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Anti-oxidant effect of ascorbic and dehydroascorbic acids in hippocampal slice culture

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Abstract

Ascorbic acid (AA) and dehydroascorbic acid (DHA) have been shown to have protective effects as anti-oxidants in experimental neurological disorder models such as stroke, ischemia, and epileptic seizures. The present study was conducted to examine the protective effects of AA and DHA on kainic acid (KA) neurotoxicity using organotypic hippocampal slice cultures. After 12 h KA treatment, significant delayed neuronal death was detected in the CA3, but not the CA1, region. Pretreatment with intermediate doses of AA and DHA significantly prevented cell death and inhibited reactive oxygen species (ROS) level, and mitochondrial dysfunction in the CA3 region. In contrast, pretreatment with low or high doses of AA or DHA was not effective. These data suggest that pretreatment with both AA and DHA has dose-dependent neuroprotective effects on KA-induced neuronal injury through inhibiting ROS generation and mitochondrial dysfunction.

Keywords: Anti-oxidants; Ascorbic acid; Dehydroascorbic acid; Kainic acid; Reactive oxygen species; Organotypic hippocampal slice culture

Excitotoxicity is widely considered to be a contributing factor in neuronal death associated with a number of neurological insults or disorders, including hypoxia, ischemia, and epileptic seizures [1,2]. Kainic acid (KA) is an agonist for kainate and α-amino-3-hydroxy-5-methyl-4-isoxasole-proprionic acid (AMPA) receptors and acts as 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].

Accumulation of KA results in the activation of presynaptic kainate receptors and the release of endogenous glutamate [6,7]. The released glutamate then acts postsynaptically on NMDA receptors, which can contribute to neuronal damage [8,9]. Excessive calcium entry is accumulated by intracellular mitochondria, causing the collapse of mitochondrial membrane potentials (MMPs) and the generation of reactive oxygen species (ROS), ultimately resulting in cytochrome C release that may induce apoptosis by activating caspase [10,11]. Several lines of recent evidence suggest that ROS play an important role in the pathogenesis of excitotoxic cell death [7,10]. Liang et al. [12] reported that systemic kainate administration specifically increases mitochondrial superoxide O_2^- radical production. Another in vitro study demonstrated free radical generation in cultured retinal neurons injured by

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kainate [13]. KA-induced neuronal damage may be prevented by certain anti-oxidants such as ascorbic acid (AA) or dehydroascorbic acid (DHA).

AA, a known potent anti-oxidant, accumulates in the brain at a much higher concentration than it does in any other organ [14]. Within the brain, however, AA levels are not homogeneous; the highest levels are found in the amygdala, hippocampus, and hypothalamus [15]. 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 [15–17]. The role of AA in protecting against oxidative stress is controversial because AA also has pro-oxidant properties in the presence of free transition metals in vitro [18]. Several studies have shown that AA induces lipid peroxide production and cell death in cortical slices [19] or PC12 cells [20]. Overall, the anti-oxidant or pro-oxidant properties of AA have not yet been fully determined.

DHA is an oxidized form of AA, and DHA treatment circumvents the pro-oxidant effects of AA [21]. DHA is taken up by glucose transporters [22] and regenerated into AA at the expense of glutathione (GSH) [23]. Pathological conditions that inhibit DHA recycling may decrease AA concentrations and thereby impair AA-dependent enzymatic and anti-oxidant activities [24]. DHA administration results in normalization of oxidative stress markers and inflammation in hyperglycemic stroke models [25]. In vitro, DHA inhibits mitochondrial damage and cell death against oxidative injury [21,26]. In primary astrocytes, we observed that DHA prevents H_2O_2-induced cell death by increasing GSH levels [27].

Organotypic hippocampal slice cultures (OHSCs) are advantageous 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 facilitate experimental manipulation that is not possible in vivo, allowing application of precise concentrations of drugs or factors at specific times and the visualization of cell morphology and function using fluorescent markers/probes within the same cultures for long periods [29]. We used OHSCs to assess the effects of AA and DHA on KA-induced neuronal death by evaluating the production of ROS and mitochondrial dysfunction.

Materials and methods

Preparation and maintenance of OHSCs. All animal experiments were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine. OHSCs were prepared by the method of Stoppini et al. [30]. 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, MO, USA) and 4 mM L-gultamine (Gibco, Grand Island, NY, USA). Transverse sections 350 μm thick were 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 6-well plates 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, 25% Hanks's balanced salt, and 25 mM HEPES supplemented with penicillin–streptomycin and D-glucose. pH was adjusted to 7.3 with 5 mM Tris and 4 mM $NaHCO_3$. The medium was changed on the first day after culture and every 3–4 days thereafter.

Drug treatment. KA (5 μM) was applied for 12 h after mature cultures were incubated in serum-free culture medium overnight. 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.

Assessment of neuronal injury. Neuronal injury was assessed with the fluorescent cell death marker propidium iodide (PI, Sigma, Saint Louis, MO, USA), a very stable dye. PI is a polar compound that 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 were captured with a digital camera under a fluorescent microscope (Model BX-51, Olympus, Tokyo, Japan) and quantified with the MetaMorph Imaging system (Universal Image Co., Downingtown, PA, USA).

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). Cultures were incubated with 5 μM DCFH-DA at 35 °C for 30 min, and then washed with fresh serum-free medium. The fluorescent DCF was measured using a fluorescent microscope, and captured by a digital camera. DCF fluorescence signals were analyzed with the MetaMorph Imaging system.

Assay of mitochondrial dysfunction. Mitochondrial dysfunction was assayed by measuring 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. R-123 was excited at 480 nm, and fluorescent images were recorded at 590 nm using a fluorescent microscope and captured by a digital camera. The R-123 fluorescence signals were analyzed using the MetaMorph Imaging system.

Statistical analysis. Data are presented as the means ± standard error of the mean (SEM). 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 OHSCs

An initial experiment was conducted to determine the relationship of KA and neuronal death in OHSCs (Fig. 1). 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 of recovery time are shown in Fig. 1A. In untreated slices, no noticeable PI fluorescence was observed (Fig. 1A). The treatment of slices with KA resulted in neuronal death, with a selective uptake of PI fluorescence in the CA3 region and faint PI staining in the CA1 region after 0 and 48 h of recovery time (Fig. 1A). The PI-stained area continued to significantly increase up to 48 h of recovery time in the CA3 region (Fig. 1B). In contrast, the CA1 region did

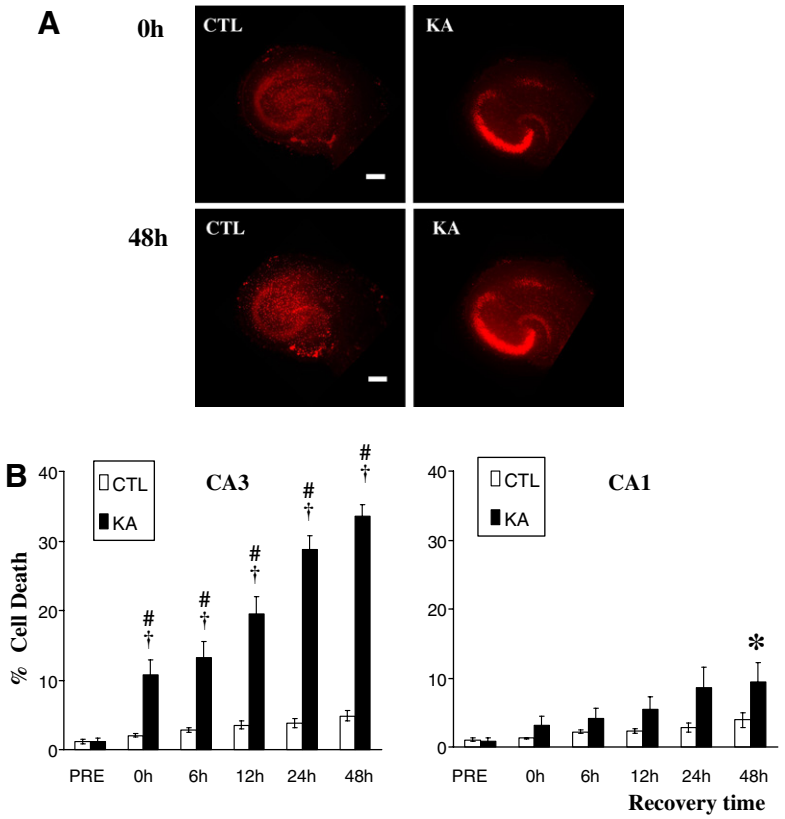

Fig. 1. KA-induced neuronal death in OHSCs. (A) Representative PI fluorescence images of OHSCs at 0 and 48 h of recovery time in fresh medium after 12 h of KA exposure (right) compared to control (left). (B) Quantification of PI-incorporated area following withdrawal of exposure to KA in the CA3 and CA1 regions at each recovery time. Data are shown as means ± SEM, with $n = 13$ in each group. $^{\dagger}p < 0.001$ vs. control (CTL), $^{*}p < 0.05$ and $^{\#}p < 0.001$ vs. PRE (before KA exposure). Scale bar, 200 μm.

not show significant cell death at every recovery time (Fig. 1B).

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 (Fig. 2). In control cultures, the PI uptake was very low, while in KA-only cultures, the area of PI-incorporated cell death was broad and deep in the CA3 region as compared to the rest of the treatments (Fig. 2A and B). Pretreatment with 500 μM AA significantly prevented cell death after 24 and 48 h of recovery in the CA3 region, but the same impact was not seen in other treatment groups (Fig. 2A). Furthermore, pretreatment with 100 μM DHA significantly prevented cell death at 12, 24, and 48 h of recovery in the CA3 region (Fig. 2B), and pretreatment with 500 μM DHA significantly reduced cell death at 24 and 48 h of recovery in the CA3 region (Fig. 2B). At the same time, low- and high-dose AA and DHA pretreatment did not prevent cell death at every recovery time.

Effects of AA and DHA on ROS generation induced by KA

We used a DCFH-DA probe to document the generation of ROS in KA-induced cell death in OHSCs (Fig. 3). In a preliminary study, we found that KA-generated ROS peaked at 0 h of recovery time and declined after that. To determine if AA and DHA have anti-oxidant effects, therefore, cultures were pretreated with increasing doses of AA and DAH for 1 h before exposure to KA, and ROS levels were measured right after withdrawal of KA (the time of the highest level of ROS production). In the KA-only group, DCF staining was widely distributed throughout the slices and more intense in the CA3 and CA1 regions than in the untreated control (Fig. 3A and B). Furthermore, with 500 μM AA and 100 and 500 μM DHA pretreatment, intense DCF fluorescence decreased throughout the entire hippocampus (Fig. 3A and B). In addition, pretreatment with 500 μM AA significantly quenched ROS, but the rest of the treatments did not (Fig. 3C). Moreover, DCF fluorescence was significantly lower after pretreatment with 100 and 500 μM DHA (Fig. 3D). Pretreatment with 100 μM DHA was more efficient than pretreatment with 500 μM DHA (Fig. 3D). Pretreatment with 1000 μM AA and DHA, however, did not have anti-oxidant effect, as the generation of ROS was not diminished (Fig. 3C and D).

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 the

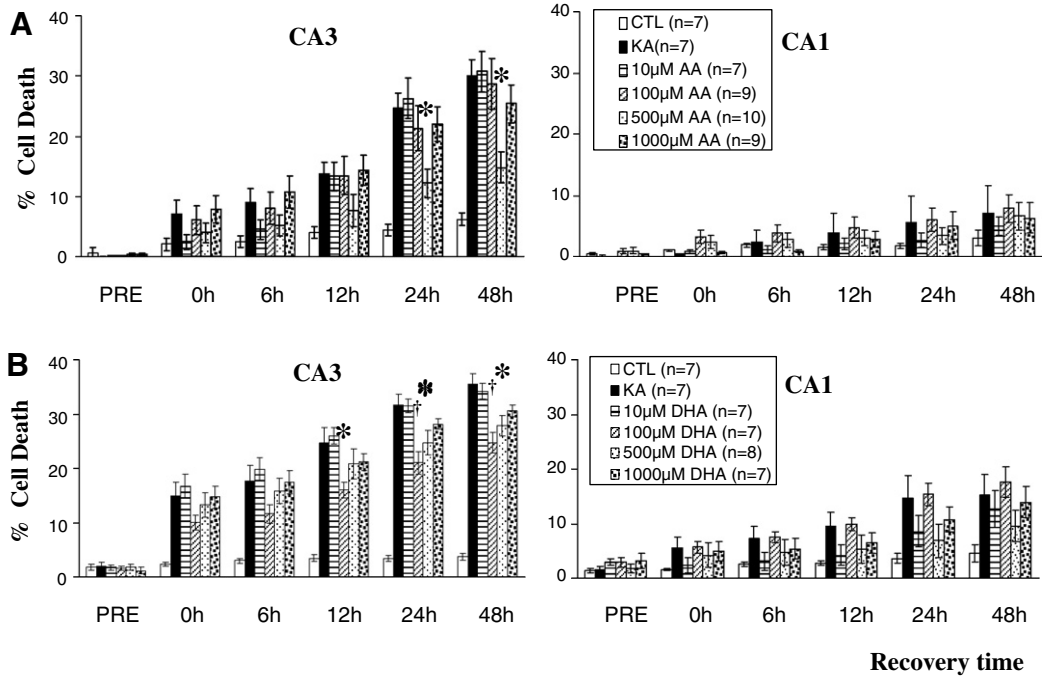

Fig. 2. The effects of AA and DHA on KA-induced PI uptake in OHSCs. (A) Quantification of PI-incorporated cell death following pretreatment with AA and withdrawal of KA in the CA3 and CA1 regions. Data are shown as means ± SEM, with $n = 7$ for CTL, KA, and 10 μM AA, $n = 9$ for 100 and 1000 μM AA, and $n = 10$ for 500 μM AA. (B) Quantification of PI-incorporated cell death following pretreatment with DHA and withdrawal of KA in CA3 and CA1 regions. Data are shown as means ± SEM, with $n = 7$ for CTL, KA, 10 and 1000 μM DHA, and $n = 8$ for 100 and 500 μM DHA. $^{*}p < 0.05$ and $^{\dagger}p < 0.001$ vs. KA.

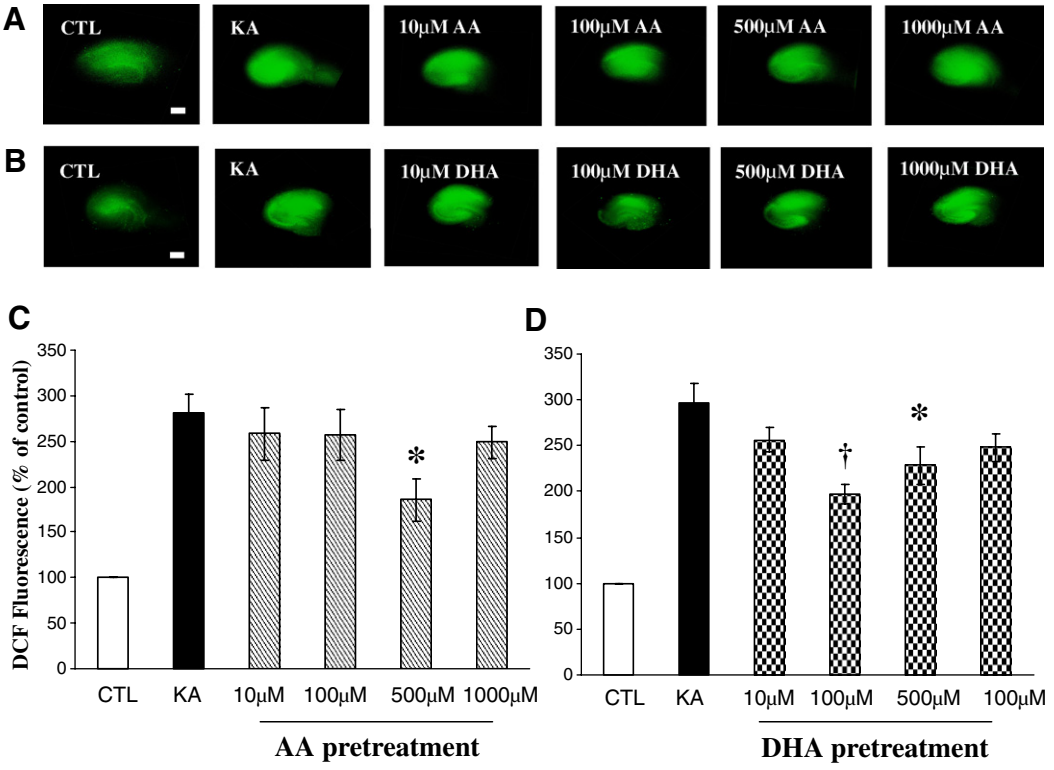

Fig. 3. Effects of AA and DHA on reactive oxygen species (ROS) production. (A and B) Representative 2′,7′-dichlorofluorescein (DCF) fluorescence images. (C and D) Quantification of DCF fluorescence in OHSCs at 0 h of recovery time following pretreatment with either AA (A and C) or DHA (B and D) and withdrawal of KA. Data are shown as means ± SEM, with $n = 8$ in each group. $^{*}p < 0.05$ and $^{\dagger}p < 0.001$ vs. KA. Scale bar, 200 μm.

fluorescent dye R-123 (Fig. 4). In control cultures, R-123 was retained inside the mitochondria as indicated by stable fluorescence images in all regions (Fig. 4A). In contrast, in KA-only cultures, the area incorporating R-123 was very small in the CA3 region as compared to other groups because the KA led to mitochondrial dysfunction (Fig. 4A). Pretreatment with 500 μM AA and 100 and 500 μM DHA intercepted the collapse of MMPs in the CA3 region (Fig. 4A). Furthermore, from 0 to 48 h of recovery time, KA caused a sudden loss of 25–16% of MMPs in the CA3 region with respect to control (100%) (Fig. 4B). Interestingly, pretreatment with 500 μM AA and 100 and 500 μM DHA significantly prevented mitochondrial dysfunction at every recovery time in the CA3 region (Fig. 4B). Pretreatment with 100 μM DHA was more effective against depolarization of the mitochondrial membrane than the other treatments (Fig. 4B).

Discussion

Our data indicate that KA specifically causes damage to the CA3 pyramidal neurons. In contrast, few CA1 pyramidal neurons were injured by KA. According to Casaccia-Bonnefil et al. [31], low concentrations of KA result in a specific loss of CA3 neurons, while high concentrations result in the complete loss of neurons. Our results are also consistent with earlier observations of a specific susceptibility of this region to KA treatment [4,5,31,32]. Specific neuronal death induced by KA has been related to the high density of KA-binding sites in the CA3 region compared to other regions [3,32,33]. KA-binding sites are known to localize 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, and this depolarization is not sufficient to induce toxicity [31]. Consequently, mossy fiber synapses are necessary for KA-induced CA3 toxicity [3,31–33].

The mechanisms of KA-induced neuronal injury are not yet well understood. ROS are an essential factor in cell death. KA-induced pyramidal cell death observed in OHSCs seems to be mediated by the generation of ROS via mitochondrial dysfunction following excessive calcium entry [4,5,10,12,13]. The involvement of ROS is supported by the observation that KA-induced neuronal injury can be prevented by certain free radical scavengers [15,34].

We found that AA and DHA pretreatment afforded protection against KA neurotoxicity in OHSCs. To determine the protective effects of AA and DHA on cell death, ROS level and mitochondrial dysfunction were measured in OHSCs. Production of ROS and loss of MMPs are important events during cell death. According to our observations, neurotoxicity by exposure to KA was significantly prevented by pretreatment with 500 μM AA and 100 and 500 μM DHA in OHSCs, although low and high doses of AA and DHA pretreatment did not prevent it. Furthermore, intermediate doses of AA and DHA inhibited ROS level and collapse of MMPs. These results indicate that AA and DHA have neuroprotective effects by means of inhibition of ROS generation and mitochondrial dysfunction.

AA is a well-known anti-oxidant which is involved in several types of protective mechanisms. AA is also known to act as a pro-oxidant, but the mechanism behind AA-induced oxidative action and apoptosis has not been estab-

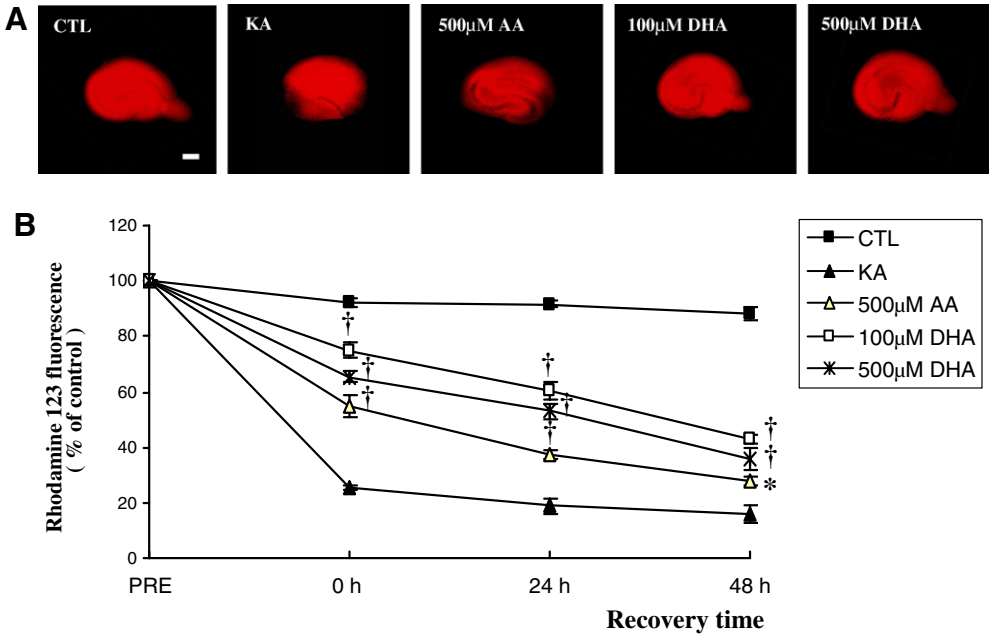

Fig. 4. Effects of AA and DHA on mitochondrial membrane potentials. (A) Representative rhodamine 123 (R-123) fluorescence images of OHSCs at 48 h of recovery following pretreatment with AA or DHA and withdrawal of KA. (B) Quantified R-123 fluorescence in the CA 3 region. Data are shown as means ± SEM, with $n = 5$ in each group. $^{*}p < 0.05$ and $^{\dagger}p < 0.001$ vs. KA. Scale bar, 200 μm.

lished. Carr and Frei demonstrated a biphasic effect of AA as an anti-oxidant or pro-oxidant [18]. We found that an intermediate dose of AA had an anti-oxidant effect in OHSCs.

Normally, AA diffuses from the cerebrospinal fluid (CSF) to the extracellular fluid of the brain and is taken up by brain cells. In rat brain, the concentration of AA in CSF is approximately 200–500 μM, compared to about 10 mM in neurons and 1 mM in glia [35]. Anterior regions such as the cerebral cortex and hippocampus consistently show higher AA levels compared to other brain structures [15]. Recent studies have shown that AA and DHA are transported into neurons and astrocytes by vitamin C recycling [36]. Continuous vitamin C recycling clearly helps to protect neuronal cell types from oxidative damage [36].

DHA has been used to circumvent the pro-oxidant effects of AA. Puskas et al. [21] reported that DHA can elevate GSH levels through stimulation of the pentose phosphate pathway. Sagun et al. [26] indicated that DHA enters mitochondria via a facilitative glucose transporter and prevents mitochondrial membrane depolarization. In the present study, pretreatment with 100 and 500 μM DHA prevented KA-induced cell death and diminished ROS level and mitochondrial dysfunction in OHSCs. Intermediate doses of DHA, therefore, exert a protective effect against KA-induced oxidative stress in OHSCs. In contrast, pretreatment with higher doses of DHA (1000 μM) did not prevent KA-induced neuronal damage.

In conclusion, we observed that KA-induced neuronal death in OHSCs is specific to the CA3 region. Pretreatment with intermediate doses of AA and DHA prevented KA-induced apoptotic cell death and inhibited ROS level and mitochondrial dysfunction. In contrast, pretreatment with low or high doses of AA and DHA did not prevent KA-induced neuronal injury. These results suggest that the anti-oxidant effects of AA and DHA pretreatment on KA-induced neuronal damage are dose-dependent and mediated by inhibiting ROS generation and mitochondrial dysfunction.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.bbrc.2007.11.050.

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