

Comparison of catalase immunoreactivity in the hippocampus between young, adult and aged mice and rats

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Abstract. Catalase (CAT) is an important antioxidant enzyme and is crucial in modulating synaptic plasticity in the brain. In this study, CAT expression as well as neuronal distribution was compared in the hippocampus among young, adult and aged mice and rats. Male ICR mice and Sprague Dawley rats were used at postnatal month (PM) 1, PM 6 and PM 24 as the young, adult and aged groups, respectively (n=14/group). CAT expression was examined by immunohistochemistry and western blot analysis. In addition, neuronal distribution was examined by NeuN immunohistochemistry. In the present study, the mean number of NeuN-immunoreactive neurons was marginally decreased in mouse and rat hippocampi during aging, although this change was not identified to be significantly different. However, CAT immunoreactivity was significantly increased in pyramidal and granule neurons in the adult mouse and rat hippocampi and was significantly decreased in the aged mouse and rat hippocampi compared with that in the young animals. CAT protein levels in the hippocampus were also lowest in the

aged mouse and rat hippocampus. These results indicate that CAT expression is significantly decreased in the hippocampi of aged animals and decreased CAT expression may be closely associated with aging.

Introduction

Reactive oxygen species (ROS) are inevitably produced during cellular energy metabolism (1), however, increased intracellular ROS production causes oxidative damage to critical macromolecules, including DNA, proteins and lipids (2-4). Oxidative stress has been considered a major aspect of aging (5-8), and is closely associated with an onset of several neurodegenerative diseases (9,10).

Numerous antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), thioredoxin (Trx) and peroxiredoxin (Prx) defend against oxidative stress by maintaining a balanced intracellular redox state (11,12); SOD converts superoxide radical ($O_2\cdot^-$) to hydrogen peroxide (H_2O_2) and water, and CAT or GPx converts H_2O_2 to water and oxygen (13).

Alterations in various molecular processes in the hippocampus during aging is a great deal of interest as hippocampus-dependent learning and memory is impaired in nearly half of the healthy elderly population over 60 years of age (14). In addition, the hippocampus is vulnerable to neurological diseases, such as cerebral ischemia, vascular dementia and Alzheimer's disease (15-17). In this regard, numerous researchers have demonstrated age-related changes in the levels of antioxidant enzymes levels in the hippocampus. In particular, it has been reported that CAT activity is unchanged or decreased in the aged hippocampus of the mouse (18) and rat (19,20).

Although certain studies have reported the age-associated changes in CAT activity in the hippocampus, it has not been fully elucidated. Therefore, in the present study, in order to show fundamental data on the change in CAT levels during normal aging, CAT expression was compared in the

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hippocampus among the young, adult, and aged mice and rats, which are good animal models in aging research (21,22), using immunohistochemistry and western blot analysis.

Materials and methods

Experimental animals. Male ICR mice and Sprague Dawley rats were purchased from Orient Bio Inc. (Seongnam, Korea). They were used at ages 1, 6 and 24 months for the young, adult and aged groups, respectively, as the ages of mice and rats are similar (23–25). The animals (n=14/group) were housed in a conventional state under adequate temperature (23°C) and humidity (60%) control with a 12-h light/dark cycle, and provided free access to food and water. Animal handling and care followed the guidelines of current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, The National Academies Press, 8th Ed., 2011) (26), and the experimental protocols were approved by the Institutional Animal Care and Use Committee of Kangwon National University (Chuncheon, Korea; approval no. KW-130424-3). All experiments were conducted with the aim of minimizing the number of animals used and avoiding animal suffering.

Tissue processing for histology. The animals (n=7/group) were anesthetized intraperitoneally 40 mg/kg pentobarbital sodium (JW Pharmaceutical Co., Ltd., Seoul, Korea) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4, Sigma-Aldrich, St. Louis, MO, USA) followed by 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M PBS (pH 7.4, Sigma-Aldrich). The brains were removed and post-fixed with the same solution for 6 h. The tissues were cryoprotected by infiltration with 30% sucrose (Sigma-Aldrich) overnight. The brain tissues were then frozen and sectioned (30 μ m) with a cryostat (CM1520, Leica Microsystems, Wetzlar, Germany), and consecutive sections were collected in six-well plates containing 0.1 M PBS.

Immunohistochemistry. To examine age-related changes in NeuN and CAT immunoreactivity in the hippocampi of the mice and rats, immunohistochemical staining and quantitative analysis of immunohistochemical data were performed according to a previous method (27). Monoclonal mouse anti-NeuN (1:800; EMD Millipore, Billerica, MA, USA; cat. no. MAB377) and polyclonal rabbit anti-CAT antibody (1:250; Abcam, Cambridge, MA, USA; cat. no. ab52477) used as primary antibodies overnight at 4°C, followed by incubation with biotinylated horse anti-mouse (1:200; cat. no. AI-2000) or goat anti-rabbit IgG (1:200; cat. no. AI-1000) obtained from Vector Laboratories, Inc., Burlingame, CA, USA) secondary antibodies for 2 h at room temperature and streptavidin peroxidase complex (1:200; Vector Laboratories, Inc.). A negative control test was conducted using 5% pre-immune serum (Vector Laboratories, Inc.) instead of primary antibody in order to establish the specificity of the immunostaining. The negative control resulted in the absence of immunoreactivity in any neurons.

According to anatomical landmarks corresponding to the mouse and rat brain atlas, seven sections with 120- μ m intervals per animal were selected to quantitatively analyze NeuN and CAT immunoreactivity, respectively. As previously

described (28), digital images of the hippocampus were captured with an Axiom1 light microscope (Carl Zeiss, Oberkochen, Germany) equipped with a digital camera (Axiocam, Carl Zeiss) connected to a PC monitor. NeuN- and CAT-immunoreactive neurons were counted in a 250x250 μ m square in the hippocampi using an image analyzing system with Optimas 6.5 (CyberMetrics, Scottsdale, AZ, USA) software. Cell counts were obtained by averaging the counts from each animal. In addition, the density of CAT-immunoreactive neurons was evaluated on the basis of optical density (OD), which was obtained after the transformation of the mean gray level using the formula: $OD = \log(256/\text{mean gray level})$. The OD of the background was taken from areas adjacent to the measured area. After the background density was subtracted, a ratio of the optical density of the image file was calibrated as a % (relative optical density, ROD) using Adobe Photoshop version 8.0 (Adobe Systems Inc., San Jose, CA, USA) and then analyzed using NIH Image J software (version 1.46; NIH Image, Bethesda, MD, USA). A ratio of the ROD was calibrated as a %, with the young group designated as 100%.

Western blot analysis. To examine changes in the CAT levels in the mouse and rat hippocampi between groups, animals at each age (n=7) were used, and western blot analysis was performed according to a previous method (27). In brief, after sacrificing the animals by cervical dislocation, the striatum was removed. The tissues were then homogenized in 50 mM PBS containing 0.1 mM ethylene glycol bis (2-aminoethyl ether)-N, N, N', N' tetraacetic acid (pH 8.0, Sigma-Aldrich), 0.2% Nonidet P-40 (Sigma-Aldrich), 10 mM ethylenediamine tetraacetic acid (pH 8.0, Sigma-Aldrich), 15 mM sodium pyrophosphate (Sigma-Aldrich), 100 mM β -glycerophosphate, (Sigma-Aldrich), 50 mM NaF (Sigma-Aldrich), 150 mM NaCl (Sigma-Aldrich), 2 mM sodium orthovanadate (Sigma-Aldrich), 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) and 1 mM dithiothreitol (DTT, Sigma-Aldrich). After centrifugation at 10,000 x g for 20 min, the protein level in the supernatants was determined using a Micro bicinchoninic acid protein assay kit (Sigma-Aldrich) with bovine serum albumin as a standard (Pierce Chemical, Rockford, IL, USA). Aliquots containing 20 μ g total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8, Sigma-Aldrich), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. The aliquots were then loaded onto a 12% polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Pall Corp., East Hills, NY, USA). To reduce background staining, the membranes were incubated with 5% non-fat dry milk (Sigma-Aldrich) in PBS containing 0.1% Tween-20 (PBST; Sigma-Aldrich) for 45 min at room temperature. Membranes were incubated with polyclonal rabbit anti-CAT antibody (1:1,000) or monoclonal mouse β -actin (1:5,000; Sigma-Aldrich; cat. no. A5316) overnight at 4°C. Following washing with PBST three times, the membranes were incubated with peroxidase-conjugated donkey anti-rabbit IgG (1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. sc-2305) or goat anti-mouse (1:1,000; Santa Cruz Biotechnology, Inc.; cat. no. sc-2031) for 1 h at room temperature. An enhanced chemiluminescence kit (Pierce ECL Western Blotting Substrate; Thermo Fisher Scientific, Inc.) was used to detect the protein expression. Western blot analysis was performed with three repetitions. The result of the western blot

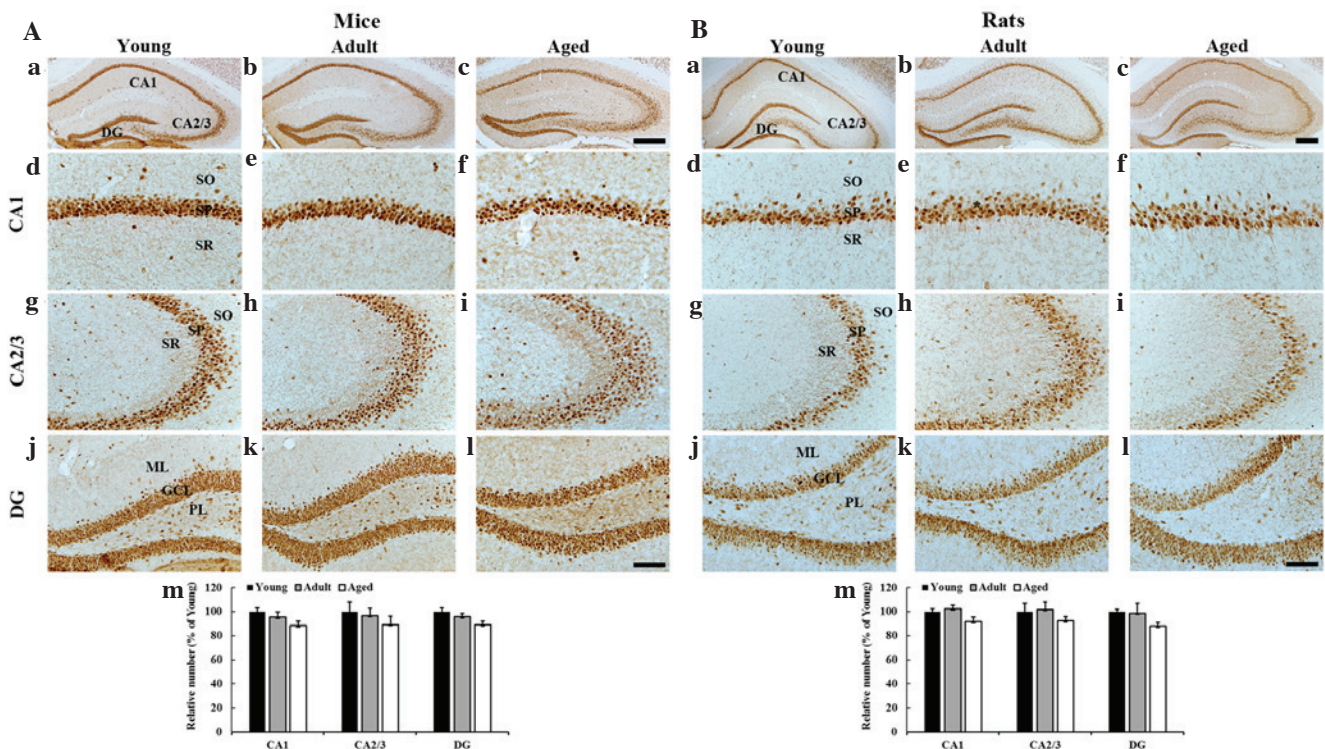


Figure 1. NeuN immunohistochemistry in the hippocampus of the (A) mice and (B) rats in the young, adult and aged groups. The distribution pattern and numbers of NeuN-immunoreactive neurons are not significantly different among all of the groups. GCL, granule cell layer; ML, molecular layer; PL, polymorphic layer; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; DG, dentate gyrus. Scale bar, 400 μ m (a-c) and 100 μ m (d-l). (m) Mean percentage of NeuN-immunoreactive neurons in the hippocampus (n=7 per group). The bars indicate the mean \pm standard error of the mean.

analysis was scanned, and densitometric analysis for the quantification of the bands was conducted using Scion Image software (version 4.0.3.2; Scion Corp., Frederick, MD, USA), which was used to count ROD. CAT levels were normalized to β -actin, used as the internal control protein, respectively. A ratio of the ROD was calibrated as %, with the young group designated as 100%.

Statistical analysis. The data shown here represent the mean \pm standard error of the mean. Differences of the means among the groups were statistically analyzed by analysis of variance with a post hoc Bonferroni's multiple comparison test in order to elucidate age-related differences among groups. Statistical analysis was performed using GraphPad InStat (version 3.05; GraphPad Software, Inc.; La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

NeuN immunoreactivity

Mice. Numerous NeuN-immunoreactive neurons were distributed in the stratum pyramidale of the hippocampus proper (CA1-3 regions) and the granule cell layer of the dentate gyrus in the young, adult and aged mice. In addition, no significant difference was identified in the mean number of NeuN-immunoreactive neurons in the hippocampus proper and dentate gyrus among the groups (Fig. 1A).

Rats. The distribution pattern of NeuN-immunoreactive neurons in the rats was similar to that in the mice. In addition,

the number of NeuN-immunoreactive neurons in the hippocampus proper and dentate gyrus was not significantly changed among the groups (Fig. 1B).

CAT immunoreactivity

Mice. CAT immunoreactivity was then analyzed in the mice (Fig. 2A). In the young mice, CAT immunoreactivity was observed primarily in the pyramidal cells of the stratum pyramidale and in certain non-pyramidal neurons in the strata oriens and radiatum of the hippocampus proper (Fig. 2Ad and g). In addition, CAT immunoreactivity was easily detected in granule cells of the granule cell layer and hilar neurons of the polymorphic layer of the dentate gyrus (Fig. 2Aj).

In the adult mice, the distribution pattern of CAT-immunoreactive neurons was similar to that in the young mice; however, the CAT immunoreactivity in all layers of the hippocampus proper and dentate gyrus was significantly increased compared with that in the young mice (Fig. 2Ae, h, k and m).

In the aged mice, CAT immunoreactivity in all layers of the hippocampus proper was significantly decreased compared with that in the young and adult mice; however, CAT immunoreactivity in the dentate gyrus was similar to the young mice (Fig. 2Af, i, l and m).

Rats. Generally, the distribution pattern of CAT-immunoreactivity in the rats (Fig. 2B) was similar to that in mice (Fig. 2A). In the adult rats, the CAT immunoreactivity was significantly increased in all layers of the hippocampus proper and dentate gyrus compared with that in the young rats (Fig. 2Be, h, k and m). However, in the aged

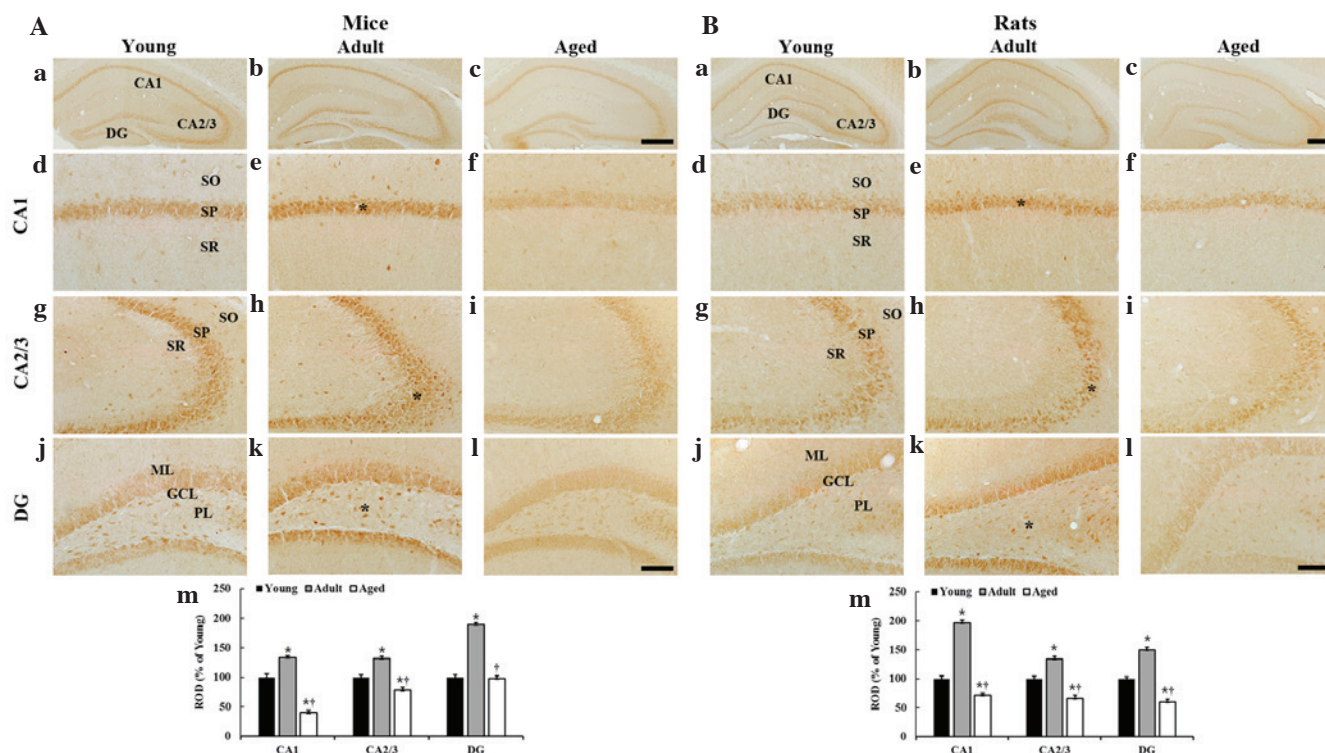


Figure 2. CAT immunohistochemistry in the hippocampus of the (A) mice and (B) rats in the young, adult and aged groups. CAT immunoreactivity is highest in the adult group and lowest in the aged group. GCL, granule cell layer; ML, molecular layer; PL, polymorphic layer; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; DG, dentate gyrus. Scale bar, 400 μm (a-c) and 100 μm (d-l). (m) ROD as % of CAT-immunoreactive structures in the hippocampus ($n=7$ per group). * $P<0.05$, compared with the young group and † $P<0.05$, compared with the adult group. The bars indicate the mean \pm standard error of the mean. ROD, relative optical density.

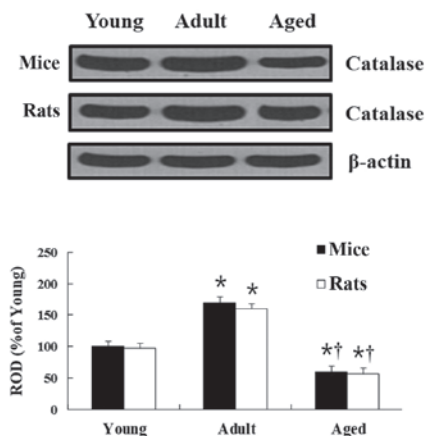


Figure 3. Western blot analysis of catalase (~60 kDa) in the hippocampus of the young, adult and aged mice and rats. The ROD as percentage of the immunoblot band is presented ($n=7$ per group). * $P<0.05$, compared with the young group, † $P<0.05$, compared with the adult group. The bars indicate the mean \pm standard error of the mean. ROD, relative optical density.

rats, CAT immunoreactivity was significantly decreased in all layers of the hippocampus proper and the dentate gyrus compared with that in the young and adult rats (Fig. 2Bf, i, l and m).

CAT protein level

Mice. In the western blot analysis, age-related changes of CAT levels in the hippocampus were observed to be similar

to those identified in the immunohistochemical results. In the hippocampus of the adult group, the CAT protein level was significantly increased compared with that in the young group. In the hippocampus of the aged group, CAT protein level was significantly decreased compared with that in the young group (Fig. 3).

Rats. Age-associated changes to the CAT protein levels in the rats were similar to that in the mice (Fig. 3).

Discussion

The present study aimed to investigate the age-related changes in neuronal distribution in the hippocampi of young, adult and aged mice and rats using NeuN immunohistochemistry. No significant changes in cell morphology and distribution patterns were identified during normal aging among all of the groups, although the mean number of NeuN-immunoreactive neurons was marginally decreased in all hippocampal subregions of the mice and rats during aging. Previously, the presence of age-related neuronal loss was reported, however, no significant difference was identified in the number of cresyl violet-positive neurons in the hippocampal CA1 region in aged gerbils compared with that in young and adult gerbils (29). In addition, the results of the present study are supported by a previous study that showed that neuronal loss was found in the hippocampi of aged rodents (30).

In the present study, CAT expression in the hippocampus was compared among young, adult, and aged mice and rats, and

it was demonstrated that CAT immunoreactivity was highest in the pyramidal and granule neurons in the adult hippocampi and lowest in the aged hippocampi. In addition, CAT protein levels in the hippocampi were lowest in the aged mice and rats. The results regarding the aged animals are consistent with those of previous studies, which reported that CAT activity was significantly diminished in the rat hippocampus during the normal aging process (20,31). However, other studies have shown inconsistent results; CAT activity in the hippocampus was unchanged in aged Fischer-344 rats (19) and C57BL/6 N mice (18). Furthermore, Sohal *et al* (32) reported that CAT was increased in the hippocampi of mice during aging. The present study hypothesized that the levels of CAT in the hippocampus from aged animals is lower than that those from young and adult animals. However, the exact mechanism underlying the decreased CAT levels in the aged hippocampus remains to be determined. It is likely that the decreased CAT expression may be associated with age-related functional changes in the hippocampus.

It is well known that CAT, an important antioxidant enzyme, protects cells from oxidative damage by ROS (33,34). In the brain, CAT exerts numerous important functions and shows a strong correlation between normal aging and increased oxidative damage (31). For example, Lee *et al* (35) reported that overexpressing CAT using a viral vector delivery system in aging rats decreased oxidative damage in the hippocampus. Furthermore, CAT is crucial in modulating synaptic plasticity. Olsen *et al* (36) demonstrated that overexpressing mitochondrial CAT in mice resulted in improvements in spatial learning and memory tested with a water maze test. In addition, a recent study showed that exogenous treatment with a synthetic CAT mimetic in aged mice ameliorated the age-related decline in cognitive impairment (37).

In conclusion, the present study shows that CAT expression was significantly decreased in the hippocampi of mice and rats from the aged group compared with that in the young and adult groups, and suggests that the decreased CAT expression may be closely associated with age-related changes in the aged hippocampus.

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