

## Differences in Lipid Peroxidation and Cu,Zn-Superoxide Dismutase in the Hippocampal CA1 Region Between Adult and Aged Dogs

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**ABSTRACT.** Reactive oxygen species have been long associated with oxidative stress relevant to many pathological damages. In brain, 4-hydroxy-2E-nonenal (HNE), a major cytotoxic end product of lipid peroxidation, is produced. In contrast, superoxide dismutase (SOD), one of the major antioxidant enzymes, protects neurons from oxidative stress. The aim of this study is to observe differences in the distribution of HNE and Cu,Zn-superoxide dismutase (SOD1) in the hippocampal CA1 region of adult (2–3 years of age) and aged (10–12 years of age) dogs. The HNE immunoreactivity and protein level in the CA1 region were significantly high in the aged dogs compared to those in the adult dogs. SOD1 immunoreactivity and its protein level were also higher in the aged dogs than those in the adult dogs. However, there were not significant differences in NeuN (a neuron-specific soluble nuclear antigen) immunoreactivity in CA1 neurons between the adult and aged dogs. These differences may be associated with oxidative stress in aged dogs compared to that in adult dogs.

**KEY WORDS:** adult and aged dog, Cu,Zn-superoxide dismutase, hippocampal CA1 region, lipid peroxidation.

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Reactive oxygen species (ROS) have been associated with oxidative stress relevant to normal aging as well as many pathological damages such as stroke, Parkinson's disease and Alzheimer's disease [3, 6]. In cellular levels, ROS may interact with many cell constituents such as nucleic acids, proteins, polysaccharides as well as fatty acids [14]. Among these, the polyunsaturated fatty acids are the most susceptible to oxidative stress because they have unsaturated bonds in their structures [7, 14]. In an organ level, brain is highly sensitive to oxidative stress because it is metabolically very active and it has high contents of polyunsaturated fatty acids, and a slight deficiency in anti-oxidative defense mechanisms in the brain [7].

4-Hydroxy-2E-nonenal (HNE) is a major cytotoxic end product of lipid peroxidation and mediates oxidative stress-induced cell death in many cell types. In addition, it is a long-chain  $\alpha$ ,  $\beta$ -unsaturated aldehyde product from the oxidation of omega-6 polyunsaturated fatty acids [11, 22, 23]. In contrast, one of major antioxidant enzymes is superoxide dismutase (SOD). It consists of three enzymes derived from three different genes that each converts superoxide radicals into hydrogen peroxide. Among SODs, Cu,Zn-SOD (SOD1) is localized in the cytoplasm and protects neurons from oxidative stress [6, 21]. Recently, we reported that the treatment with SOD1 into ischemic brains protected neu-

rons in the hippocampus from oxidative stress [10, 13].

The hippocampus is important for learning and memory and it, especially CA1 region, is one of the first regions of the brain to suffer damage, memory problems in several disorders such as Alzheimer's disease and stroke. Dogs are accepted as a model of brain aging because the canine brain in aging stage produces neuronal loss and senile plaques in the cortex and other gray matter structures. In addition, dogs develop a well characterized cognitive deficit syndrome during aging [8, 16, 19, 20]. However, there were few studies about the age-related lipid peroxidation and SOD1 level in the hippocampal CA1 region. In the present study, therefore, we investigated HNE and SOD1 immunoreactivities and protein levels in the hippocampi of adult and aged dogs.

### MATERIALS AND METHODS

*Experimental animals:* The present study used the progeny of German shepherd obtained from the SWAT, South Korea. Male dogs ( $n=8$  at each age) were used at 2–3 years of age for adult group and 10–12 years of age for aged group. These animals had not show any clinical and other signs of neural disorders. The procedures for handling and caring for the animals adhered to the guidelines, which are in compliance with the current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996), and they were approved by the Institutional Animal Care and Use Committee at Hallym's Medical Center. All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used

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in this study.

**Tissue processing for histology:** For the histological analysis, adult and aged dogs ( $n=5$  at each age) were anesthetized with ketamine and xylazine mixture and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brains were removed and postfixed in the same fixative for 6 hr. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter the frozen tissues were serially and transversely sectioned on a cryostat (Leica, Wetzlar, Germany) into 30- $\mu\text{m}$  coronal sections, and the sections were then collected into six-well plates containing PBS.

**Immunohistochemistry for NeuN, HNE and SOD1:** Immunohistochemistry was performed under the same conditions in the hippocampal CA1 region in adult and aged dogs ( $n=5$  at each group) in order to examine whether the degree of immunohistochemical staining was accurate. The sections were sequentially treated with 0.3% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in PBS for 30 min and 10% normal goat serum or normal rabbit serum in 0.05 M PBS for 30 min. The sections were next incubated with diluted mouse anti-NeuN (1:1,000, Chemicon, Temecula, CA), diluted mouse anti-HNE (1:1,000, Alexis Biochemicals, San Diego, CA) or sheep anti-SOD1 antibody (1:1,000, Calbiochem, San Diego, CA) overnight at 4°C and subsequently exposed to biotinylated goat anti-mouse IgG or rabbit anti-sheep IgG and streptavidin peroxidase complex (1:200, Vector, Burlingame, CA). And they were visualized with 3,3'-diaminobenzidine tetrachloride (Sigma, St Louis, MO) in 0.1 M Tris-HCl buffer and mounted on the gelatin-coated slides. After dehydration the sections were mounted with Canada balsam (Kanto, Tokyo, Japan). Negative control tests were carried out using pre-immune serum instead of primary antibody in order to establish the specificity of the immunostaining. The negative control resulted in the absence of immunoreactivity in any structures.

**Western blot analysis:** To confirm changes in HNE and SOD1 levels in the hippocampal CA1 region of adult and aged dogs, 3 animals in each group were sacrificed and used for Western blot analysis. After sacrificing them and removing the brain, the hippocampal CA1 region were then dissected with a surgical blade. The tissues were homogenized in 50 mM PBS (pH 7.4) containing EGTA (pH 8.0), 0.2% NP-40, 10 mM EDTA (pH 8.0), 15 mM sodium pyrophosphate, 100 mM  $\beta$ -glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM PMSF and 1 mM DTT. After centrifugation, the protein level was determined in the supernatants using a Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, Rockford, IL). Aliquots containing 50  $\mu\text{g}$  of total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. The aliquots were then loaded onto a 7.5% polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Pall Crop, East Hills, NY). To reduce background staining, the

membranes were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min, followed by incubation with diluted sheep anti-SOD1 antibody (1:1,000) or diluted mouse anti-HNE (1:1,000), peroxidase-conjugated goat anti-mouse IgG or rat anti-sheep (Sigma, St Louis, MO) and an ECL kit (Pierce Chemical, Rockford, IL).

**Quantification of data and statistical analysis:** All measurements were performed in order to ensure objectivity in blind conditions, by two observers for each experiment, carrying out the measures of experimental samples under the same conditions.

Fifteen sections per animal were randomly selected from the corresponding areas of the dentate gyrus in order to quantitatively analyze HNE and SOD1 immunoreactivity in the hippocampal CA1 region. The corresponding areas in the hippocampal CA1 region were measured on the monitor at a magnification of 25–50 $\times$ . Images of all HNE and SOD1 immunoreactive structures taken from 3 layers (stratum radiatum, pyramidale and oriens) of hippocampal CA1 region were obtained through a BX51 light microscope (Olympus, Tokyo, Japan) equipped with a digital camera (DP71, Olympus, Tokyo, Japan) connected to a PC monitor. The images were processed into an array of 512  $\times$  512 pixels corresponding to a tissue area of 140  $\times$  140  $\mu\text{m}$  (40 $\times$  primary magnification). Each pixel resolution was 256 gray levels. The staining intensity of all HNE and SOD1 immunoreactive structures was evaluated on the basis of an optical density (OD), which was obtained after the transformation of the mean gray level using the formula:  $\text{OD} = \log(256/\text{mean gray level})$ . The OD of background was taken from areas adjacent to the measured area. After the background density was subtracted, a ratio of the OD of image file was calibrated as % (relative optical density, ROD) using Adobe Photoshop version 8.0 and then analyzed using NIH Image 1.59 software. The result of the Western blot analysis was scanned, and the quantification of the Western blotting was done using Scion Image software (Scion Corp., Frederick, MD), which was used to count the ROD.

The data shown here represent the means  $\pm$  SEM of experiments performed for each hippocampal CA1 region. Differences among the means were statistically analyzed by student *t*-test analysis of variance to elucidate differences between adult and aged groups. Statistical significance was considered at  $P < 0.01$ .

## RESULTS

**NeuN immunoreactivity:** NeuN immunoreactive neurons in the adult group were distributed mainly in the stratum pyramidale of the hippocampal CA1 region (Fig. 1A). In the aged group, NeuN immunoreactive neurons were similar to those in the adult group; however, NeuN immunoreactivity in the aged group was slightly weaker than that in the adult group (Fig. 1D).

**HNE immunoreactivity:** In the adult group, HNE immunoreactivity was detected mainly in the stratum pyramidale

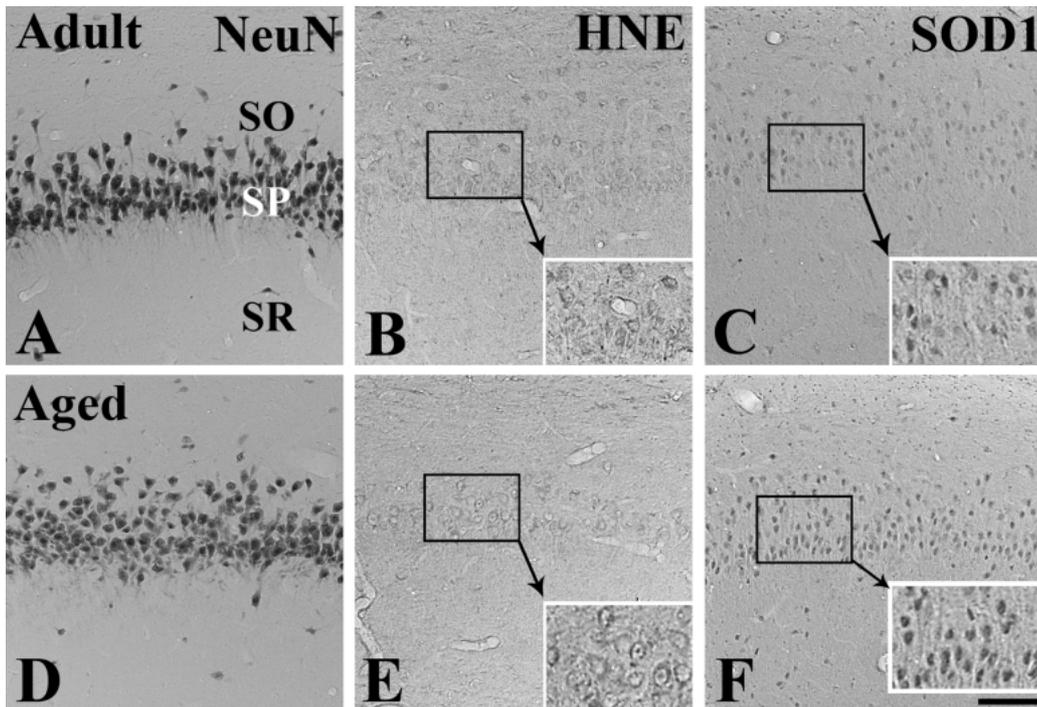


Fig. 1. Immunohistochemistry for NeuN (A and D), 4-hydroxy-2*E*-nonenal (HNE) (B and E) and Cu,Zn-superoxide dismutase (SOD1) (C and F) in the hippocampal CA1 region of adult (A, B and C) and aged dogs (D, E and F). NeuN immunoreactivity in the adult group is somewhat weaker than that in the aged group. HNE and SOD1 immunoreactivity in the stratum pyramidale (SP) in the aged group (see the arrowed-boxes) is higher than that in the adult group. Bar = 100  $\mu$ m.

of the CA1 region. The HNE immunoreactivity was observed mainly in the periphery of cytoplasm (Fig. 1B). In the aged group, HNE immunoreactivity was also detected in neurons in the CA1 region (Fig. 1E) and was higher than that in the adult group (Fig. 2). In the aged group, HNE immunoreactivity was also detected in the nucleus as well as cytoplasm (Fig. 1E).

**SOD1 immunoreactivity:** SOD1 immunoreactivity in the hippocampal CA1 region of the adult group was detected mainly in the stratum pyramidale (Fig. 1C). In this group, SOD1 immunoreactivity was also observed in some cells in the strata oriens and radiatum (Fig. 1C). In the aged group, SOD1 immunoreactivity was markedly increased in the stratum pyramidale (Figs. 1F and 2). In this group, SOD1 immunoreactivity was very strong in the nucleus. In addition, SOD1 immunoreactivity in the aged group was increased in the stratum radiatum (Fig. 1F).

**HNE and SOD1 protein levels:** Western blot analysis for HNE and SOD1 levels in the hippocampal CA1 region of adult and aged dogs concurred with immunohistochemical results. Increased HNE protein level in the aged dogs was detected between 37.1 kDa and 64.2 kDa when compared to that in the adult dogs (Fig. 3A). SOD1 protein levels in hippocampal CA1 homogenates were found to be significantly high in aged dogs compared to that in adult dogs (Fig. 3B).

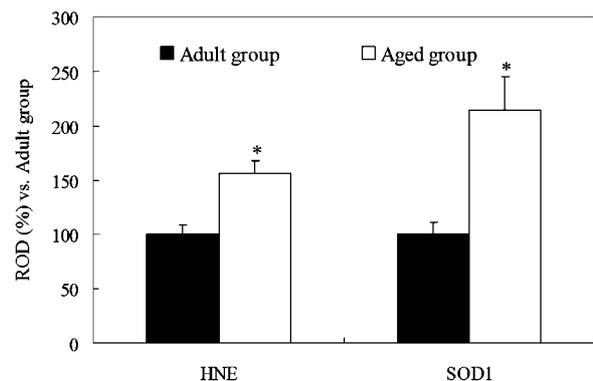


Fig. 2. Relative optical density (ROD) in the hippocampal CA1 region of adult and aged dogs quoted as a percentage of HNE and SOD1 immunoreactivity in the adult group. Differences between means were analyzed using the student's *t*-test ( $n=5$  per group;  $*P<0.01$ , significantly different from the adult group). Bars indicate means  $\pm$  SEM.

## DISCUSSION

It has been reported that oxidative stress increases with age [14]. Aging significantly increases superoxide radical levels in the hippocampus of Wistar Kyoto rats [1]. It is now known that the reaction of  $O_2^-$  and nitric oxide (NO)

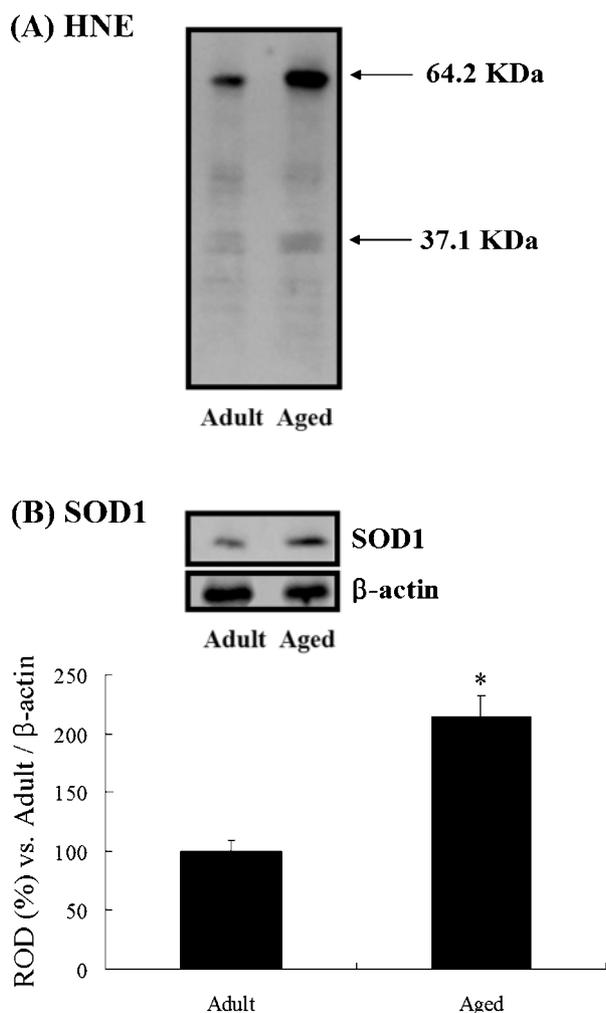


Fig. 3. Western blot analysis of HNE and SOD1 in the hippocampal CA1 region of adult and aged dogs. Relative optical density (ROD) is quoted as a percentage of HNE and SOD1 protein band in adult group ( $n=3$  per group; \*  $P<0.01$ , significantly different from the adult group). The bars indicate means  $\pm$  SEM.

results in the formation of peroxynitrite ( $\text{ONOO}^-$ ), a powerful and toxic oxidant [2]. SOD prolongs the half-life of NO via the inhibition of reaction with  $\text{O}_2^-$  to protect neurons from exogenous damage.

HNE is an aldehyde that is generated during lipid peroxidation which can impair cellular function [12]. In this study, we investigated HNE and protein levels in the hippocampal CA1 region of adult and aged dogs. The HNE immunoreactivity and protein level were significantly increased in the aged dogs compared to that in the adult dogs. Especially, increased HNE protein level in the aged dogs was detected between 37.1 kDa and 64.2 kDa when compared to that in the adult dogs. However, in the present study, NeuN positive neurons in the CA1 region were similar in both adult and aged groups. The increase of HNE may be associated with the age-related increase of lipid peroxidation in these

molecular weights in the hippocampal CA1 homogenates.

In brain, ROS can induce the peroxidation of lipids and generate highly reactive aldehydes [9] which can easily diffuse and attack intracellular molecules such as polyunsaturated fatty acids. In this process, free radicals generate several aldehydes such as HNE. These aldehydes have high reactivity with sulfhydryl groups of cysteine, histidine, and lysine [9] which are the main components of neuron survival enzymes including  $\text{Na}^+/\text{K}^+$  ATPase [17], glucose, and glutamate transporters [18]. HNE is neurotoxic and present at increased concentrations in brain tissue and cerebrospinal fluid of Alzheimer's disease patients, in the spinal cord of amyotrophic lateral sclerosis patients, and is found in Lewy bodies in brain stem neurons in Parkinson's disease patients [25].

In this study, we also observed SOD1 immunoreactivity and its level in hippocampal CA1 region of adult and aged dogs. The SOD1 immunoreactivity and protein level were high in the aged dogs compared with the adult dogs. This result was supported by our previous and other studies that SOD1 immunoreactivity, mRNA and enzyme activity were increased in the aged brains compared to that in the adult brains [4, 5, 24]. It was reported that aging in spontaneously hypertensive stroke prone rats (SHRSP) significantly increased brain superoxide level compared with that in Wistar Kyoto rats [1]. They also suggested that the excess cortical superoxide level in the SHRSP may be associated with a down-regulation of SOD1 but is not related to a decrease in estrogen.

In brief, HNE protein level in hippocampal CA1 homogenates in aged dogs significantly increased between 37.1 kDa and 64.2 kDa compared that in adult dogs. In addition, SOD1 immunoreactivity in the CA1 region of aged dogs was highly strong nuclei. These results suggest that neurons in the hippocampal CA1 region of aged brains may be susceptible to oxidative stress compared with adult brains regardless of neuronal loss.

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