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RESEARCH ARTICLE

Effect of ischemic preconditioning on antioxidant status in the gerbil hippocampal CA1 region after transient forebrain ischemia

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Abstract

Ischemic preconditioning (IPC) is a condition of sublethal transient global ischemia and exhibits neuroprotective effects against subsequent lethal ischemic insult. We, in this study, examined the neuroprotective effects of IPC and its effects on immunoreactive changes of antioxidant enzymes including superoxide dismutase (SOD) 1 and SOD2, catalase (CAT) and glutathione peroxidase (GPX) in the gerbil hippocampal CA1 region after transient forebrain ischemia. Pyramidal neurons of the stratum pyramidale (SP) in the hippocampal CA1 region of animals died 5 days after lethal transient ischemia without IPC (8.6% (ratio of remanent neurons) of the sham-operated group); however, IPC prevented the pyramidal neurons from subsequent lethal ischemic injury (92.3% (ratio of remanent neurons) of the sham-operated group). SOD1, SOD2, CAT and GPX immunoreactivities in the sham-operated animals were easily detected in pyramidal neurons in the stratum pyramidale (SP) of the hippocampal CA1 region, while all of these immunoreactivities were rarely detected in the stratum pyramidale at 5 days after lethal transient ischemia without IPC. Meanwhile, their immunoreactivities in the sham-operated animals with IPC were similar to (SOD1, SOD2 and CAT) or higher (GPX) than those in the sham-operated animals without IPC. Furthermore, their immunoreactivities in the stratum pyramidale of the ischemia-operated animals with IPC were steadily maintained after lethal ischemia/reperfusion. Results of western blot analysis for SOD1, SOD2, CAT and GPX were similar to immunohistochemical data. In conclusion, IPC maintained or increased the expression of antioxidant enzymes in the stratum pyramidale of the hippocampal CA1 region after subsequent lethal transient forebrain ischemia and IPC exhibited neuroprotective effects in the hippocampal CA1 region against transient forebrain ischemia.

Key Words: *nerve regeneration; ischemic preconditioning; neuroprotection; transient forebrain ischemia; pyramidal neurons; hippocampus; antioxidant enzymes; neural regeneration*

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Introduction

The pathogenesis of cerebral ischemia/reperfusion injury is very complex. Among many mechanisms of neuronal damage/death, oxidative stress greatly affects signaling pathway in ischemia-induced cell death (Chan, 1994; Numagami et al., 1996; Zhang et al., 2011). Reactive oxygen species (ROS) such as superoxide anion radical (O_2^-), hydroxyl radical (HO), and hydrogen peroxide (H_2O_2), are generated when exposure of excessive oxygen occurs after ischemia/reperfusion injury and promote the generation of free radicals from mitochondria (Zhang et al., 1990; Ali et al., 1992; Christophe and Nicolas, 2006). The overproduction of ROS in abnormal conditions, such as ischemia/reperfusion, induces oxidative injury that involves changes in cellular proteins, nucleic acids, lipids, and DNA (Radi et al., 1993; Chan, 2001). Furthermore, many studies have demonstrated that ROS is associated with the pathogenesis of various neurological disorders and degenerative diseases (Floyd, 1999; Jenner, 2003; Sugawara et al., 2004; Mohsenzadegan and Mirshafiey, 2012).

ROS are transformed to nontoxic compounds by free-radical-scavenging enzymes. For instance, superoxide dismutase (SOD) detoxifies O_2^- and produces H_2O_2 and O_2, and catalase (CAT) and glutathione peroxidase (GPX) converts H_2O_2

to H_2O and O_2 (Kehrer, 2000; Crack et al., 2003; Sugawara et al., 2004; Slemmer et al., 2008). Various antioxidants have been known to be helpful in the treatment of several neurodegenerative diseases and neurological disorders including cerebral ischemia (Floyd, 1999; Delanty and Dichter, 2000).

Among neurological disorders, ischemic insults, which lead to permanent disability or death, occur due to the block or severe reduction of cerebral blood flow by several situations, such as cerebral ischemia, cardiac arrest, and cardiovascular surgery (White et al., 1993; Block, 1999). Transient global cerebral ischemia occurs because of temporal block of cerebral blood flow and gives rise to neuronal damage or death in some vulnerable regions such as the striatum, cerebral neocortex, and hippocampus (Lipton, 1999). In particular, the vulnerability of neurons in the hippocampus varies with hippocampal subregions; the most vulnerable subregion to transient forebrain ischemia is the hippocampal CA1 region (Schmidt-Kastner and Freund, 1991). Neuronal death in the hippocampal CA1 region by transient forebrain ischemia happens several days after transient ischemic insult, and most of pyramidal cells in the stratum pyramidale in the CA1 region are dead. This neuronal death is called "delayed neuronal death" (Kirino, 1982).

Ischemic preconditioning (IPC) is a precondition of sublethal transient ischemia and has been applied to neuroprotection against subsequent lethal ischemic insults (Kirino et al., 1996). IPC has a resistance in various organs including the brain and this phenomenon has been called "ischemic tolerance" (Moraru et al., 2005; Nakamura et al., 2006).

Many possible explanations regarding the neuroprotective effects of IPC against cerebral ischemic insults have been suggested (Lee et al., 2014, 2015; Kim et al., 2015), however, to the best of our knowledge, few studies have been reported about the expression of antioxidant enzymes and their changes in ischemic hippocampus with IPC. In this study, we detected the changes in antioxidant enzyme expression such as SOD1, SOD2, CAT and GPX in the hippocampus of a gerbil model of transient forebrain ischemia subjected to IPC using immunohistochemistry and western blot assay (Liu et al., 2014a, b; Shen et al., 2015).

Materials and Methods

Experimental animals

As previously described (Kim et al., 2015), 56 male Mongolian gerbils, aged 6 months, weighing 65–75 g, were divided into four groups: (1) sham-operated group (n = 7; bilateral exposure of the common carotid arteries but no ischemia); (2) ischemia group (n = 21; lethal ischemia, *i.e.*, 5-minute transient forebrain ischemia); (3) IPC + sham-operated group (n = 7; IPC followed by 2-minute transient ischemia (sublethal ischemia)); and (4) IPC + ischemia group (n = 21; IPC followed by lethal ischemia). The animals were examined at 1, 2 and 5 days after lethal ischemia, because CA1 pyramidal neurons do not die until 3 days and commenced to die 4 days after 5 minutes of transient forebrain ischemia (Kirino, 1982). All the experimental methods were approved (approval number: KW-130424-1) by the Institutional Animal Care and Use Committee (IACUC) at Kangwon National University, South Korea and adhered to guidelines that are in compliance with the current international laws and policies (Guide for the Care and Use of Laboratory Animals, The National Academies Press, 8th Ed., 2011).

Surgery of transient forebrain ischemia

As previously described (Kim et al., 2015), in brief, the animals were anesthetized with 2.5% isoflurane (Baxtor, Deerfield, IL, USA). The common carotid arteries were ligated bilaterally for 2 (for sublethal ischemia) or 5 minutes (for lethal ischemia). Body (rectal) temperature was controlled under normothermic (37 ± 0.5°C) conditions during the surgery.

Tissue preparation

As we previously described (Kim et al., 2015), briefly, the animals (n = 7 in each group at each time point) were anesthetized with pentobarbital sodium at the designated times and they were perfused transcardially with 4% paraformaldehyde. The forebrain tissues were serially cut into 30-μm coronal sections.

Cresyl violet (CV) staining

For cellular distribution in the gerbil hippocampus, as we previously described (Lee et al., 2014), in brief, 1% of cresyl violet acetate (Sigma, MO, USA) and 0.28% of glacial acetic acid were used for CV staining.

Immunohistochemistry for neuronal nuclei (NeuN)

To investigate neuronal damage in the gerbil hippocampus after transient forebrain ischemia, NeuN immunohistochemistry was carried out according to our published procedure (Kim et al., 2013). In brief, the brain sections were incubated with diluted mouse anti-NeuN (a neuron-specific soluble nuclear antigen) (diluted 1:1,000, Chemicon International, Temecula, CA, USA) overnight at 4°C and incubated in biotinylated goat anti-mouse IgG (diluted 1:250, Vector, Burlingame, CA, USA) and streptavidin peroxidase complex (Vector) for 2 hours at room temperature. Finally, they were visualized with 3,3′-diaminobenzidine.

Fluoro-Jade B (F-J B) histofluorescence staining

To examine neuronal death, F-J B, a marker for neuronal degeneration) histofluorescence staining was carried out using a previously published method (Candelario-Jalil et al., 2003). Briefly, the hippocampal sections were immersed in 1% sodium hydroxide solution, transferred to 0.06% potassium permanganate solution and 0.0004% F-J B (Histochem, Jefferson, AR, USA) solution. Neuronal damage was examined using a fluorescence microscope (Carl Zeiss, Göttingen, Germany).

Immunohistochemistry for SOD1, SOD2, CAT and GPX

In brief, according to our published procedure (Kim et al., 2013), immunohistochemical staining was carried out with sheep anti-copper, zinc-SOD1 (1:1,000, Calbiochem, Darmstadt, Germany), sheep anti-mangan-SOD2 (SOD2, 1:1,000, Calbiochem), rabbit anti-CAT (1:1,000, Calbiochem) and sheep anti-GPX (1:1,000, Calbiochem) overnight at 4°C. Thereafter the tissues were exposed to biotinylated goat anti-rabbit IgG (diluted 1:250, Vector), goat anti-sheep IgG (diluted 1:250, Vector) and streptavidin peroxidase complex (Vector) for 2 hours at room temperature.

Western blot analysis

For changes in SOD1, SOD2, CAT and GPX protein levels in the CA1 region, according to our published procedure (Lee et al., 2014), brain tissues (n = 7 in each group at each time point) were used at 2 and 5 days after transient forebrain ischemia. In brief, the protein levels of SOD1, SOD2, CAT and GPX in the hippocampal CA1 region were determined in the supernatants using a Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, Rockford, IL, USA). Aliquots containing 20 μg of total protein were loaded onto a 12.5% polyacryamide gel. The gels were transferred to nitrocellulose transfer membranes (Pall Crop, East Hills, NY, USA) after electrophoresis. The membranes were incubated with goat anti-SOD1 (diluted 1:1,000, Calbiochem) and goat anti-SOD2 (diluted 1:1,000, Calbiochem), rabbit anti-CAT (diluted 1:500, Lab-Frontier, Seoul, Korea), and sheep anti-GPX (diluted 1:500, Chemicon International) overnight at 4°C and incubated in peroxidase-conjugated horse anti-goat IgG, goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 hours at room temperature and subsequently exposed to an ECL kit (Amersham, UK).

Data analysis

Data were analyzed according to our published procedure (Kim et al., 2015). In brief, 20 brain sections were chosen in each animal with 120 μm interval, and cell counts were carried out by averaging the counts. NeuN- and F-J B-positive cells in the stratum pyramidale were taken through an AxioM1 light microscope (Carl Zeiss) equipped with a digital camera (Axiocam, Carl Zeiss) connected to a PC monitor. The mean number of the cells was counted in a 200 × 200 μm^2 area at the center of the CA1 region. Cell counts were analyzed as a percent, with the sham-operated group designated as one hundred percent. To quantitatively analyze antioxidant enzyme immunoreactivities in the stratum pyramidale, the density of SOD1, SOD2, CAT and GPX-immunoreactive structures was assessed on the basis of an optical density (OD) that was obtained after the transformation of the mean gray level using the formula: OD = log (256/mean gray level). A ratio of the OD of an image file was calibrated in Adobe Photoshop 8.0 (Adobe Systems, San Jose, CA, USA) and analyzed as a percent, with the sham-operated group designated as one hundred percent in NIH Image 1.59 (National Institutes of Health, Bethesda, MD, USA). The protein levels of SOD1, SOD2, CAT and GPX were quantified using Scion Image software (Scion Corp., Frederick, MD, USA) that was used for relative optical density (ROD): A ratio of the ROD was calibrated as percentage compared to the control.

Statistical analysis

All data are presented as the mean ± SEM. Differences of the means among the groups were analyzed statistically by one-way analysis of variance (ANOVA) with a *post hoc* Bonferroni's multiple comparison test to elucidate ischemia-related differences among the groups. Statistical significance was considered at $P < 0.05$.

Results

CV-positive cells

CV-positive cells were easily observed throughout the hippocampus of the animals from the sham-operated group (**Figure 1A** and **a**). In the ischemia group, the number and morphology of CV-positive cells were similar to those in the sham-operated group until 2 days after transient forebrain ischemia (**Figure 1C**, **c**, **E** and **e**), and the numbers of CV-positive cells were significantly decreased in the stratum pyramidale in the hippocampal CA1 region at 5 days after transient forebrain ischemia (**Figure 1G** and **g**).

In the IPC + sham-operated group, the distribution pattern of CV-positive cells was similar to that in the sham-operated group (**Figure 1B** and **b**). In the IPC + ischemia group, the distribution pattern of CV-positive cells in the stratum pyramidale of the CA1 region was not significantly different from that in the IPC + ischemia group after transient ischemia (**Figure 1H** and **h**).

NeuN-immunoreactive neurons

NeuN-immunoreactive neurons were easily observed in the stratum pyramidale of the hippocampal CA1 region in the sham-operated group (**Figure 2A**). In the ischemia group, NeuN-immunoreactive neurons were present in the stratum pyramidale of the hippocampal CA1 region until 2 days after transient forebrain ischemia (**Figure 2C**, **E** and **I**), however, NeuN-immunoreactive neurons were hardly observed in the stratum pyramidale at 5 days after transient forebrain ischemia (**Figure 2G** and **I**).

The appearance of NeuN-immunoreactive neurons in the hippocampal CA1 region of animals from the IPC + sham-operated group was similar to that in the sham-operated group (**Figure 2B** and **I**). In the IPC + ischemia groups, NeuN-immunoreactive pyramidal neurons in the CA1 region were well observed after transient forebrain ischemia (**Figure 2D**, **F**, **H** and **I**); NeuN-immunoreactive neurons were still present 5 days after transient forebrain ischemia.

F-J B-positive cells

No F-J B-positive cells were detected in any layer of the hippocampal CA1 region of animals until 2 days after transient ischemia as well as in the sham-operated group (**Figure 2J**, **L**, **N** and **R**). However, at 5 days after transient ischemia, many

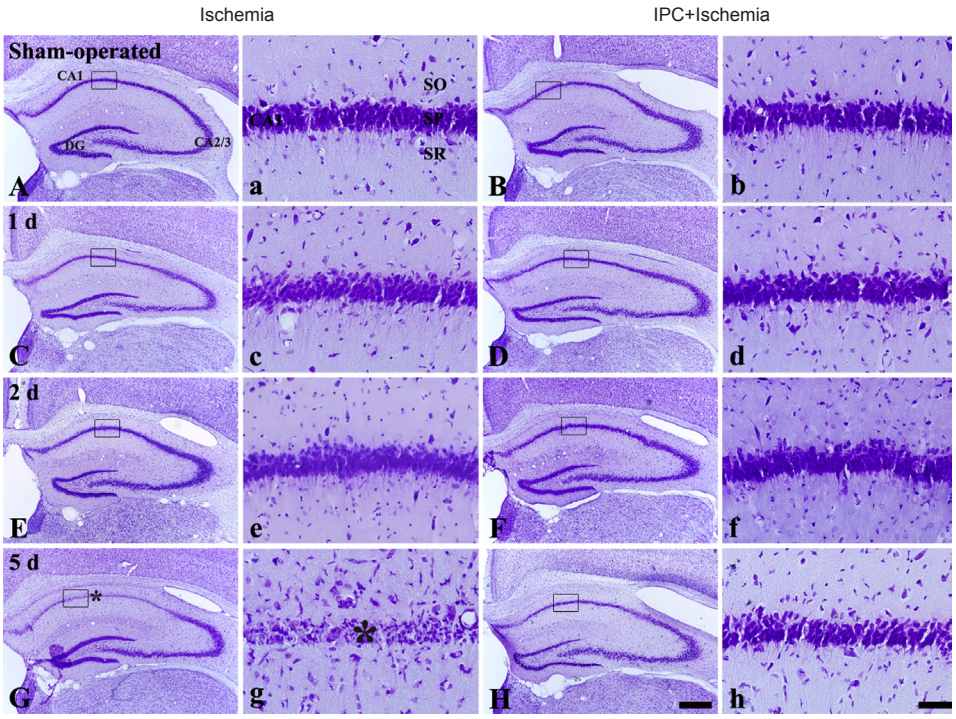

Figure 1 Cresyl violet (CV) staining in the hippocampus of gerbils from the sham-operated (A, a), IPC + sham-operated (B, b), ischemia (C, c, E, e, G, g) and IPC + ischemia (D, d, F, f, H, h) groups.
In the ischemia group, a few CV-positive cells were found in the hippocampal CA1 stratum pyramidale (asterisk) only 5 days after transient forebrain ischemia. However, the distribution pattern of CV-positive cells in the IPC + ischemia group was similar to that in the sham-operated group. IPC: Ischemic preconditioning; CA: cornus ammonis; DG: dentate gyrus; SO: stratum oriens; SR: stratum radiatum. Scale bars: 200 μm (low magnification, A–H), 60 μm (a–h: high magnification of boxes in A–H).

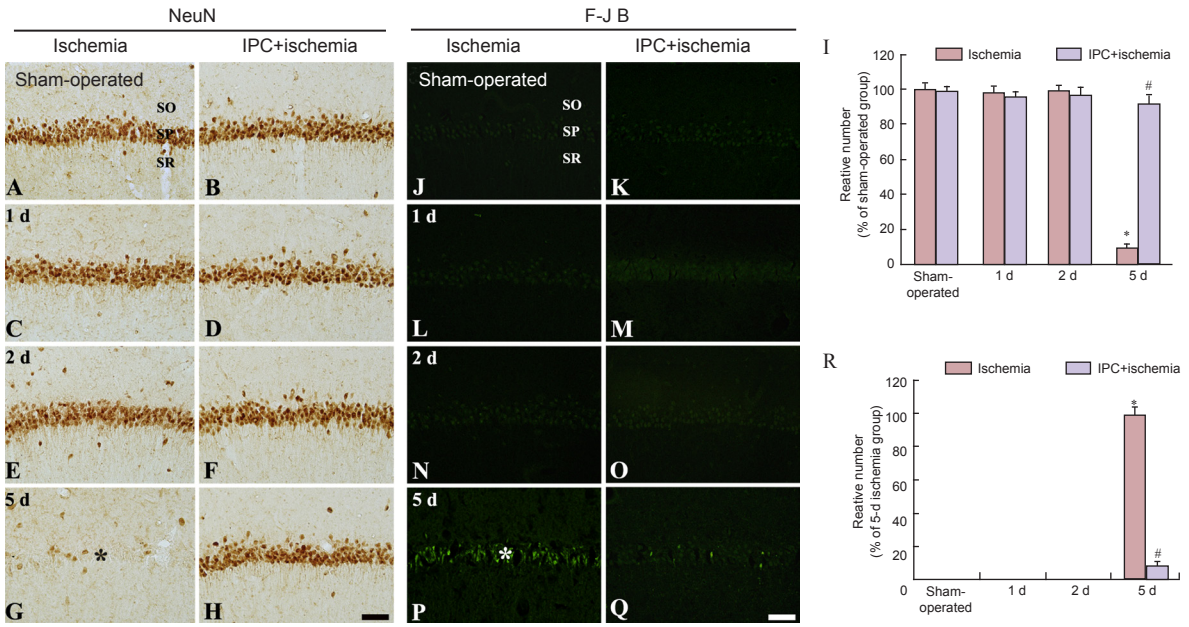

Figure 2 NeuN immunohistochemistry and F-J B histofluorescence staining in the hippocampal CA1 region of gerbils from the sham-operated (A, J), IPC + sham-operated (B, K), ischemia (C, E, G, L, N, P) and IPC + ischemia (D, F, H, M, O, Q) groups.
NeuN-immunoreactive neurons were hardly observed in the SP (asterisk) of gerbils in the ischemia group 5 days after ischemia/reperfusion injury. NeuN-immunoreactive neurons in the SP of hippocampal CA1 region of animals in the IPC + ischemia group were still present 5 days after transient forebrain ischemia. Many F-J B-positive cells were detected in the gerbil hippocampal SP (asterisk) in the ischemia group only 5 days after transient forebrain ischemia; at this time, however, F-J B-positive cells in the IPC + ischemia group were rarely observed. Scale bars: 60 μm. (I, R) Relative analysis as percent in the mean number of NeuN-immunoreactive neurons and F-J B-positive cells in the SP of the gerbil hippocampal CA1 region ($n = 7$ gerbils at each group); *$P < 0.05$, *vs.* sham-operated group; #$P < 0.05$, *vs.* ischemia group; one-way analysis of variance with a *post hoc* Bonferroni's multiple comparison test). The bars indicate the mean ± SEM. NeuN: Neuronal nuclei; IPC: ischemic preconditioning; SO: stratum oriens; SP: stratum pyramidale; SR: stratum radiatum; d: day(s).

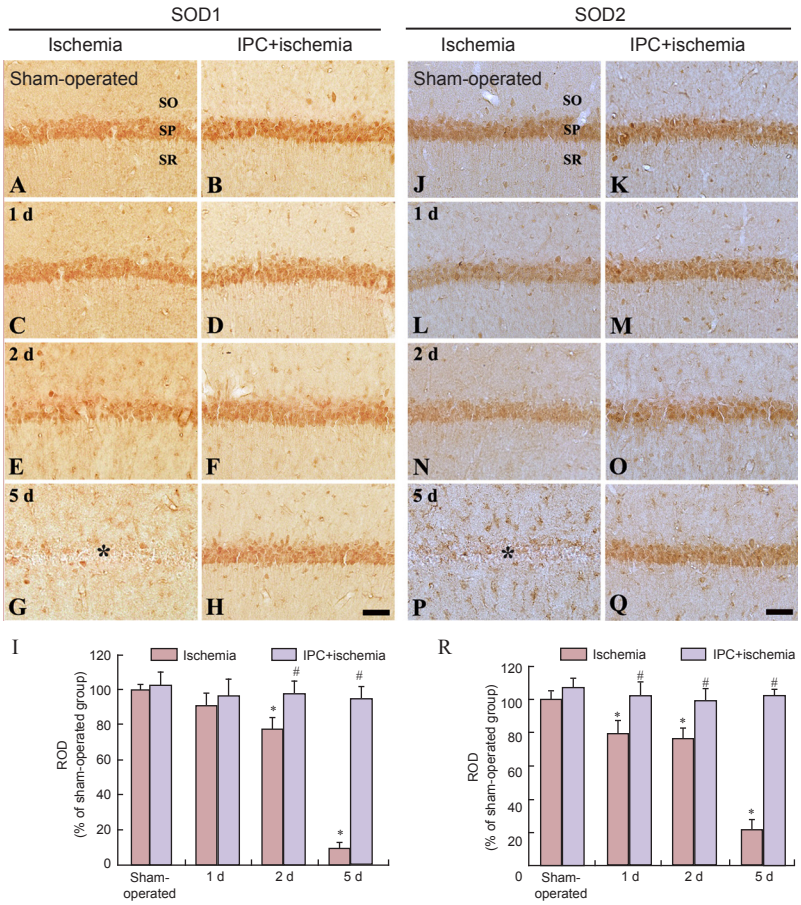

Figure 3 SOD1 and SOD2 immunohistochemistry in the hippocampal CA1 region of gerbils in the sham-operated (A and J), IPC + sham-operated (B and K), ischemia (C, E, G, L, N and P) and IPC + ischemia (D, F, H, M, O and Q) groups.
SOD1 immunoreactivity was well detected in the SP. In the ischemia group, SOD1 immunoreactivity was significantly decreased in the SP (asterisk) 5 days after transient ischemia. However, SOD1 immunoreactivity in the IPC + ischemia group was similar to that in the sham-operated group. SOD2 immunoreactivity was also detected in the SP and the change of SOD2 immunoreactivity in all groups was similar to that of SOD1 immunoreactivity. Scale bars: 60 μm. (I, R) ROD as percent values of SOD1 and SOD2 immunoreactivity in the SP of animals in all groups ($n = 7$ animals in each group; $^*P < 0.05$, *vs.* sham-operated group, $\#P < 0.05$ *vs.* ischemia group; one-way analysis of variance with a *post hoc* Bonferroni's multiple comparison test). The bars indicate the mean ± SEM. SOD: Superoxide dismutase; IPC: ischemic preconditioning; SP: stratum pyramidale; ROD: relative optical density; d: day(s).

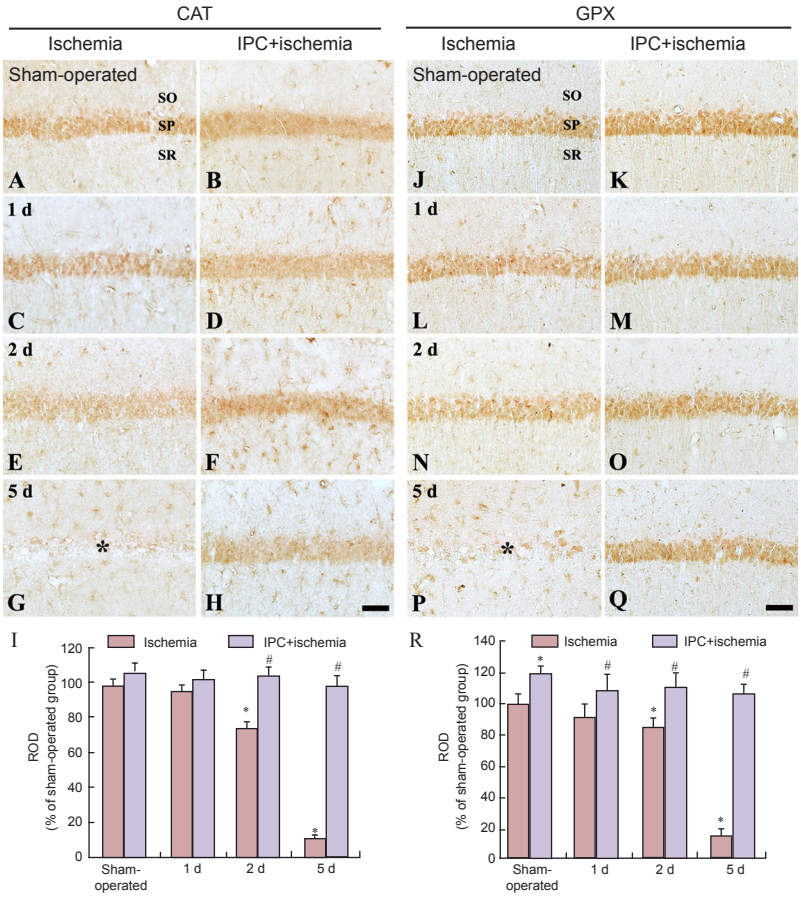

Figure 4 CAT and GPX immunohistochemistry in the hippocampal CA1 region of gerbils in the sham-operated (A and J), IPC + sham-operated (B and K), ischemia (C, E, G, L, N and P) and IPC + ischemia (D, F, H, M, O and Q) groups.
CAT immunoreactivity was easily observed in the SP of animals in the sham-operated group. CAT immunoreactivity was markedly decreased in the SP (asterisk) 5 days after transient ischemia. In the IPC + ischemia group, CAT immunoreactivity in the SP was steadily maintained without change. GPX immunoreactivity was also detected in the SP and its change pattern after transient ischemia was similar to the change pattern of CAT immunoreactivity. However, GPX immunoreactivity in the IPC + sham-operated and IPC + ischemia groups was significantly increased than in the sham-operated-group. Scale bars: 60 μm. (I, R) ROD as percent values of CAT and GPX immunoreactivity in the SP in all of the groups ($n = 7$ animals in each group; $^*P < 0.05$, *vs.* sham-operated group; $\#P < 0.05$, *vs.* ischemia group; one-way analysis of variance with a *post hoc* Bonferroni's multiple comparison test). The bars indicate the means ± SEM. CAT: Catalase; GPX: glutathione peroxidase; IPC: ischemic preconditioning; SO: stratum oriens; SP: stratum pyramidale; SR: stratum radiatum; ROD: relative optical density; d: day(s).

F-J B-positive cells were increased in the stratum pyramidale of the hippocampal CA1 region (**Figure 2P** and **R**). No F-J B-positive cells were detected in any layer of the CA1 region of gerbils in the IPC + sham-operated group and in the IPC + ischemia group until 2 days after transient forebrain ischemia (**Figure 2K**, **M**, **O** and **R**). Furthermore, F-J B-positive cells were rarely observed in the hippocampal CA1 region of gerbils in the IPC + ischemia group 5 days after transient ischemia (**Figure 2Q** and **R**).

SOD1 immunoreactivity

SOD1 immunoreactivity was easily detected in the stratum pyramidale of the hippocampal CA1 region of animals in the sham-operated group (**Figure 3A**). SOD1 immunoreactivity in the CA1 stratum pyramidale was decreased from 1 day after transient ischemia (**Figure 3C**, **E** and **I**); at 2 days after transient ischemia, SOD1 immunoreactivity was significantly reduced (**Figure 3E**), and SOD1 immunoreactivity was hardly detected in the CA1 stratum pyramidale 5 days after transient ischemia (**Figure 3G**). In the IPC + sham-operated group, SOD1 immunoreactivity in the CA1 stratum pyramidale was similar to that in the sham-operated group (**Figure 3B** and **I**). In the IPC + ischemia group, SOD1 immunoreactivity in the stratum pyramidale was not significantly changed until 5 days after transient ischemia compared to that in the IPC + sham-operated group (**Figure 3D**, **F**, **H** and **I**).

SOD2 immunoreactivity

SOD2 immunoreactivity was detected in pyramidal neurons in the CA1 stratum pyramidale of animals in the sham-operated group (**Figure 3J**). SOD2 immunoreactivity in the CA1 stratum pyramidale of animals was significantly reduced 1 and 2 days after transient ischemia (**Figure 3L**, **N** and **R**). Five days after transient ischemia, SOD2 immunoreactivity in the CA1 stratum pyramidale was low; however, at this time, SOD2 immunoreactivity was detected in CA1 non-pyramidal cells (**Figure 3P** and **R**). SOD2 immunoreactivity in the CA1 stratum pyramidale was slightly, but not significantly, increased in the IPC + sham-operated group than in the sham-operated group (**Figure 3K** and **R**). Furthermore, in the IPC + ischemia group, SOD2 immunoreactivity in the CA1 stratum pyramidale was consistently maintained after ischemia/reperfusion (**Figure 3M**, **O**, **Q** and **R**).

CAT immunoreactivity

CAT immunoreactivity in the sham-operated group was easily detected in the CA1 stratum pyramidale (**Figure 4A**). CAT immunoreactivity in the CA1 stratum pyramidale was significantly decreased 2 days after transient ischemia (**Figure 4C**, **E** and **I**), and at 5 days after transient ischemia, CAT immunoreactivity in the CA1 stratum pyramidale was hardly detected (**Figure 4G** and **I**). CAT immunoreactivity in the IPC + sham-operated group was also detected in the CA1 stratum pyramidale and the CAT immunoreactivity was not different from that in the sham-operated group (**Figure 4B**). Furthermore, CAT immunoreactivity in the CA1 stratum pyramidale of animals in the IPC + ischemia group was well detected after transient ischemia (**Figure 4D**, **F**, **H** and **I**).

GPX immunoreactivity

GPX immunoreactivity was well detected in the CA1 stratum pyramidale of animals in the sham-operated group (**Figure 4J**). In the ischemia-operated group, GPX immunoreactivity in the CA1 stratum pyramidale was decreased from 1 day after transient ischemia and hardly detected 5 days after transient ischemia (**Figure 4L, N, P** and **R**). In the IPC + sham-operated group, GPX immunoreactivity in the CA1 stratum pyramidale was significantly incerased compared to the sham-operated group (**Figure 4K** and **R**). In addition, GPX immunoreactivity in the CA1 stratum pyramidale of animals in the IPC + ischemia group was steadily maintained after transient ischemia (**Figure 4M, O, Q** and **R**).

SOD1, SOD2, CAT and GPX protein expression

The result of western blot analysis for SOD1, SOD2, CAT and GPX in the CA1 stratum pyramidale was similar to immunohistochemical data (**Figure 5**).

Discussion

Transient forebrain ischemia leads to the death of the CA1 pyramidal neurons several days after transient ischemic insults, and this phenomenon is "selective vulnerability" in the brain (Kirino and Sano, 1984). We, in the present study, used Mongolian gerbils as excellent animal models of transient cerebral ischemia subjected to IPC (Duszczyk et al., 2009; Durukan and Tatlisumak, 2010; Pires et al., 2011; Makarewicz et al., 2014). In the ischemia group, a significant loss of CA1 pyramidal neurons was detected using CV staining, NeuN immunohistochemistry and F-J B histofluorescence staining. The present findings are consistent with previous findings from studies involving gerbils (Kim et al., 2013, 2014).

We investigated the neuroprotective effect of IPC using CV staining, NeuN immunohistochemistry, and F-J B histofluorescence staining and found that IPC protected pyramidal neurons in the CA1 region of the gerbil hippocampus from subsequent lethal transient forebrain ischemia. This finding has been reported using gerbils (Lee et al., 2014, 2015). IPC is a stimulation of brief transient cerebral ischemia and evokes neuronal tolerance to a following longer or lethal period of transient brain ischemia (Gidday, 2006; Lehotsky et al., 2009).

One of possible mechanisms of neural cell death induced by transient ischemic injury is cellular event associated with ROS-mediated oxidative damage (White et al., 2000; Moro et al., 2005). Excessive ROS production is followed by membrane lipid peroxidation, DNA damage, dysfunctions of membrane receptors and ion channels, important redox-sensitive enzymes, and cytochrome c release from mitochondria (Valko et al., 2007). Therefore, neuronal

defense mechanisms against ischemia-mediated oxidative stress have been focused on antioxidant systems in many studies (Blomgren and Hagberg, 2006; Allen and Bayraktutan, 2009). The antioxidant system is tightly regulated to maintain the redox balance and endogenous antioxidants that can be rapidly used for defense against oxidative stress (Chan, 2001; Sugawara et al., 2002). Along these lines, antioxidants have been expected to display a neuroprotective effect against ischemic injury in the brains (Fujimura et al., 2005; Rodrigo et al., 2013).

SOD1 has been shown to exhibit a protective role in controlling ischemia-induced cellular damage through some mechanisms including prevention of the early loss of DNA repair enzymes following focal cerebral ischemia in mice (Kim et al., 2001; Noshita et al., 2001) and attenuation of DNA fragmentation after global cerebral ischemia in rats (Chan et al., 1998). SOD2 as a scavenger of superoxide anions is also important in neuroprotection. There is evidence that mitochondrial susceptibility to oxidative stress exacerbates brain infarction following permanent focal cerebral ischemia in mutant mice with SOD2 deficiency (Murakami et al., 1998). Armogida et al. (2011) reported that CAT showed a protective role in the brains after transient ischemia although CAT level in neurons was lower compared with other organs (Sugawara et al., 2004). Furthermore, some researchers reported that GPX, which is important in detoxifying H_2O_2 after ischemia/reperfusion (de Haan et al., 1998; Weisbrot-Lefkowitz et al., 1998), was related to decreases in oxidative stress and DNA fragmentation and showed neuroprotective effects in global cerebral ischemia (Sharma and Gupta, 2007).

In the present study, SOD1, SOD2, CAT and GPX immunoreactivities in the ischemia group were decreased with time after transient ischemic insults and hardly detected in CA1 pyramidal neurons 5 days after transient ischemia. However, a distinct increase in GPX immunoreactivity and slight increases in SOD1, SOD2 and CAT immunoreactivities were found in CA1 pyramidal neurons of animals in the IPC + sham-operated group than in the sham-operated group. Furthermore, their immunoreactivities were steadily maintained until 5 days after transient ischemia in the IPC + ischemia group. This result indicates that IPC is able to increase the expression of antioxidant enzymes, which affect neuroprotection after subsequent ischemic insults.

An important molecular mechanism underlying the neuroprotective effect of IPC is the activation of antioxidant gene expression (Stroev et al., 2005). Our results also showed that the increased activity of SOD1 and SOD2 by IPC lead to increased production of hydrogen peroxide and that the cooperative action of CAT and GPX after IPC reduced oxidative stress after severe ischemia/reperfusion (Choi et al., 2007). Alkan et al. (2008) demonstrated that hypoxic preconditioning exhibited strong endogenous protection against subsequent lethal hypoxia and its neuroprotective mechanism was related to the up-regulation of antioxidant enzymes including SOD and GPX, which reduced the oxidative stress associated with ischemia/reperfusion.

In brief, we demonstrated in the present study that the immunoreactivities of antioxidant enzymes (SOD1, SOD2, CAT and GPX) were significantly reduced in CA1 pyramidal neurons with time after transient cerebral ischemia; however, IPC increased GPX expression and maintained SOD and CAT expression in the CA1 pyramidal neurons after subsequent lethal transient ischemia. These results indicate that IPC-mediated increase or maintenance of antioxidant enzymes provides an evidence to explain the neuroprotective effects of IPC in the hippocampus after subsequent transient forebrain ischemia.

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Conflicts of interest: *None declared.*
Plagiarism check: *This paper was screened twice using Cross-Check to verify originality before publication.*
Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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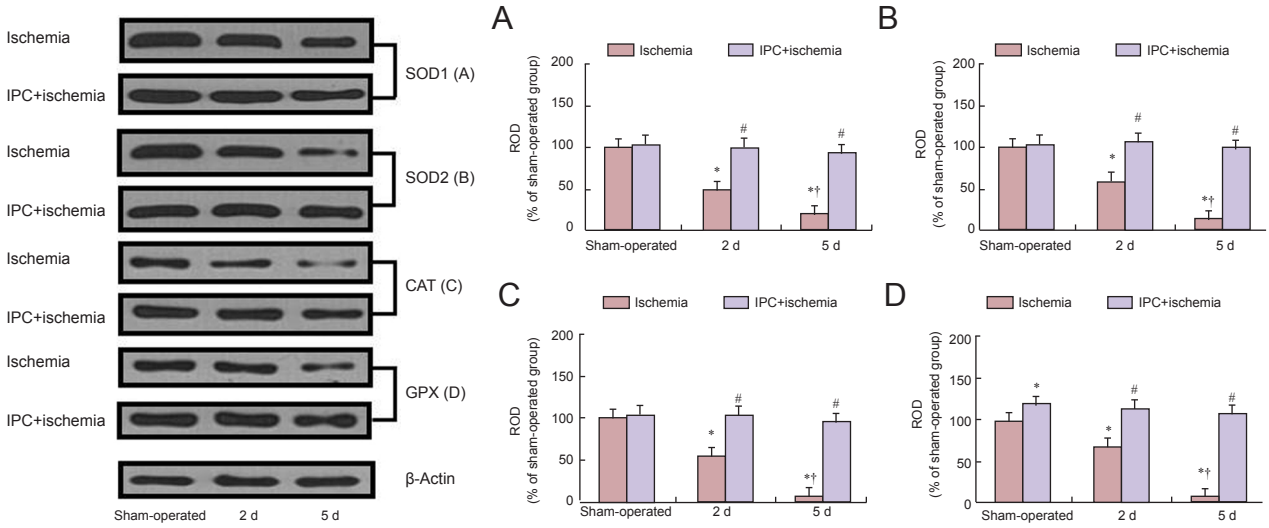

Figure 5 Western blot analysis of SOD1, SOD2, CAT and GPX in the hippocampal CA1 region of gerbils in all groups.
(A–D) Protein expression levels of SOD1, SOD2, CAT and GPX. ROD as percent of the immunoblot band is presented (*$P < 0.05$, *vs.* sham-operated group, #$P < 0.05$, *vs.* ischemia group, †$P < 0.05$, *vs.* the respective pre-time point group; one-way analysis of variance with a *post hoc* Bonferroni's multiple comparison test). Bars indicate the mean ± SEM from 7 animals per group. SOD: Superoxide dismutase; CAT: catalase; GPX: glutathione peroxidase; IPC: ischemic preconditioning; d: day(s).

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