The role of superoxide dismutase 1 in the selected vulnerability of hippocampal subfield after status epilepticus

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The role of superoxide dismutase 1 in the selected vulnerability of hippocampal subfield after status epilepticus

Directed by Professor Kyoung Heo

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Yang-Je Cho

December 2014

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December 2014

ACKNOWLEDGEMENTS

With love, this doctoral dissertation is dedicated to my parents who have taught me the faithfulness and meaning of hard working.

With my great gratitude, this doctoral dissertation is dedicated to my mentor Kyoung Heo who has taught me the way of devotion as a doctor.

I would like to thank every people for the great help in preparation of the doctoral dissertation, especially to the professor Baik-Lin Eun, Hoon Chul Kang, Chul Hoon Kim, Ki Taek Nam, and to all members of the laboratory, especially professor Byung In Lee and Won-Joo Kim.

December 2014

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ABSTRACT

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Selected vulnerability of hippocampal neuron after epileptic insults is a well-known phenomenon. Pyramidal neurons in the Cornus Ammonis (CA) 1 and 3 are known as particularly vulnerable; however, the granule cells in the dentate gyrus (DG) as well as neurons in the CA2 are known to be resistant to the various insults including status epilepticus (SE) in the animal models or even in the human temporal lobe epilepsy. The molecular basis for this different susceptibility remains controversial, but different defense mechanisms against exogenous insults have been suggested.

Excessive reactive oxygen species (ROS) production has been reported as a responsible process for neural cell death in the various neurological disorders such as ischemic stroke, Alzheimer's disease, or Huntington disease. In addition, the

pathological overproduction of ROS has been suggested as a crucial process in the neuronal death by SE. However, the role of superoxide anion ($O2^{\bullet-}$), one of the most potent ROS, in the seizure-induced neuronal death has remained unclear.

I hypothesized that the pathological excessive production of $O2^{\bullet-}$ may contribute to hippocampal neuronal death after SE, and different activation of superoxide dismutase 1 (SOD1), the selective inhibitor of $O2^{\bullet-}$, in the hippocampal subfields might be responsible for this selected vulnerability after SE.

Adult male C57BL/6J mice were given injections of pilocarpine to induce SE, which was confirmed by visual inspection and electroencephalography. Hippocampal neuronal death was assessed by both cresyl-violet and TUNEL staining. The temporal and spatial production of $O2^{\bullet-}$ in each hippocampal subfield was investigated using *in situ* detection of oxidized hydroethidine (HEt). Western blot, activity assay, and immunohistochemical staining of SOD1 in the each hippocampal subfield were performed to investigate the role of specific defense system of $O2^{\bullet-}$. Lamotrigine (LTG), a neuroprotectant in the various animal models by modulating ROS, was treated to see its effect on SOD1. Inhibitor of SOD1, diethyldithiocarbamate (DDC) was treated to identify the effect of SOD1 inhibition.

Neuronal cell death and TUNEL-positive cells increased significantly in the hippocampal CA1 and CA3 compared to DG after SE. $O2^{\bullet-}$ measured by oxidized HEt significantly increased after pilocarpine-induced SE, especially in the CA1 and CA3 compared to DG. In the normal control, SOD1 expression in the DG was

significantly higher than those of CA1 and CA3. The expression and activity of SOD1 significantly increased in the DG compared to CA1 and CA3 as early as 12 hours after SE. Treatment of LTG significantly increased SOD1 activity, expression, and decreased $O2^{\bullet-}$ production, thus ameliorated the selective neuronal death in the CA1 and CA3. DDC treatment resulted in marked decrease of SOD1 activity, increased production of $O2^{\bullet-}$, and subsequent extensive neuronal cell death in the DG after SE.

This study confirmed that the excessive production of $O2^{\bullet-}$ after SE resulted in different neuronal cell death according to the hippocampal subfields, to less degree in the DG compared to CA1 and CA3. Increased expression and activity of SOD1 in the DG and marked neuronal death by SOD1 inhibition were confirmed. The difference defense mechanism against $O2^{\bullet-}$ by SOD1 in each hippocampal subfield may have a pivotal role in the selected vulnerability of hippocampal subfields after SE.

Key words: Epilepsy, Superoxide anion, Superoxide dismutase 1, Status epilepticus, Hippocampus, Dentate gyrus, Selective vulnerability

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I. INTRODUCTION

Epilepsy is one of the most common neurological disorders and defined as an enduring predisposition of spontaneous seizure recurrences by cerebral dysfunction.[1, 2] Mechanisms of underlying development of epilepsy, the epileptogenesis, are very complicated and remain still unrevealing. The process usually starts following acute neuronal injury, commonly called as initial precipitating injury (IPI) under influence of different genetic susceptibility. After IPI, complex histological and molecular processes such as receptor changes, synaptic reorganization, gliosis, and inflammation continuously undergo, and these changes finally result in pathologically altered hyperexcitable neuronal networks, leading to a pathologic condition characterized with spontaneous and recurrent seizure generation, the epilepsy. The IPI occurs in the diverse clinical situations

such as complex febrile seizure, encephalitis, traumatic brain injury, stroke, various perinatal insults as well as status epilepticus (SE).[1, 2] SE is defined as sustained seizure activity for prolonged period or two or more consecutive seizures without fully regaining of consciousness. SE can induce extensive neuronal cell death by continuous seizure activity through the mechanisms of excitotoxicy, acute inflammatory reaction, energy failure, and pathological overproduction of reactive oxygen species (ROS), and one of the most vulnerable brain regions by uncontrolled SE is hippocampus.[1-5]

Hippocampus can be easily damaged by prolonged seizure activity in both human epilepsy and the animal SE models. The resultant hippocampal sclerosis (hippocampal neuronal cell death with extensive gliosis) is the most common pathology responsible for human mesial temporal lobe epilepsy, the most common focal epilepsy in human.[6, 7] Interestingly, there is selective vulnerability reacting to various IPI within hippocampal subfields, highly vulnerable pyramidal neuron in the Cornus Ammonis (CA) 1, 3, and 4, but highly resistant granule cells in the dentate gyrus (DG) as well as neurons in the CA2. These patterns are relatively consistent finding across human epilepsy and various animal epilepsy models. Hippocampal sclerosis in human is a well-known example showing this discrepancy, characterized by selective loss of neuron in the CA1, CA3, and CA4.[6, 7] Although this unique susceptibility difference to injury within the hippocampus has been known as early as in the late nineteenth century, the molecular and cellular basis of this selectivity has been extensively debated and remained unclear for

decades. Different intrahippocampal vasculature, selective neuronal vulnerability to excitotocixity, different Ca^{2+} homeostasis, selective loss of GABAergic interneuron, and different ROS formation by mitochondrial activities are known as possible candidates.[7, 8]

ROS, under normal physiological condition, do various physiologic roles such as signal processing, and its production within cells is precisely controlled by diverse antioxidant defense system to prevent any excessive oxidative stresses resulting neuronal cell death.[9, 10] This neutralizing antioxidant system consists of various free radical scavenging enzymes, such as catalase, kinds of superoxide dismutases (SOD), glutathione peroxidase as well as numerous non-enzymatic antioxidants such as glutathione.[9-11] The balance between ROS and its scavenging systems is disturbed in the various pathologic conditions, including both acute and chronic neurodegenerative diseases such as hypoxia, ischemic stroke, Alzheimer disease, Parkinson disease, and Huntington disease.[4, 11] Extensive neuronal death in the hippocampus was also associated with increased lipid peroxidation, free radical formation, and decreased glutathione content in the various animal SE models.[3, 4, 11-13] During SE, ROS production in the hippocampal region is rapidly and extensively increased by repetitive seizure activity in concordance with impairment of defense mechanism, leading to acute neuronal cell death in this structure.[5, 12-14] Given that seizure activity within the hippocampus is generally similar across subfields, the different net effect between ROS and its defense mechanism among different hippocampal subfields may explain the selective vulnerability of hippocampal

subregions.

Superoxide anion ($O2^{\bullet-}$) is the most potent free radical and a key molecule of ROS-mediated cell death. It is rapidly reversed by various SODs. The role of $O2^{\bullet-}$ and SOD in hippocampal neuronal death during SE has not been extensively studied, and remained to be elucidated.[13, 15-25]

In this study, I hypothesized and tried to reveal that the pathological excessive production of $O2^{\bullet-}$ after SE may contribute to hippocampal neuronal cell death, and the different activation or expression of SOD1, the selective inhibitor of $O2^{\bullet-}$, in the different hippocampal subfields might be responsible for this selected vulnerability after SE.

II. MATERIALS AND METHODS

1. Pilocarpine-induced SE in mice and seizure assessment

All procedures were approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Adult male C57BL/6 mice (20 to 25 g, Orientbio, Gyeonggi, Korea) were used in this study. Mice were housed under a 12 hours light/dark cycle with food and water *ad libitum*. Three to five mice were used in each experimental group at each time point. Mice were pretreated by intraperitoneal (i.p.) injection with methyl scopolamine (1 mg/kg, i.p.; Sigma, St. Louis, MO, USA) to reduce peripheral cholinergic effects. Thirty minutes after methyl scopolamine injection, mice were given injections of pilocarpine hydrochloride (325 mg/kg, i.p.; Sigma) or the same volume of normal saline as a vehicle control. All animals treated with pilocarpine started focal motor seizures and progressively fell into SE within 1 hour. Only mice experienced sustained SE showing more than Racine stage 4 seizures were included to the study.[26] Diazepam (10 mg/kg, i.p.; Samjin pharmacy, Seoul, Korea) was administered to stop seizures two hours after the SE onset for controlling the seizure duration.[27-29] Electroencephalography (AS40, Grass technologies, West Warwick, RI, USA) was done to confirm electrographic seizures in the selected animals (Fig. 1).

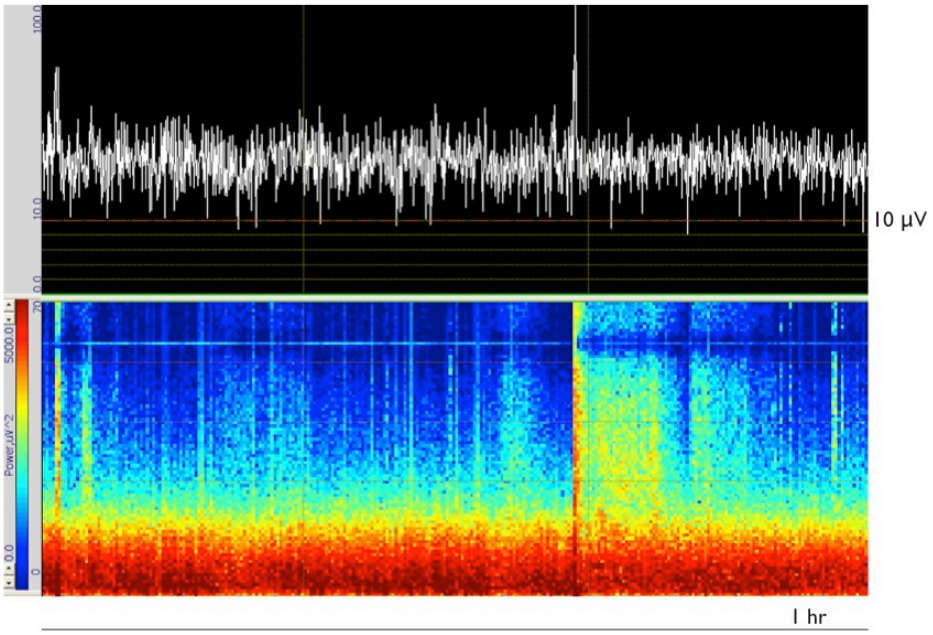

Figure 1. Electroencephalography (EEG) monitoring during status epilepticus (SE). Epidural electrode EEG monitoring during SE in the selected mice confirmed continuous seizure activity after pilocarpine injection. Both raw tracing of EEG (upper panel) and compressed spectral array (lower panel) were displayed.

2. Tissue preparation for histological assessment

Animals were anesthetized and transcardially perfused with heparinized saline. Following perfusion, fresh dorsal hippocampus was dissected and used for Western blot analysis and activity assay. For histological analysis brains were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) after the perfusion with heparinized saline and then isolated. They were additionally post-fixed using the same fixatives overnight at 4°C and then sectioned coronally in 16 μm thickness using a cryostat. For histological assessment of hippocampal pyramidal damages, cresyl violet staining was performed. Sections were immersed in water and stained in 0.2% cresyl fast violet acetate for five minutes. Then the sections were dipped well in absolute alcohol and rinsed with water, and they were cleaned and mounted with mounting solution.[27-29]

3. Detection of $O2^{\bullet-}$ free radicals by *in situ* hydroethidine assessment

To assess the temporal and spatial production of ROS after SE, *in situ* detection of oxidized hydroethidine (HEt, Molecular Probes, Eugene, OR, USA) was performed. HEt is oxidized to ethidium reacting $O2^{\bullet-}$ free radicals. HEt (stock solution 100 mg/ml in dimethyl sulfoxide) was diluted to 1 mg/ml in PBS just before use, transferred to a 1 ml foiled syringe, and sonicated in a water bath. A total of 200 μl of HEt was administrated intravenously through the jugular vein one hour before sacrifice. The animals were killed and transcardially perfused with saline with heparin and 3.7% formaldehyde in PBS. The brain sections were incubated

with 2.5 x 10^{-3} mg/ml Hoechst 33258 (Molecular Probes, USA) in PBS for 5 min in a dark chamber and then washed and mounted with VectorShield (Vector Labs, Burlingame, CA, USA).[29] The prepared sections were observed with a microscope and computerized digital camera system under fluorescent light (excitation 510 to 550 nm and emission > 580 nm; BX51; Olympus, Japan). To semi-quantify $O2^{\bullet-}$ production, cells in the cytosol with oxidized HEt were counted and averaged in the entire field after random determination of four different sites under high magnification (x 400) using the image analysis program. The percentage of these cells to the total cells stained with Hoechst 33258 nuclear staining was calculated.

4. Fluorescent labeling for DNA fragmentation

To identify DNA fragmentation in the degenerating neuron, terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphatebiotin nick end-labeling (TUNEL) staining was performed using a kit (Roche Diagnostics GmbH, Penzberg, Germany). Sections were incubated with TUNEL mixture for an hour at 37°C in a dark chamber. After washing, the sections were counter-stained with Hoechst 33258 and examined under a confocal laser-scanning microscope (LSM 700; Carl Zeiss, Thornwood, NY, USA).[27, 28]

5. Immunohistochemical and immunofluorescent staining of SOD1

For immunohistochemical staining, mice were perfused with heparinized saline followed by 3.7% formaldehyde in PBS. The brains were isolated and post-fixed in

the same fixative overnight at 4°C. The fixed sections were stained with specific antibodies for SOD1 (Calbiochem, Darmstadt, Germany) using biotinylated secondary antibody, ABC kit, and avidin fluorescein or avidin texas red (Vector Labs). For immunofluorescent staining, sections were blocked with PBS containing 5% BSA at room temperature for 1 hour and reacted with the primary antibody, sheep anti-SOD1 (1:200; Merck Millipore, Darmstadt, Germany).[20, 30] As a negative control, the sections were incubated without a primary antibody. Sections were washed with PBS, and reacted with the secondary antibody FITC-conjugate (1:200, Jackson Immuno Research Laboratories, West Grove, PA, USA) at room temperature for 1 hour and the stained sections were observed under LSM 700 confocal laser scanning microscopy. Intensity (optical density) in high-magnification field and expression patterns of the expression of SOD1 were analyzed with computerized analysis system and program (Image j; Molecular Devices).

6. Western blot analysis

Dissected hippocampal tissues were homogenized in the lysis buffer (20 mM Tris–HCl, pH 7.4, at 4°C; 137 mM NaCl; 25 mM β-glycerophosphate; 2 mM NaPPi; 1 mM Na3VO4; 1% Triton X-100; 10% glycerol; 2 mM benzamidine; 0.5 mM DTT; 1 mM phenylmethylsulfonyl fluoride). Homogenates were added to the sample buffer (125 mM Tris/HCl, 2% SDS, 10% glycerin, 1 mM DTT, and 0.002% bromphenol blue, pH 6.9) and boiled for 5 min. Proteins were resolved on 8% SDS-

poly acrylamide gels and blotted onto polyvinylidinedifluoride membranes (PVDF, Millipore, Bedford, MA, USA). Membranes were washed with TBS-T (50 mM Tris/HCl, 140 mM NaCl, pH7.3 containing 0.1% Tween 20) before blocking non-specific binding with TBS-T plus 5% skim milk for 1 hour. Membranes were incubated for 1 hour with the following polyclonal antibodies: sheep anti-SOD1 (1:140; Merck Millipore, Darmstadt, Germany). After washing, blots were incubated with secondary antibodies conjugated with horseradish peroxidase (1:5000 in TBS-T plus 5% skim milk) for 1 hour, followed by ECL plus (Amersham Biosciences, Piscataway, NJ, USA) detection.[27]

7. SOD1 activity assay

SOD1 activity was measured with a modified method as described by commercial SOD Assay Kit (Dojindo Molecular Technologies, Temecula, MD USA). Experimental animals were decapitated at various times after SE. The hippocampi were quickly removed and taken. The tissues were homogenized in cold sucrose buffer (0.25 M sucrose, 10 mM Tris, 1 mM EDTA, pH 7.4) using Teflon homogenizer. The homogenized the tissues were centrifuged at 10,000 ×g for 60 minutes at 4℃ and the cytosolic samples collected carefully to the new tubes. 2-methoxyestradiol (SOD2 inhibitor) was added to the cytosolic samples and was used in assay. SOD1 can be measured by blocking the SOD2 activity using 2-methoxyestradiol. The assay of SOD1 activity was based on the rate of WST-1 reduction by $O2^{\bullet-}$ related with the xanthine oxidase activity and its inhibition by

SOD1. One unit of SOD1 activity was defined as the amount that reduced the absorbance at 450 nm by 50% inhibition concentration of SOD1 and colorimetric method was used.[30]

8. Lamotrigine treatment

Lamotrigine (LTG) is an established antiepileptic drug and has been reported as a neuroprotectant by modulating ROS.[31-34] LTG was provided by courtesy of GlaxoSmithKline (GSK plc. Brentford, Middlesex, United Kingdom). LTG was dissolved in 20% β-cyclodextrin (β-CD) in distilled water and injected intraperitoneally at the different doses (30 mg/kg and 60 mg/kg) and times after diazepam treatment for controlling SE.[31, 32]

9. Inhibition of SOD1

To see the effect of SOD1 inhibition, diethyldithiocarbamate (DDC), a SOD1 inhibitor was given and compared with vehicle-control. 250 μg of DDC was given 1 hour after SE by *per os* (p.o.) route, and observed at the selected time points.[35]

10. Statistical analysis

Data are expressed as mean ± SE. The statistical comparisons among multiple groups were made using ANOVA followed by Fisher's *post hoc* protected least-significant difference test, whereas comparisons between two groups were performed using the unpaired t-test (StatView, version 5.01; SAS Institute Inc, Cary,

NC, USA). The level of significance was set at $p^* < 0.05$.

III. RESULTS

1. Different neuronal cell death in the hippocampal subfields after SE

Cresyl violet staining was performed to detect neuronal damage in hippocampus (Fig. 2). In normal control group, neuronal structures were well preserved in the pyramidal cell layer of CA1, CA3, and granule cells in the DG subfields. In the pilocarpine-injected group, pyramidal neurons in CA1 and CA3 regions were damaged and decreased in numbers at one day after SE, but granule cells in DG showed less neuronal cell death compared to CA1 and CA3. This pattern became more prominent three days after SE.

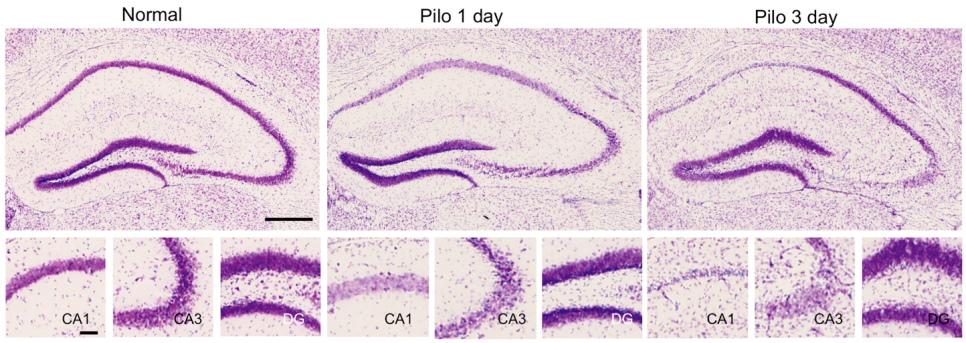

Figure 2. Cresyl violet staining in hippocampus after pilocarpine-induced SE. In the pyramidal layers of CA1 and CA3, neuronal cell death was prominent and reached maximum three days after pilocarpine-induced SE; however, the neurons in the DG showed less prominent changes. Pilo, pilocarpine-induced SE; 1 day, 1 day after SE; 3 day, 3 days after SE; CA, Cornus Ammonis; DG, dentate gyrus. Scale bar = 500, 100 μm.

2. Different DNA fragmentation in the hippocampal subfields after SE

Hippocampal neuronal damage by DNA fragmentation after SE was observed by TUNEL staining in the different subfields (Fig. 3). In the normal control group, TUNEL-positive cell (red) was not detected. TUNEL-positive cells significantly increased in the CA1 and the CA3 subfields three days after SE. However, the TUNEL-positive granule cells in the subfield DG were rarely detected.

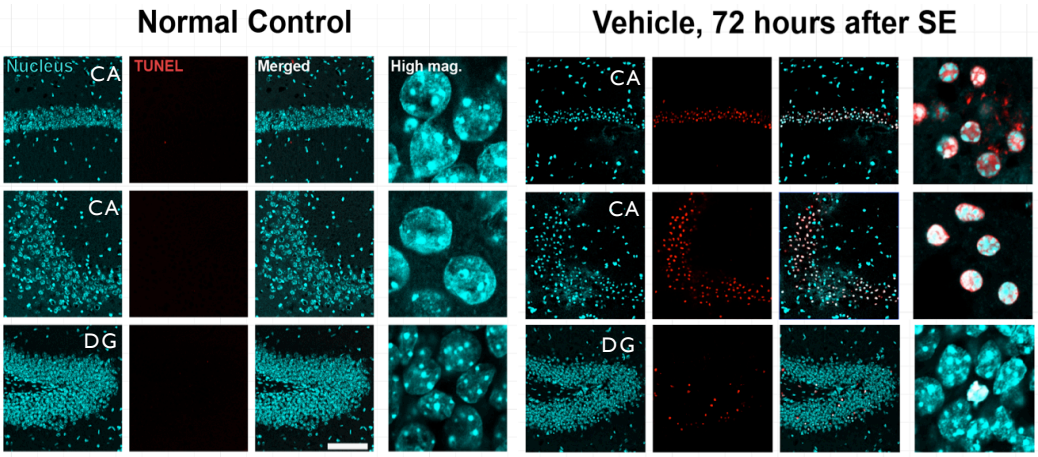

Figure 3. TUNEL staining of hippocampal subfields after SE**.** TUNEL positive-cells (red) markedly increased in the CA1 and CA3 hippocampal regions compared to the DG three days after SE. Blue, Hoechst 33258; Red, TUNEL; Scale bar = 100 μm.

3. Different $O2^{\bullet-}$ production among the hippocampal subfields

In the pilocarpine–induced SE group, signal of oxidized HEt (red) by $O2^{\bullet-}$ significantly increased in the CA1, CA3, and hilus regions; while the activity was less prominent in the CA2 and DG compared to the CA1 and CA3 regions. In the semiquantitative analysis, the intensity of oxidized HEt was significantly higher in the CA1, CA3, and hilus regions compared to those of the CA2 and DG subfields (Fig. 4) three days after SE ($p < 0.001$). The intensity of oxidized HEt markedly increased in the all hippocampal subfields compared to the normal control one day after SE, but the signal was returned to close to normal three days after SE in the CA2 and DG subfields, but not in the others.

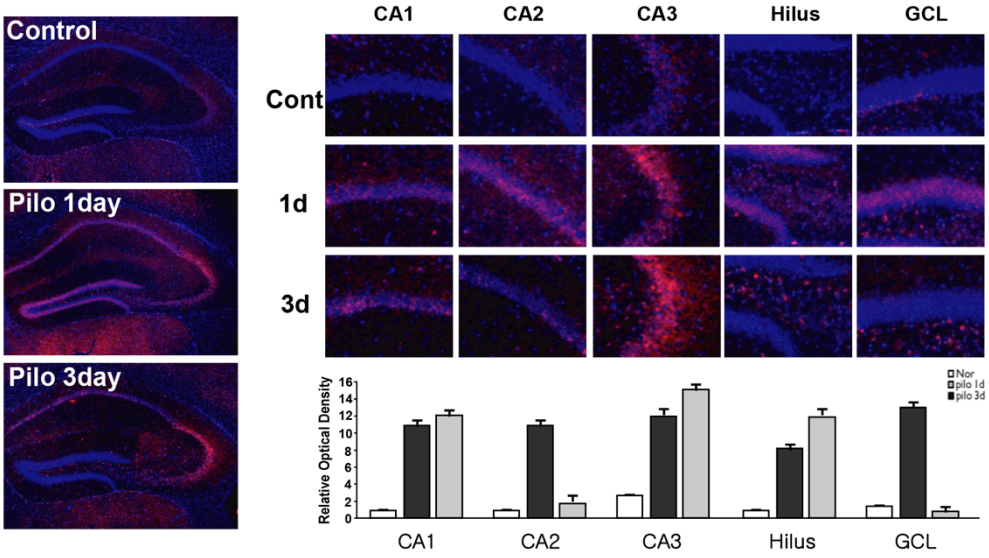

Figure 4. Different $O2^{\bullet-}$ production according to the hippocampal subfields after SE. The $O2^{\bullet-}$ signal, measured by oxidized HEt, markedly increased in CA1, CA4, and hilus one day after SE, and persisted three days after SE. The oxidized HEt signal in the DG and CA2 was less prominent and became normalized three days after SE. (Left) Spatial distribution was shown with low magnification over whole hippocampus. (Upper) Individual subfields with higher magnification. (Lower) Semiquantitative analysis. Pilo, pilocarpine-induced SE; Cont, normal control; GCL, granule cell layer of DG; Blue, Hoechst 33258; Red, oxidized HEt.

4. Different spatial expression of SOD1 among the hippocampal subfields

Immunohistochemical staining of SOD1 showed a significant increase of SOD1 in the cytosol of neurons in the CA2 and DG compared to the normal control as early as 8 hours (CA2) and 1 day (DG) after SE ($p = 0.021$ and $p = 0.017$, respectively). The SOD1 intensity was not changed significantly (CA1) or even decreased (CA3 and hilus) in the CA1, CA3, and hilus (Fig. 5).

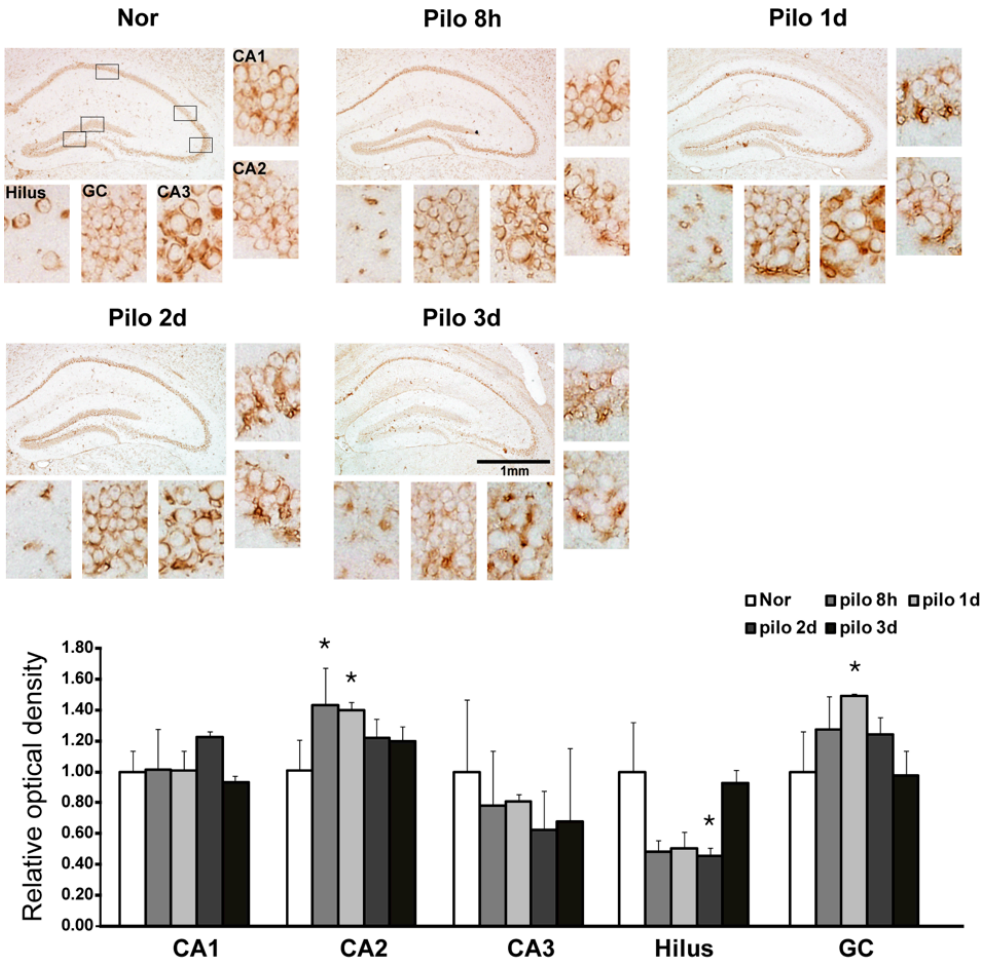

Figure 5. SOD1 level using immunohistochemical staining. SOD1 did not significantly increase (CA1) or decreased in the subfield CA3 and hilus. In the CA2 and GC, the SOD1 level increased as early as 8 hours, reached plateau at one day after SE, and slowly decreased thereafter. (Upper) Immunohistochemical staining. (Lower) Semi-quantitative analysis of SOD1. Nor, normal control; GC, granule cell of DG; Pilo, pilocarpine-induced SE.

5. Different SOD1 expression among the hippocampal subfields

For quantitative assessment of SOD1 expression, Western blot analysis was performed in the individual hippocampal subfields. Mice treated with vehicle only (normal control) showed higher SOD1 protein level in the subfield of DG compared to CA1 ($p = 0.025$), and there was no significant difference between CA1 and CA3 (Fig. 6). After pilocarpine-induced SE, there was no significant change of SOD1 expression in the CA1 and CA3 subregions; however, the SOD1 expression increased significantly in the DG subfield as early as 12 hours and reached maximum one day after SE ($p = 0.011$ and $p = 0.012$, respectively).

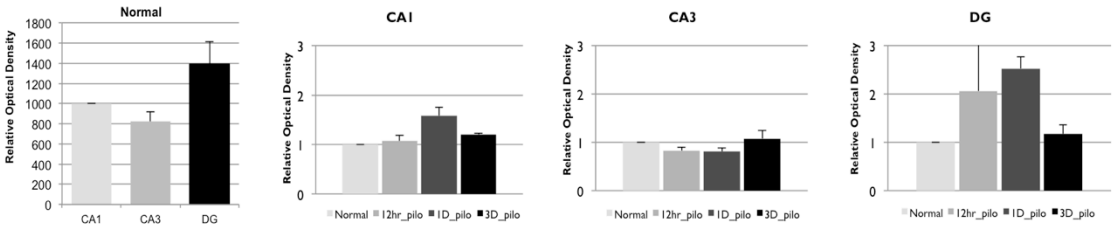

Figure 6. Different SOD1 expression in the normal control and its change after pilocarpine-induced SE among different hippocampal subfields. Higher amount of SOD1 expression was found in the DG compared to the CA1 and CA3 in the normal control (left panel). Time-dependent changes of SOD1 expression among various hippocampal subfields after pilocarpine-induced SE (right three panels). Significant increase of SOD1 was evident in the DG subfield as early as 12 hours after SE, but not in the CA1 and CA3, respectively.

6. Different SOD1 activity among the hippocampal subfields

To determine its enzymatic activity, SOD1 activity assay was performed. The SOD1 activity of the normal control was different among hippocampal subfields, significantly higher in the DG than CA1 or CA3 ($p = 0.021$). The activity of SOD1 increased significantly overall after SE, but significant change was found in CA1 and DG compared to normal control, not in the CA3 12 hours after SE ($p = 0.037$, $p = 0.041$, and $p = 0.649$, respectively) (Fig. 7).

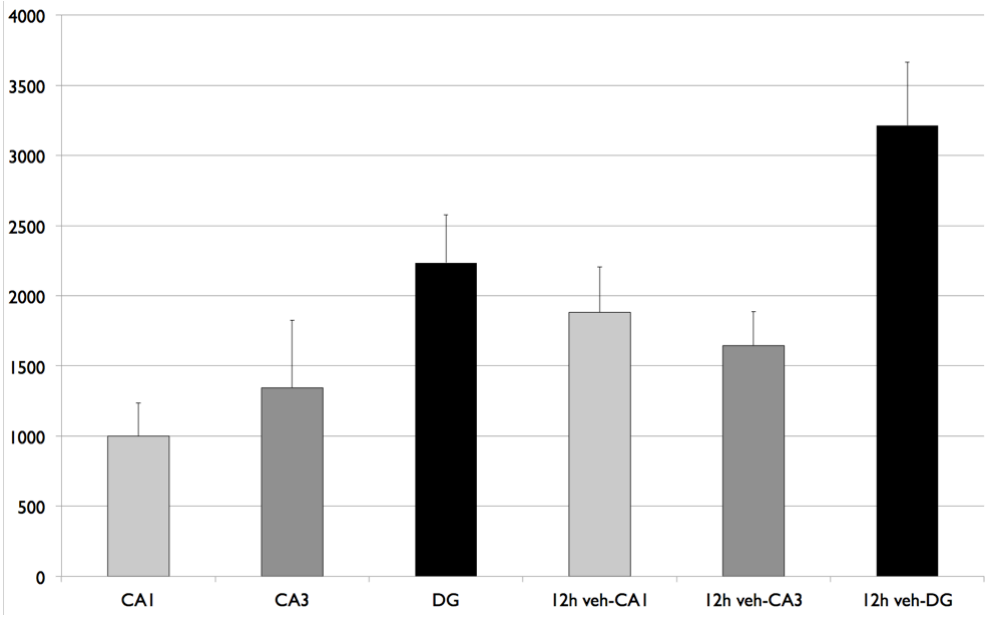

Figure 7. Enzymatic activity assay of SOD1 after pilocarpine-induced SE. Increased SOD1 activity was prominent in the DG subfield compared the CA1 and CA3 in the normal hippocampus (left three bars). SOD1 activity increased significantly after SE in the CA1 and DG, but not in the CA3 subfield (right three bars).

7. Effect of LTG treatment

To further confirm the neuroprotective effect of SOD1 affecting the different vulnerability of hippocampal subfields, the effect of LTG was tested. Cresyl violet staining showed a prominent neuronal cell death in the CA1 and CA3 subfields compared to DG in vehicle-treated mice, which was markedly reduced by LTG treatment (60 mg/kg, i.p. 2 hours after SE) (Fig. 8A). DNA fragmentation was also significantly reduced by LTG treatment (Fig. 8B). $O2^{\bullet-}$ production was also reduced by LTG treatment (Fig. 8C), and SOD1 immunofluorescent staining revealed increase of SOD1 activity compared to the vehicle-treated SE mice (Fig. 8D).

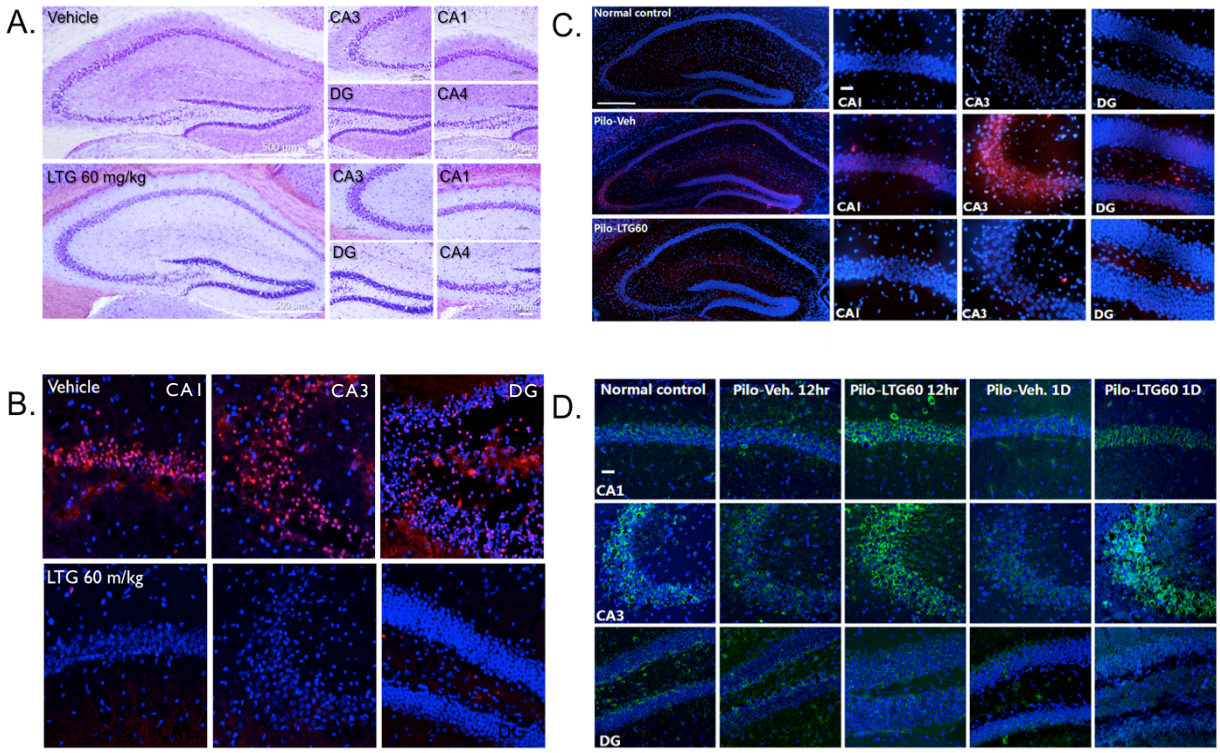

Figure 8. The effect of lamotrigine (LTG) treatment on hippocampal neuronal cell death, DNA fragmentation, superoxide anion production, and SOD1 expression. Prominent neuronal cell death (A) and DNA fragmentation (B) in the CA1 and CA3 subfields compared to DG was markedly reduced by LTG. $O2^{\bullet -}$ production was also reduced by LTG (C), and marked increase of SOD1 activity by LTG was found, compared to the vehicle-treated SE (D). Blue, Hoechst; red, TUNEL (B); blue, Hoechst; red, oxidized HEt; large panel scare bar = 500 μm, small panel scare bar = 100 μm (C); blue, Hoechst; green, SOD1; scare bar = 100 μm (D).

8. DDC treatment induced marked neuronal death in DG after SE

To further identify the neuroprotective effect of SOD1 on the different vulnerability of hippocampal subfields, the effect of SOD1 inhibitor, DDC was examined after pilocarpine-induced SE. Activity assay of SOD1 confirmed markedly decreased SOD1 activity compared to the normal control as early as 1 hour after SE (Fig. 9). Oxidized HEt increased markedly in the DDC-treated SE mice in the all subfields including DG and CA2 where oxidized HEt was weakly detected in the vehicle-treated SE mice (Fig. 10, upper panels). The resultant hippocampal neuronal death by cresyl violet staining increased markedly in the DG and CA2 subfields compared to the CA1 and CA3, although there was still considerable amount of cell death in CA1 and CA3 (Fig. 10, lower panels).

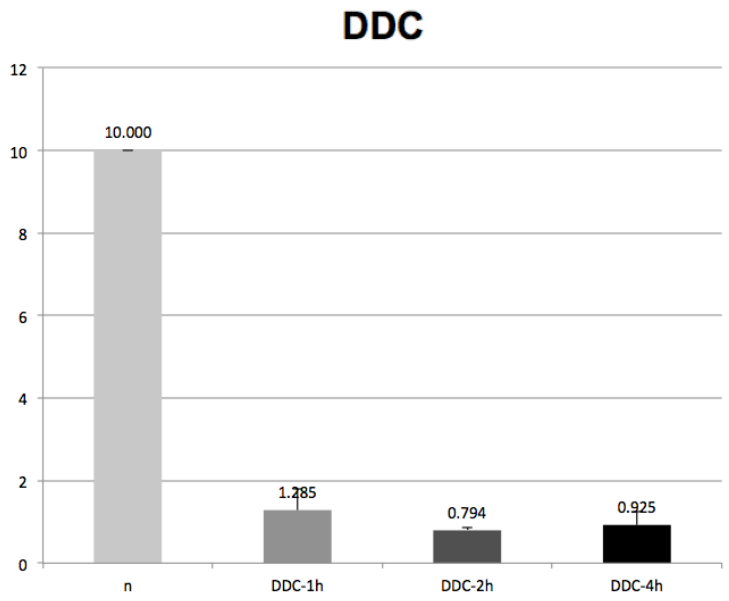

Figure 9. SOD1 activity assay after diethyldithiocarbamate (DDC) treatment. Compared to the normal control, SOD1 activity was significantly decreased by DDC as early as one hour after SE. n, normal control; DDC, diethyldithiocarbamate.

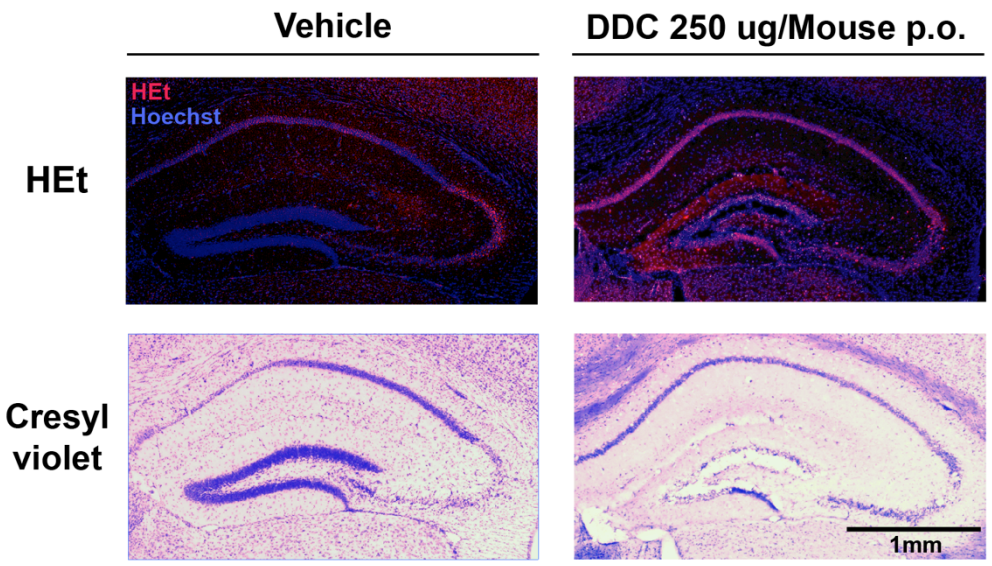

Figure 10. The effect of DDC on $O2^{\bullet-}$ production and neuronal cell death after SE. Three days after SE, the oxidized HEt in the DDC-treated mice increased markedly in the whole subfields compared to vehicle-treated mice, especially in DG and CA2 (upper panels). Cresyl-violet staining revealed very severe neuronal cell death in the DG, CA2, and CA3 as well as CA1 to less degree compared to vehicle-treated mice three days after SE (lower panels).

IV. DISCUSSION

Principal findings in this study can be summarized here, 1) increased production of $O2^{\bullet-}$ after pilocarpine-induced SE in mice was confirmed, more severe in the CA1 and CA3 subfields compared to the DG and CA2; 2) this increased production of $O2^{\bullet-}$ was well correlated with the pattern of different hippocampal neuronal death, more severe in CA1 and CA3 subfields compared to DG subfield; 3) SOD1 expression and activity was inversely correlated with the above mentioned pattern of cell death and $O2^{\bullet-}$ production within hippocampus. Higher SOD1 expression and activity in the DG than those of CA1 and CA3 was found in normal mouse brain as well as in the mice brain after pilocarpine-induced SE; 4) LTG treatment showed its neuroprotective property through increased SOD1 expression and decrease of $O2^{\bullet-}$ against oxidative stress after pilocarpine-induced SE; 5) Treatment of DDC, the SOD1 inhibitor resulted in marked neuronal cell death in the DG subfield through disinhibition of overproduction of $O2^{\bullet-}$.

The hypothesis in this study was the selective vulnerability within hippocampus is dependent on different intrahippocampal ROS formation under influence of its counterpart scavenging system. The different susceptibility of hippocampal subfields to the injury has been well recognized[1, 2, 6], but the cellular mechanisms that account for this variability remain unclear. The earliest hypothesis that different intrahippocampal vasculatures are responsible for this selective vulnerability of CA[36] is now unlikely to be primary mechanism for selective hippocampal vulnerability,[7, 8] and the following studies have suggested that complex interaction

of different mechanisms such as different glutamate-induced excitotoxicity[37-40], Ca^{2+} homeostasis[39], selective loss of GABAergic interneuron[40, 41], and different ROS formation by mitochondrial activities could explain such selective vulnerability.[38, 42]

ROS is well recognized for its dual role as both deleterious and beneficial effect. Under normal physiological condition, ROS involves various important physiologic roles such as signal processing, defense against infectious agents, induction of a mitogenic response, and even cellular protection through maintaining redox balance.[9, 11] When the balance between ROS and its defense mechanism is disturbed in pathologic condition, the overproduction of ROS results in cellular dysfunction and death and subsequently contributes the development of various acute and chronic neurodegenerative disorders.[3, 4, 11-14] During SE, ROS production in the hippocampal region is rapidly and extensively increased by repetitive seizure activity in concordance with impairment of defense mechanism, leading to acute neuronal cell death in this structure.[5, 12-14]

I chose $O2^{\bullet-}$ among various ROS involved in the neuronal death during SE because $O2^{\bullet-}$ has been known as the most potent free radical and a central mediator of ROS-mediated neuronal cell death in the various neurodegenerative disorders.[4, 9-11] This study results showed a consistent and strong pattern of increased $O2^{\bullet-}$ production in the hippocampus after pilocarpine-induce SE in mice. The pattern of intrahippocampal $O2^{\bullet-}$, however, differed according to the hippocampal subfields, and showed strong increase of $O2^{\bullet-}$ in the CA1, CA3, and CA4 compared to DG and CA2. Also the findings that increased production of $O2^{\bullet-}$ in the CA1 and CA3

subfields was well correlated with the more neuronal cell death and DNA fragmentation in the same subfields may support my hypothesis that $O2^{\bullet-}$ increases in the hippocampus after SE, and this increased $O2^{\bullet-}$ as well as coherent intrahippocampal neuronal death may suggest ROS being the primary mechanism of neuronal damage in the pilocarpine-induced SE model. The role of $O2^{\bullet-}$ in the SE, however, has not been extensively investigated so far, and the results were inconclusive.[15-19, 21, 23] Although the role of $O2^{\bullet-}$ in the development of various neurological degeneration is generally accepted, because of its methodological difficulty most studies adopted only indirect methods in detecting $O2^{\bullet-}$ such as reduction of the SOD-inhibitable nitroblue tetrazolium,[15, 16] dihydrorhodamine 123 fluorescence,[19] aconitase inactivation,[21] and lucigenin-enhanced chemiluminescence.[17] Only two studies adopted the direct measure of $O2^{\bullet-}$ using HEt, in which HEt is oxidized selectively by $O2^{\bullet-}$.[18, 23] *In situ* detection of oxidized HEt for detection of $O2^{\bullet-}$ has been adopted in the lots of studies and now regarded as a standard method.[18, 23, 27, 28] In this study, I adopted the direct measuring method of $O2^{\bullet-}$ using oxidized HEt, and this will strengthen my study results favoring the role of $O2^{\bullet-}$ in selective hippocampal neuronal death after pilocarpine-induced SE in mice. In addition, the method of intravenous HEt injection enables accurate measurement of temporal and spatial pattern of $O2^{\bullet-}$ under microscope, as compared with other indirect methods described above those used the homogenate of brain sample for analysis.[15-17, 19, 21]

Different intrahippocampal pattern of $O2^{\bullet-}$ production; however, was only

supported by a few studies reporting contrast results. Peterson et al.[23] reported that diffuse cytosolic distribution of ethidium fluorescence was detected in the neocortex and thalami after SE, but not in the hippocampus and amygdala using HEt detection, the same method used in this study. This finding was quite different from my results. In contrast, Chuang et al.[17] reported increase of $O2^{\bullet-}$ in the CA3 compared to the sham or vehicle-treated group in the kainic acid-treated SD rat model, and another study confirmed increase of $O2^{\bullet-}$ in the CA1, CA3 and DG of the hippocampus using HEt.[18] These two studies support my finding that $O2^{\bullet-}$ increase after SE, but different $O2^{\bullet-}$ increase pattern was not evident in those studies because of different study design they focused.[17, 18]

Given that the seizure activity during SE evoked by systemic administration of proconvulsant is too widespread (also evident by observation of generalized seizure) to induce localized difference of $O2^{\bullet-}$ production, the hypothesis that the main mechanism underlying selected vulnerability might be its controlling system of $O2^{\bullet-}$ seems attractive. ROS production within cells is precisely controlled by diverse antioxidant defense system to prevent any excessive oxidative stresses resulting neuronal cell death.[9-11] In this study, SOD1 expression and activity increased in the resistant hippocampal subfield (DG and CA2) and decreased in the vulnerable regions, CA1, CA3, CA4 after SE. This finding was consistent across three different modalities: immunohistochemical staining, Western blot, and activity assay. The increase of SOD1 in response to SE in the most resistant hippocampal subfield (DG) may suggest that SOD1 may play an important role in this different

vulnerability within the hippocampus after SE. Several studies reported the change of SODs in the various model of SE, but the results were quite different among studies.[13, 20-22, 24, 25] No change of SOD activity were reported in two studies,[22, 25] and other studies reported SOD2 not SOD1 decreased after SE.[13, 21, 24] Differential intrahippocampal pattern was not studied in those studies. Only one study reported that SOD1 level significantly decreased in the CA1 and CA3 in the kainic acid-treated SD rat and was closely related with neuronal cell death.[20] Similarly with my results, the SOD1 immunoreactivity decreased over seven days in CA1, CA3, and CA4, but no significant change in the DG subfield, suggesting the neuroprotective role of SOD1.[20]

Another interesting finding of my study was that the SOD1 expression was constantly increased in the DG subfield in the normal control hippocampus without seizure activity in addition to reactive increase of SOD1 by seizure activity. SOD consists of three types of isoenzymes encoded by three different genes. The copper/zinc SOD (cytoplasmic SOD or SOD1) is found in the cytosol, whereas manganese SOD (mitochondrial SOD or SOD2) is mainly located in the mitochondrial matrix. The last type of SOD, being called extracellular SOD (SOD3), is expressed at low level in the extracellular fluids.[43] Although SOD1 has been extensively studied in the various neurodegenerative models such as global or focal ischemia, amyotrophic lateral sclerosis, Huntington disease, and Parkinson disease, it has been known to be constant rather than reactive to the various insults.[43, 44] In contrast, SOD2 is rich in the mitochondria and has been known easily

activated to the exogenous insults.[43] Liang and Patel [21] found SOD2, and to less extent SOD3 increase in response to local application of kainic acid, but not SOD1. My finding that SOD1 increased in the normal DG may suggest that without robust reactivity like SOD2 to the exogenous insults, constantly increased SOD1 in the normal DG (together with reactive increase of SOD1) might be sufficient to protect DG granule cell from the ROS-mediated injury leading to neuronal cell death. This finding, in part, is supported by only one study by Bergeron et al.[44] in 1996 in which they found constitutively increased SOD1 level in CA2 and CA3, but decreased SOD1 in CA1 using *in situ* hybridization in the human brain samples of amyotrophic lateral sclerosis, Alzheimer disease, and Parkinson disease. Further study in this regard will be required in the near future.

LTG has been reported as having neuronal protective effect in the various neurodegenerative models such as focal and global ischemia, various seizure models, hypoxia, and depression-stress models.[31-34] It was found that LTG decrease NO and lipid peroxidation, increase glutathione s-transferase expression and activity, and glutathione peroxidase activity[31-34] In this study, LTG was found to increase SOD1 expression and activity, ameliorated $O2^{\bullet-}$ production, and induced subsequent decrease of neuronal cell death and DNA fragmentation. These findings further supported my hypothesis that SOD1 have neuroprotective effect through inhibition of $O2^{\bullet-}$ after SE, and inter-subfield difference of those mechanisms may underlie the different vulnerability of hippocampal subfield after SE.

Treatment of SOD1 inhibitor, DDC, induced very extensive neuronal cell death

in the DG and CA2 subfield, to less extent in the CA3 and CA1. This unique phenomenon might be resulted through strong inhibition of SOD1 and subsequent increase of $O2^{\bullet-}$, confirmed by both SOD1 activity assay and intense increase of oxidized HEt after DDC treatment. Interestingly, the degree of neuronal cell death was inversely severe, maximum in the DG, to less degree in the CA3, and least in CA1 subfield. This finding may suggest that SOD1 is the major mechanism against $O2^{\bullet-}$-induced neuronal death in the DG subfield, but not in the CA1 or CA3 subfields. Presence of other protective mechanisms beside SOD1 in the CA1 or CA3 subfields would be another possible explanation for this phenomenon. Further investigation should be required to answer this.

In conclusion, excessive production of $O2^{\bullet-}$ after SE resulted in neuronal cell death, but different according to the hippocampal subfields, to less degree in the DG compared to CA1 and CA3. Increased expression and activity of SOD1 in the DG and marked neuronal death by its inhibition may suggest the difference of SOD1 expression as a pivotal role in the selected vulnerability of hippocampal subfields.

V. CONCLUSION

This study demonstrated that increased production of superoxide anion after pilocarpine-induced SE in mice in the CA1 and CA3 subfields, but less degree in the DG subfield. This increased production of superoxide anion was well correlated with the pattern of different hippocampal neuronal death, more severe in CA1 and CA3 subfields compared to DG subfield, suggesting the pivotal role of superoxide anion in the different intrahippocampal neuronal death after pilocarpine-induced SE. SOD1 expression and activity was inversely correlated with the pattern of neuronal cell death and superoxide anon production within hippocampus. SOD1 expression increased in the constant manner in the DG subfield in normal mouse brain and SOD1 expression and activity significantly increased in the DG subfield reacting to pilocarpine-induced SE. This difference of SOD1 and superoxide anion coupling according to the hippocampal subfields may play a key role in the selected vulnerability. Treatment of DDC, the SOD1 inhibitor resulted in marked neuronal cell death in the DG subfield through disinhibiting overproduction of superoxide anion. This finding may support that SOD1 is the major mechanism against ROS-induced neuronal death in the DG subfield after pilocarpine-induced SE.

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ABSTRACT (In Korean)

간질중첩증 후 해마 내 선택적 세포사멸현상에 미치는 superoxide dismutase 1의 역할

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조양제

간질중첩증 이후 해마 내 신경세포의 선택적 세포사멸현상은 잘 알려져 있다. 해마 내 암몬 뿔(Cornus Ammonis, CA) 1 구역과 CA3 구역의 피라미드세포들은 간질중첩증과 같은 여러 손상자극에 매우 취약한 반면 CA2 구역이나 치아이랑(dentate gyrus, DG)의 과립세포(granule cell) 들은 이러한 자극에 저항성을 갖고 있다. 이러한 해마 내 저항성의 차이는 비록 다양한 기전이 제시되고 있음에도 아직 정확한 기전이 알려져있지 않으며, 외부 자극에 반응하여 발생하는 세포독성기전과 이를 방어하는 기전들의 구역별 차이에 의해 발생하는 것으로 추측되고 있다.

활성산소종(reactive oxygen species, ROS)은 이러한 세포독성기전의 하나로

허혈성 뇌경색, 알츠하이머병, 헌팅톤병과 같은 다양한 급성 및 만성 퇴행성 뇌질환의 발생에 중요한 역할을 하는 것으로 보고되고 있으며, 마찬가지로 간질중첩증에 의해 과다하게 발생하는 활성산소종은 간질중첩증 후 세포사멸현상에 중요한 역할을 한다. 특히 초과산화물 음이온(superoxide anion, $O2^{\bullet-}$)은 가장 강력한 활성산소종의 하나로 다양한 뇌신경질환모델에서 세포사멸현상에 중요한 역할을 한다. 하지만, 아직까지 간질중첩증모델에서 그 역할은 잘 알려져 있지않으며 특히 해마 내 선택적 세포사멸현상에서 더욱 그러하다.

따라서 이 연구에서 나는 간질중첩증 이후 과도한 초과산화물 음이온 생성이 해마 내 신경세포사멸에 중요한 역할을 할 것이며, 초과산화물 음이온을 선택적으로 억제하는 효소인 초과산화물 디스뮤타아제(superoxide dismutase, SOD) 1의 불균등한 작용이 해마내 선택적 신경세포 사멸현상에 핵심적인 역할을 할 것이라는 가설을 세우고 이를 입증하고자 하였다.

본 연구에서는 성인 수컷 C57BL/6J 생쥐에 pilocarpine을 투여함으로써 간질중첩증모델을 만들었으며, 행동학적 관찰 및 뇌파를 통해 이를 확인하였다. 해마 내 신경세포사멸현상을 관찰하기 위해 cresyl violet 염색과

DNA 분절현상을 확인하는 TUNEL 염색을 시행하였다. 초과산화물 음이온에 선택적으로 반응하여 형광을 나타내는 hydroethidine (HEt) 을 정맥 내에 주입하여, 초과산화물 음이온의 해마 내 시공간 분포를 살펴보았으며, 초과산화물 음이온을 억제하는 SOD1의 상태를 해마 내 세부공간별로 웨스턴 블롯, 효소활성도 정량, 그리고 면역조직화학염색법을 통해 다각도로 평가하였다. SOD1 이 간질중첩증 이후 해마 내 선택적 신경세포사멸에 미치는 영향을 더욱 확실히 알아보기 위해 SOD1을 증가시키는 라모트리진(lamotrigine, LTG)과 SOD1을 억제시키는 diethyldithiocarbamate (DDC) 를 각각 투여함으로써 SOD1의 증감이 해마 내 선택적 신경세포사멸에 어떠한 미치는지 알아보고자 하였다.

간질중첩증 이후 cresyl violet 염색과 TUNEL 염색 모두 해마 내 신경세포의 선택적 사멸현상이 뚜렷하게 관찰되었는데, CA1과 CA3에서는 세포사멸이 증가하였던 반면 DG에서는 이러한 현상이 비교적 적었다. 초과산화물 음이온은 간질중첩증 이후 CA1과 CA3에서는 뚜렷하게 증가하였던 반면 DG에서는 상대적으로 적은 증가를 보였다. 정상 해마조직 내에서 SOD1 의 단백질 발현이 CA1과 CA3보다 DG에서 유의하게 높았으며, 간질중첩증 이후 SOD1이 DG에서 유의하게 증가를 보였으나 CA1이나 CA3에서는

SOD1이 감소하거나 유의한 차이를 보이지 않았다. LTG 투여 이후 SOD1의 활성도와 단백발현이 증가하였으며, 초과산화물 음이온이 유의하게 감소하였고 CA1이나 CA3에서의 선택적 신경세포사멸현상이 완화되는 것을 확인할 수 있었다. 간질중첩증 이후 SOD1 억제제인 DDC를 투여하였더니, 전반적으로 초과산화물 음이온의 생성이 크게 증가하였고 이전에 세포사멸이 잘 관찰되지 않던 DG에서 광범위한 신경세포사멸현상을 관찰할 수 있었다.

본 연구에서는 간질중첩증 이후 과다하게 생성된 초과산화물 음이온이 해마 내 소구역에 따라 다른 신경세포사멸을 보였는데 CA1과 CA3에서는 심한 반면 DG에서는 상대적으로 적었다. 이러한 선택적 해마 내 취약성은 초과산화물 음이온의 발생정도와 정비례하는 모습을 보여서 초과산화물 음이온과 연관이 있음을 밝혔으며, 반대로 SOD1은 DG에서 단백발현과 활성도 모두 가장 높았고 CA1과 CA3에서는 상대적으로 감소 혹은 큰변화를 보이지 않아서 이러한 SOD1의 차이가 선택적 해마 내 취약성 현상과 밀접한 연관이 있음을 밝혔다. 이러한 소견은 SOD1 억제제와 활성제를 투여함으로써 다시 한번 그 효과를 확인할 수 있었다.

결론적으로 해마 내 소구역별로 SOD1의 활성도 및 발현 차이가 초과산화물 음이온의 발생 정도를 조절함으로써 이러한 간질중첩증 이후

해마 내 소구역의 선택적 세포사멸현상에 중요한 역할을 하는 것으로 생각된다.

핵심되는 말: 간질, 초과산화물 음이온, 초과산화물 디스뮤타아제, 간질중첩증, 해마, 치상이랑, 선택적 취약성