로 해마에서 흥분독소로 작용한다고 알려져 있다. 일련의 연구에 의하면 KA가 kainate receptor에 작용하게 되어 세포 내 칼슘의 과다 축적을 유도하여 미토콘드리아의 free radical이 생성되게 된다. 이러한 많은 양의 활성산소(reactive oxygen species)는 세포자멸(apoptosis)을 일으킨다. Ascorbic acid(AA)는 활성산소와 독성을 제지하는 잘 알려진 항산화제이다. 다른 조직에 비해 뇌에 높은 농도로 존재한다고 알려져 있다. 그러나 최근에 free transition metal이 존재하면 prooxidant 효과를 보인다는 보고가 있어 AA의 산화된 형태인 dehydroascorbic acid(DHA)를 AA의 대체물로 사용하고 있다. AA와 DHA는 간질, 뇌졸중과 허혈 모델 실험에서 free radical의 생성을 줄여 신경세포 손상으로부터 조직을 보호한다고 알려져 있다.

이에 본 연구에서는 organotypic hippocampal slice cultures(OHSC) 모델을 이용하여 항산화제인 AA와 DHA를 전 처리

한 후 KA로 손상을 주어 해마의 CA1과 CA3 구역의 후속적인 신경세포의 손상과 활성산소 생성을 측정하고, 세포자멸과 관련해서 미토콘드리아의 기능장애와 caspase-3의 활성을 알아보았다. 또한, AA의 prooxidant 효과가 철 이온과 관련이 있는지 알아보기 위해 iron chelator를 이용한 실험을 실시하였다.

KA를 12시간 동안 처리한 후 새로운 배지로 갈아준 후에 후속적인 신경세포 사멸이 CA3 구역에서는 의미하게 나타났으나 CA1 구역에서는 나타나지 않았다. 반면 500 μM의 AA와 100과 500 μM의 DHA를 전 처리 하였을 경우, CA3 구역에서 세포사멸과 활성산소 생성을 막아내고 미토콘드리아의 기능장애와 caspase-3의 활성을 억제하였으나, 다른 그룹에서는 어떠한 억제나 방지 효과를 볼 수 없었다. 1000 μM의 AA를 전 처리하면 prooxidant 효과를 보인다고 생각되나, free transition metal과 관련 되어있지 않다고 여겨진다. 중간 농도의 AA와 DHA 전 처리하였을 경우, OHSC에서 KA에 의해 유발된 신경세포 손상을 막을 수 있었지만, 고농도의 AA와 DHA를 전 처리하면 KA에 의해 유발된 세포 사멸을 방지하지 못했다. 왜냐하면, 그들은 prooxidant 효과를 가지기 때문이다. 따라서, 이러한 결과들은 AA와 DHA의 전 처리는 KA에 의해 유발된 신경세포 손상에서 농도의 정도에 따라서 항산화와 prooxidant 효과를 모두 가지고 있는 것으로 생각된다.

핵심되는 말: 항산화제, ascorbic acid, dehydroascorbic acid, kainic acid, 활성산소, organotypic hippocampal slice culture