Ischemic preconditioning in the rat hippocampus increases antioxidant activities but does not affect the level of hydroxyl radicals during subsequent severe ischemia

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Abstract

Several studies have demonstrated that ischemic preconditioning increases superoxide dismutase activity, but it is unclear how ischemic preconditioning affects events downstream of hydrogen peroxide production during subsequent severe ischemia and reperfusion in the hippocampus. To answer this question, we investigated whether ischemic preconditioning in the hippocampal CA1 region increases the activities of antioxidant enzymes glutathione peroxidase and catalase, resulting in a decrease in the level of hydroxyl radicals during subsequent severe ischemia-reperfusion. Transient forebrain ischemia was induced by four-vessel occlusion in rats. Ischemic preconditioning for 3 min or a sham operation was performed and a 15-min severe ischemia was induced three days later. Ischemic preconditioning preserved the CA1 hippocampal neurons following severe ischemia. The concentration of 2,3-dihydroxybenzoic acid, an indicator of hydroxyl radical, was measured using *in vivo* microdialysis technique combined with HPLC. The ischemia-induced increase in the ratio of 2,3-dihydroxybenzoic acid concentration relative to baseline did not differ significantly between preconditioned and control groups. On the other hand, activities of the antioxidant enzymes glu-tathione peroxidase-1 and catalase were significantly increased at 3 days after ischemic preconditioning in the hippocampus. Our results suggest that, in preconditioned rats, while hydrogen peroxide is generated from severe ischemia, the activity of catalase and glutathione peroxidase-1 is correspondingly increased to eliminate the excessive hydrogen peroxide. However, our results show that the enhanced activity of these antioxidant enzymes in preconditioned rats is not sufficient to decrease hydroxyl radical levels during subsequent severe ischemia-reperfusion.

Keywords: brain ischemia; catalase; glutathione peroxidase; hippocampus; hydroxyl radical; ischemic preconditioning

Introduction

Transient forebrain ischemia induces delayed neuronal death in the hippocampal CA1 region. Oxygen free radicals have been suggested to play a significant role in this neuronal death (Piantadosi and Zhang, 1996; Yamamoto et al., 1997; Kim et al., 1999, 2006; Song et al., 2007). It is particularly interesting that superoxide generation increases during cerebral ischemia-reperfusion (Chan et al., 1998; Sugawara et al., 2005). Superoxide reportedly induces damage in the endoplasmic reticulum (Hayashi et al., 2005). Also, superoxide can form another toxic radical, peroxynitrite, through a reaction with nitric oxide which is specifically accumulated in regions that are vulnerable to ischemic insults (Hicks et al., 1999; Calapai et al., 2000; Choi et al., 2004). Overexpression of copper/zinc superoxide dismutase (Cu/Zn-SOD) minimizes superoxide toxicity, resulting in reduction of cell death (Sugawara et al., 2002). Thus, understanding radical generation and subsequent toxicity can lead to therapeutic tools to prevent brain injury including ischemic insult.

Kitagawa et al. (1990) first showed that short periods of ischemia (ischemic preconditioning) protect vulnerable neurons against subsequent ischemic injury. There is much evidence that ischemic preconditioning increases the expression level of SOD, an enzyme that converts superoxide into

hydrogen peroxide (Kato *et al.*, 1995; Wada *et al.*, 2001). Increased activity of SOD results in a reduction in both superoxide and peroxynitrite toxicity. Downregulation of Cu/Zn-SOD increases cell death via peroxynitrite formation (Troy *et al.*, 1996). In addition, overexpression of Cu/Zn-SOD minimizes superoxide toxicity, resulting in reduction of cell death (Sugawara *et al.*, 2002). It is possible that increased expression of SOD may result in the accumulation of hydrogen peroxide. After severe ischemia-reperfusion in preconditioned rats, this SOD-induced increase in hydrogen peroxide could cause increased production of hydroxyl radical which has been known as one of the most toxic free radicals both *in vivo* and *in vitro* excitotoxic conditions including ischemia (Christensen *et al.*, 1994; Zhang and Piantadosi, 1994; Kil *et al.*, 1996; Yamamoto *et al.*, 1997). Hydroxyl radical may damage cells through the development of DNA fragmentation and lipid peroxidation (Gutteridge, 1984; Tsang *et al.*, 1996; Rollet-Labelle *et al.*, 1998). Thus, it is critical to understand how ischemic preconditioning affects events downstream of hydrogen peroxide production.

In the present study, we investigated whether ischemic preconditioning modulates hydroxyl radical levels during severe ischemia and reperfusion, and examined the activities of antioxidant enzymes catalase and glutathione peroxidase (GPx)-1 in the rat hippocampus.

Materials and Methods

Time-schedule of forebrain ischemia

Age-matched, adult male Sprague-Dawley rats (250-300 g, Koatech, Pyeongtaek, Korea) were used. The time-schedule of this experiment was summarized in Figure 1. Severe ischemia or ischemic preconditioning was induced by four-vessel occlusion according to Pulsinelli and Brierley (1979) with minor modification (Lee *et al.*, 2002). In brief, rats were anaesthetized with chloral hydrate (400 mg/kg, i.p.) and the vertebral arteries were electrocauterized (Day 1). After 24 h (Day 2), ischemic preconditioning was induced by occlusion of both common carotid arteries for 3 min. As a control group for ischemic preconditioning, animals were subjected to the same anesthesia and surgical procedures, yet with a sham manipulation rather than artery occlusion. Three days later (Day 5), severe ischemia was induced for 15 min in all rats of both groups. Body temperature was maintained at $37.5 \pm 0.3^{\circ}C$ with a heat lamp during and after the global ischemia. All experimental procedures performed on the animals were conducted with the approval of the Ethics Committee of the Catholic University of Korea, and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996).

In vivo microdialysis

In each group (preconditioned and sham), six rats were used. For the determination of hydroxyl radical levels, an *in vivo* microdialysis technique was used. Two days after ischemic preconditioning or sham manipulation (Day 4), a guide cannula was stereotaxically implanted (AP: -4.1 mm, ML: -2.8 mm, DV: -0.9 mm) using the atlas of Paxinos and Waston (1998) and fixed with dental cement on the skull surface. To locate the microdialysis probe along the pyramidal cell layer of hippocampal CA1, the guide cannula was implanted with
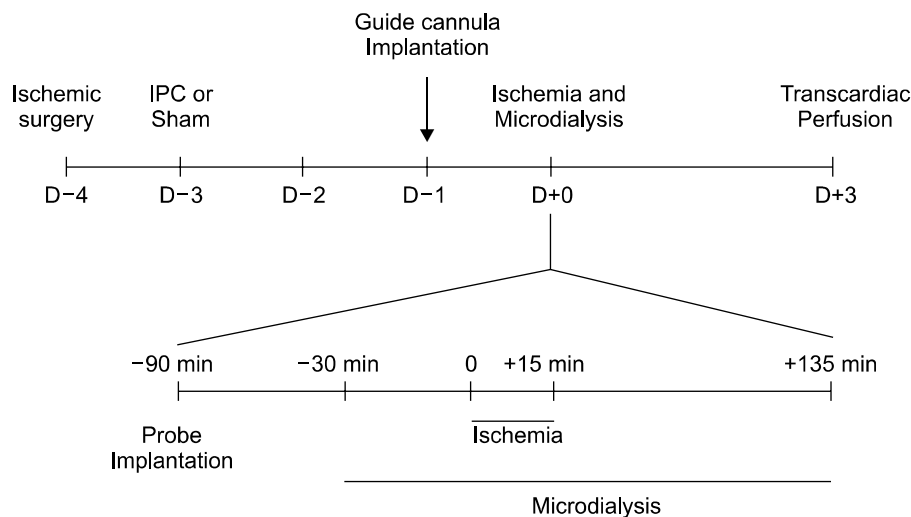

Figure 1. Summary of experimental protocol. Ischemic preconditioning (IPC) was induced for 3 min. Three days later severe ischemia was induced for 15 min. A microdialysis probe was implanted 90 min before severe ischemia induction and Ringer solution containing salicylate was perfused. After stabilizing baseline condition for 60 min, perfusate was collected every 15 min until 2 h after reperfusion. In a second group of rats, hippocampi were harvested at time D + 0 to measure the activities and expression levels of antioxidant enzymes prior to subsequent ischemia-reperfusion.

a 40° gradient from lateral to medial side. After 24 h (Day 5), a microdialysis probe (diameter: 240 μm, length: 2 mm, CMA/11, Carnegie Medicine, Stockholm, Sweden) was placed inside the guide cannula 90 min before severe ischemia induction and perfused with Ringer solution (NaCl 148.2 mM, KCl 4 mM, $CaCl_2$ 2.3 mM, pH 7.0) containing 5 mM sodium salicylate (Sigma, St Louis, MO) at a rate of 2 μl/min. One hour of recovery time was allowed following the implantation of probe before basal values were monitored for 30 min. From 30 min before severe ischemia induction until 120 min after reperfusion, microdialysates were collected every 15 min into eppendorf tubes containing 30 μl of 0.1 mM perchloric acid and frozen at $-70^{o}C$ until use (Obata, 1997).

HPLC

To measure the concentration of 2,3-dihydroxy-benzoic acid (DHBA) as an indicator of hydroxyl radical, HPLC system with an electrochemical detector was used. The system consisted of a Waters 510 pump (Waters, Milford, MA), autosampler (Spark Holland, Emmen, Netherlands), DHBA-250 column (5 μm, 250 × 3.0 mm, ESA, Chelmsford, MA), electrochemical detector (M5200A, ESA), dual analytical cell (M5011, ESA), guard cell (M5020, ESA) and integrator (Spectra-Physics analytical, Mountain View, CA). The detector potentials were set at + 250 mV (E1), + 750 mV (E2) and + 775 mV (guard cell). The mobile phase contained 5% isopropyl alcohol, 15% methanol, 50 mM sodium acetate and 50 mM citric acid, and was adjusted to a final pH of 2.5 with 85% phosphoric acid. The eluent was delivered with a flow rate of 0.5 ml/min.

Tissue processing

Three days after severe ischemia and reperfusion (Day 8), rats were deeply anesthetized with chloral hydrate (600 mg/kg; Merck, Darmstadt, Germany) and perfused with cold saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brain was then removed and postfixed in the same fixative for 4 h. After cryoprotection with 30% sucrose in 0.1 M PB, 25 μm sections were cut using a cryostat (Beckman, Fullerton, CA). To identify cell viability, tissues were stained with cresyl violet. In brief, sections were mounted onto a gelatin-coated slide and dried. After rehydration in 95, 90, 80 and 70 % ethanol and distilled water for 3 min each, sections were stained with 0.3% cresyl violet solution. After destaining in 95% ethanol containing 0.1% acetic acid glacial, sections were dehydrated and covered with permount.

Enzyme activity test

For catalase or GPx activity determinations, rats were killed 3 days after ischemic preconditioning or sham manipulation. For each group (preconditioned and sham), six rats were used. Immediately after decapitation, the hippocampus was removed on an ice-chilled plate, weighed and stored at $-70^{o}C$. Tissues were ultrasonically homogenized in 1 ml of 0.05 M phosphate buffer, pH 7.0 and the protein concentration was measured. We assayed catalase activity in the hippocampus with a kit that uses H_2O_2 as a substrate (Sigma). The reaction mixture containing homogenate was incubated with 0.2 M H_2O_2 at $25^{o}C$ for 1 min and the reaction was stopped by adding stop solution containing sodium azide. After mixing with color reagent containing 0.25 mM 4-aminoantipyrine and 2 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, we measured absorbance at 520 nm. We assayed GPx activity in the hippocampus with a kit using tert-butyl hydroperoxide as substrate (Sigma). The reaction mixture containing homogenate was incubated with assay reagent containing NADPH and 30 mM tert-butyl hydroperoxide solution for 30 s and absorbance was measured at 340 nm.

Western blot analysis

Ten microgram of hippocampus homogenate was mixed with an equal volume of sample buffer. The samples were heated for 5 min at $95^{o}C$. The proteins were analyzed by electrophoresis on 8 or 12% polyacrylamide gels for catalase or GPxs, respectively. Proteins were electrophoretically transferred from the SDS-polyacrylamide gel to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membrane was blocked in 1% casein for 1 h. The blot was then incubated with rabbit anti-GPx-1 (Labfrontier, Seoul, Korea) or mouse anti-catalase antibodies (Sigma) overnight at $4^{o}C$. Each antibody was identified with goat-anti rabbit IgG or goat-anti mouse IgG, conjugated with HRP, and incubated for 1 h at room temperature. The signal was visualized by using Renaissance chemiluminescent HRP substrate (PerkinElmer Life Sciences, Wellesley, MA). As a control for equal loading of protein across the gel, membranes were stripped and probed for β-tubulin (Sigma) expression using the same method.
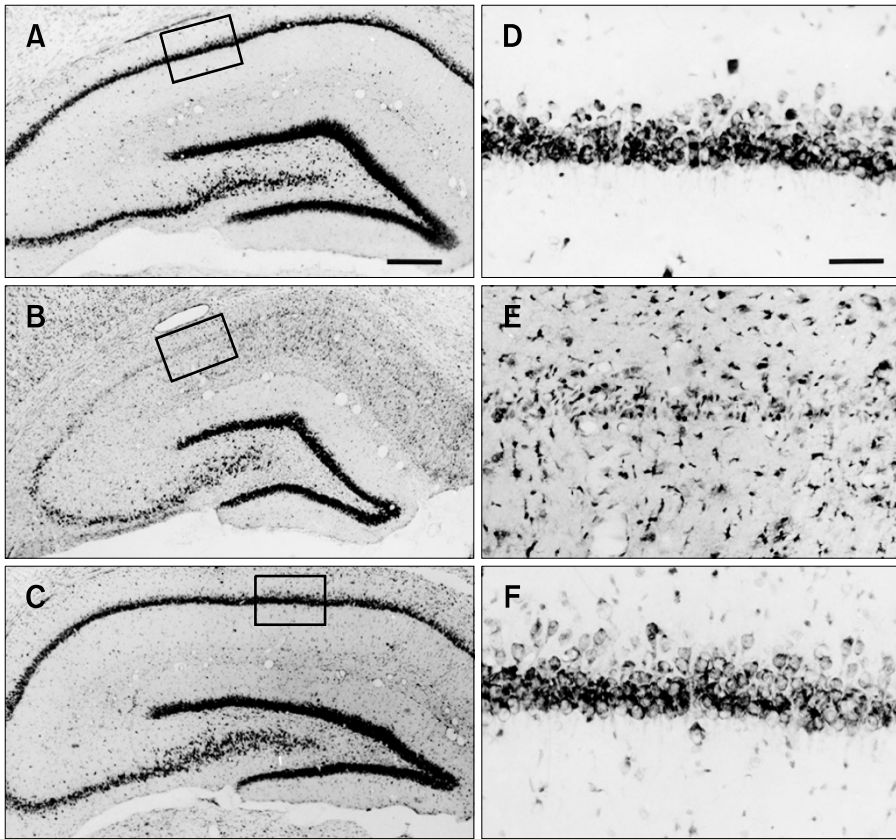
Figure 2. Histopathological observations of the hippocampus. Control (A, B) three days after forebrain ischemia for 15 min in the rat without (C, D) and with (E, F) ischemic preconditioning. Preconditioning was induced by 4-vessel occlusion for 3 min followed by reperfusion for 3 days. Hippocampal CA1 neurons were destroyed in the rat without preconditioning (C, D), but preserved with preconditioning (E, F). Scale bars, 1,500 μm for A-C; 400 μm for D-F.

Statistics

Statistical analysis was done using Student's *t*-test, and significance was accepted at $P < 0.05$.

Results

Identification of ischemic tolerance in the hippocampal CA1 region

The experimental time-course is summarized in Figure 1. At three days after severe ischemia, rats that did not undergo ischemic preconditioning had few surviving pyramidal neurons in the CA1 region of the hippocampus compared to the control group (Figure 2A and 2B). However, ischemic preconditioning in this experimental system successfully prevented neuronal loss induced by severe ischemia (Figure 2C).

The lack of effect of ischemic preconditioning on hydroxyl radical levels in the hippocampal CA1 area after severe ischemia

Representative chromatograms of 2,3- and 2,5-DHBA are shown in Figure 3A and 3B. Figure 3A shows a chromatogram from a standard solution containing 25 nM 2,3- and 2,5-DHBA and Figure 3B shows the chromatogram from *in vivo* micro-dialysate containing 31.1 nM 2,3-DHBA during the ischemic period. Previous reports showed that 2,5-DHBA can be formed *in vivo* by the action of cytochrome P450 (Ingelman-Sundberg *et al.*, 1991). Therefore, we chose 2,3-DHBA as an indicator of hydroxyl radical in the hippocampus. Figure 3C shows the concentration of 2,3-DHBA in micro-dialysate from 30 min before ischemia to 120 min after reperfusion. The basal concentration of 2,3-DHBA was calculated as the mean concentration of 2,3-DHBA in two microdialysates obtained during the pre-ischemic period. The basal concentrations of 2,3-DHBA were 30.1 ± 7.7 nM and 28.6 ± 8.1 nM in control and preconditioned groups, respectively. A transient forebrain ischemia significantly increased 2,3-DHBA production in both groups (56.5 ± 12.2 % and 54.8 ± 8.9 % in control and preconditioned groups, respectively). These data show that ischemic preconditioning does not have a significant effect on hydroxyl radical levels in the hippocampus during subsequent severe ischemia and early reperfusion.
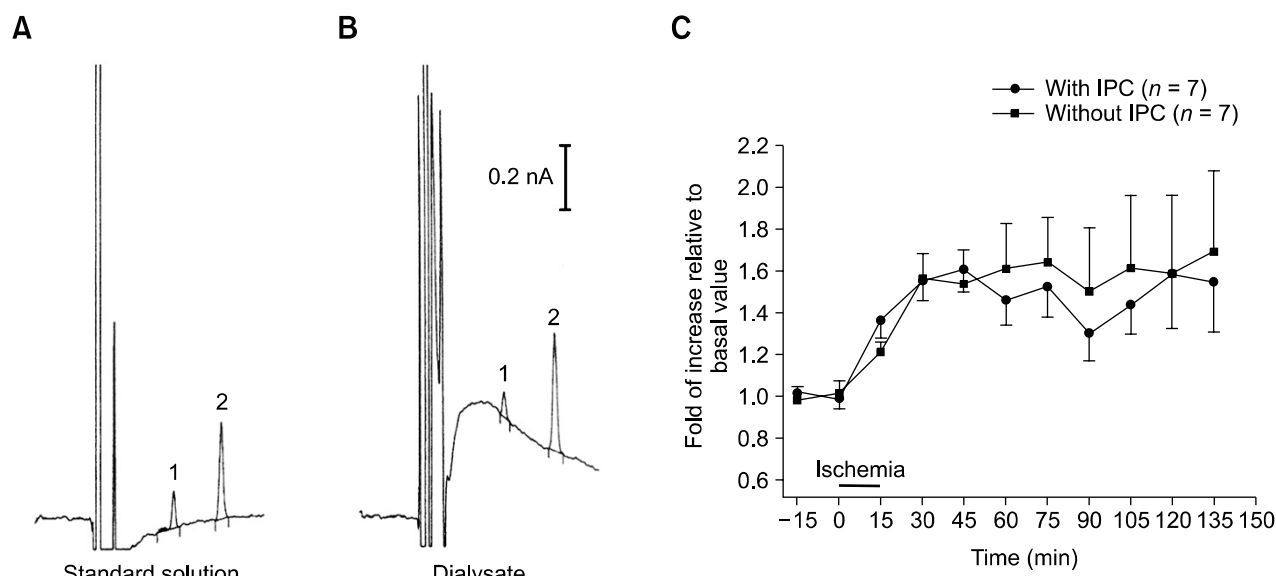
A   **B**   **C**

Standard solution   Dialysate

Figure 3. Effects of ischemic preconditioning on hydroxyl radical levels during severe ischemia-reperfusion in the rat hippocampus. (A-B) Representative HPLC chromatograms. Each chromatogram indicates the result of standard solution (A, 2,3-DHBA of 25 nM) and a dialysate (B, 2,3-DHBA of 31.1 nM) from the hippocampal CA1 area during the ischemic period. 1 and 2 represent 2,5-DHBA and 2,3-DHBA, respectively. (C) In both control and preconditioned groups, extracellular 2,3-DHBA concentrations were increased during ischemic and reperfusion periods. Data were obtained from 6 rats in each group and presented as mean $\pm$ SEM.
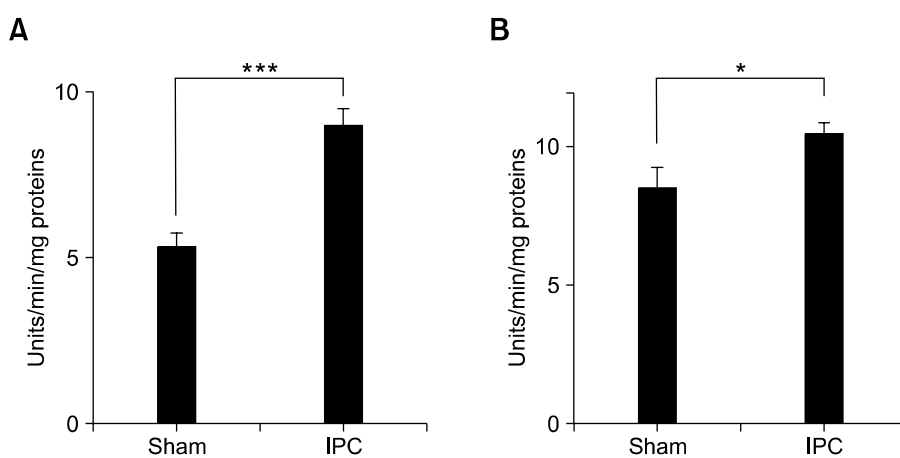
A   **B**

Figure 4. Enzymatic activities of catalase (A) and glutathione peroxidase-1 (GPx-1, B) in the hippocampus after ischemic preconditioning. Hippocampal tissue was isolated 3 days after ischemic preconditioning or sham manipulation and the activity of each enzyme was measured. (A) Ischemic preconditioning for 3 min significantly increased enzymatic activity of catalase in the hippocampus at 3 days post-treatment. One unit of catalase decomposes 1.0 μmol of hydrogen peroxide into oxygen and water per minute at pH 7.0 at 25°C at a substrate concentration of 10 mM hydrogen peroxide. (B) Ischemic preconditioning for 3 min significantly increased enzymatic activity of GPx-1 in the hippocampus at 3 days post-treatment. One unit of GPx-1 catalyzes the formation of 1.0 mmol of NADP^{+} from NADPH per minute at pH 8.0 at 25°C. Values are presented as mean $\pm$ SEM. Six animals were used in each group (pre-conditioned and sham). *$P <$ 0.05, ***$P <$ 0.001 compared to sham-manipulated rats.

Determination of antioxidant enzyme activity after ischemic preconditioning

As mentioned above, increased expression of manganese-SOD (Mn-SOD) by ischemic preconditioning could increase production of hydrogen peroxide. Therefore, we asked whether ischemic preconditioning increases the activity of enzymes that convert hydrogen peroxide into water molecules. To examine this, we examined the activity of
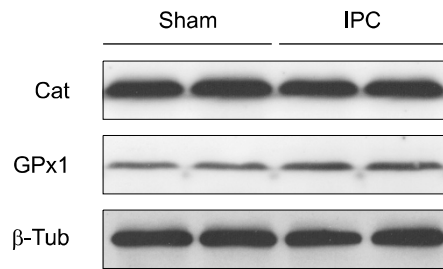
Figure 5. Western blot analysis of catalase and glutathione peroxidase-1 in the rat hippocampus 3 days after ischemic preconditioning or sham manipulation. Cat, catalase; GPx1, glutathione peroxidase-1; IPC, ischemic preconditioning; β-Tub, β-tubulin.

catalase and GPx-1 at 3 days after either ischemic preconditioning or sham manipulation. As shown in Figure 4, both enzyme activities were significantly increased following ischemic preconditioning (increases of 66.9% and 22.6% for catalase and GPx-1, respectively). These data suggest that ischemic preconditioning significantly increases the activity of antioxidant enzymes that convert hydrogen peroxide into water molecules.

Expression of antioxidant enzymes after ischemic preconditioning

To examine whether the activity of antioxidant enzymes such as catalase and GPx-1 is associated with their expression, we did western blot analysis. We harvested hippocampal tissue at 3 days after either ischemic preconditioning or sham manipulation. We found no significant difference in enzyme expression between the two groups (Figure 5).

Discussion

The results of the present study show that 1) ischemic preconditioning does not have a significant effect on production of 2,3-DHBA, an indicator of hydroxyl radical, in the hippocampal CA1 region during subsequent severe ischemia and early period of reperfusion, and 2) ischemic preconditioning enhances the activity of H_2O_2-degrading enzymes such as GPx-1 and catalase.

Brain ischemia results in a significant increase in the production of superoxide anions (Chan *et al.*, 1998; Sugawara *et al.*, 2005), which are converted into hydrogen peroxide by SOD. In turn, hydrogen peroxide is broken down into either hydroxyl radical via the Fenton reaction, or into water molecules via the activity of catalse or GPx. Increased generation of hydroxyl radicals could

play a pivotal role in the delayed neuronal death seen after ischemic insult. For example, the concentration of hydroxyl radical increases in the hippocampus following ischemia-reperfusion injury (Christensen *et al.*, 1994; Zhang and Piantadosi, 1994), and the inhibition of hydroxyl radical generation prevents the delayed neuronal death (Kil *et al.*, 1996; Yamamoto *et al.*, 1997). In the present study, the production of 2,3-DHBA significantly increased in the hippocampal CA1 region during severe ischemia and reperfusion, in accordance with earlier reports (Zhang and Piantadosi, 1994; Kil *et al.*, 1996; Negishi *et al.*, 2003).

Ischemic preconditioning before severe ischemia preserved the neuronal signal (Figure 2), suggesting that ischemic preconditioning induces ischemic tolerance in the animal models used in this study. An increased level of antioxidants is considered a critical step in the development of ischemic tolerance. For instance, ischemic preconditioning enhances synthesis of Mn-SOD in the hippocampus (Kato *et al.*, 1995; Stroev *et al.*, 2004). Increased activity of Mn-SOD through ischemic preconditioning would cause increased production of hydrogen peroxide and subsequently hydroxyl radicals, through the Fenton reaction. Thus, in an animal that underwent preconditioning, the generation of hydroxyl radicals following subsequent severe ischemia would depend upon the activity of H_2O_2-degrading enzymes, including GPx and catalase. Thus, we determined whether ischemic preconditioning affects the levels of hydroxyl radicals during the severe ischemia-reperfusion period. Unexpectedly, our results showed that there was no significant difference between preconditioned and control groups in terms of the 2,3-DHBA levels during severe ischemia-reperfusion in the hippocampal CA1 area. However, since we only examined the hydroxyl radical levels for 120 min after reperfusion, we cannot rule out the possibility that hydroxyl radical concentrations differ in later phase of reperfusion between the two groups.

In both mature and neonatal brain, studies with GPx-1-overexpressing mice have shown significant neuroprotection against focal cerebral ischemia as well as hypoxia (Weisbrot-Lefkowitz *et al.*, 1998; Furling *et al.*, 2000; Ishibashi *et al.*, 2002; Sheldon *et al.*, 2004). Truelove *et al.* (1994) also provided evidence that microinfusion of catalase reduces neuronal damage following repetitive ischemic insult. Interestingly, our data indicates that the activity of GPx-1 and catalase is significantly increased in the hippocampus 3 days after ischemic preconditioning, although the level of protein expression does not increase concomitantly. This

temporal pattern overlaps with the period during which ischemic preconditioning can provide protection against subsequent ischemia (Kato *et al.*, 1994; Dooley and Corbett, 1998; Zhou *et al.*, 2004). This suggests that the increase in GPx-1 and catalase activity induced by ischemic preconditioning may contribute to degrading hydrogen peroxide generated by subsequent severe ischemia-reperfusion. Considered together with results showing increased SOD activity in the ischemically preconditioned animals (Kato *et al.*, 1995; Stroev *et al.*, 2004), the increased rate of both synthesis and elimination of hydrogen peroxide after subsequent severe ischemia-reperfusion in preconditioned animals seems to be balanced, probably resulting in no significant change in hydroxyl radical levels in this situation.

It is known that levels of catalase and GPx activity do not necessarily correlate with protein expression (Brunelli *et al.*, 2001; Sneddon *et al.*, 2003; Drew *et al.*, 2005). For example, nitric oxide can form a transient complex with resting catalase, thus reducing its activity. In addition, posttranslational modifications, such as phosphorylation, have been suggested to regulate GPx activity. c-Abl and Arg tyrosine kinases phophorylate GPx1 on a tyrosine residue (Tyr-96), inhibiting its activity (Cao *et al.*, 2003). Our data do not provide any evidence regarding the regulation of enzyme activities. Thus, further study is needed to determine the mechanisms by which antioxidant enzyme activity is enhanced by ischemic preconditioning.

In summary, our data suggests that the cooperative actions of antioxidant enzymes including catalase and GPx in the hippocampus after ischemic preconditioning reduce oxidative stress following severe ischemia and reperfusion, and play a role in the development of ischemic tolerance.

Acknowledgement

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