Neuroprotective effects of ischemic preconditioning on hippocampal CA1 pyramidal neurons through maintaining calbindin D28k immunoreactivity following subsequent transient cerebral ischemia

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Abstract
Ischemic preconditioning elicited by a non-fatal brief occlusion of blood flow has been applied for an experimental therapeutic strategy against a subsequent fatal ischemic insult. In this study, we investigated the neuroprotective effects of ischemic preconditioning (2-minute transient cerebral ischemia) on calbindin D28k immunoreactivity in the gerbil hippocampal CA1 area following a subsequent fatal transient ischemic insult (5-minute transient cerebral ischemia). A large number of pyramidal neurons in the hippocampal CA1 area died 4 days after 5-minute transient cerebral ischemia. Ischemic preconditioning reduced the death of pyramidal neurons in the hippocampal CA1 area. Calbindin D28k immunoreactivity was greatly attenuated at 2 days after 5-minute transient cerebral ischemia and it was hardly detected at 5 days post-ischemia. Ischemic preconditioning maintained calbindin D28k immunoreactivity after transient cerebral ischemia. These findings suggest that ischemic preconditioning can attenuate transient cerebral ischemia-caused damage to the pyramidal neurons in the hippocampal CA1 area through maintaining calbindin D28k immunoreactivity.

Key Words: nerve regeneration; transient cerebral ischemia; ischemic tolerance; neuroprotection; hippocampus; pyramidal neurons; calcium binding protein; neural regeneration

Introduction
Calcium ions play a complex and fatal role in nerve cell function (Cai et al., 2015). Evidence exists that uncontrolled increase in intracellular calcium ion concentrations results in excessive cell activation, injury, and eventually cell death, and increased calcium ion concentrations lead to calcium binding by regulatory proteins, which are called calcium binding proteins including calbindin, parvalbumin and calretinin (Baimbridge et al., 1992; Verdaguer et al., 2015).

Calbindin-D28k (CB), a member of the EF-hand family of calcium binding proteins, is easily found in neuronal cells in the central nervous system and performs function that is able to buffer toxic intracellular calcium ions induced by various insults including ischemic stroke (Baimbridge et al., 1992; Klapstein et al., 1998; Yenari et al., 2001). CB overexpression shows resistance to ischemic insults in vitro and in vivo (Yenari et al., 2001; Fan et al., 2007; Freimann et al., 2010); and exogenous CB reduces oxidative stress, preserves mitochondrial function (Guo et al., 1998), and delays the onset of cell death following excitotoxic stimulation (D’Orlando et al., 2001).

Transient global cerebral ischemia, which is able to happen due to the blockage or lack of cerebral blood flow induced by cardiac arrest or cardiovascular surgery, leads to death of vulnerable neurons such as pyramidal neurons in the hippocampal CA1 area (Ohk et al., 2012; Lee et al., 2013a; Kim et al., 2014). Ischemic preconditioning (IPC), which is a technique for producing resistance to the loss of blood supply, can be elicited by non-fatal brief occlusion of blood flow, has been demonstrated as a therapeutic strategy against subsequent fatal ischemic insults (Lehotsky et al., 2009; Liu et al., 2009; Thompson et al., 2013; Kovalska et al., 2014). IPC can activate certain cellular pathways that are able to help alleviate fatal damage induced by subsequent ischemic insults,
In the ischemia group, a few CV-positive cells were found in the stratum pyramidale (SP, asterisks) of the CA1 area from 4 days post-ischemia. However, CV-positive CA1 pyramidal cells in the IPC+ ischemia group were well preserved after ischemia/reperfusion. CA: Cornu ammonis; DG: dentate gyrus; SO: stratum oriens; SR: stratum radiatum; IPC: ischemia preconditioning. Scale bars: 200 μm for A–L (low magnification photos); 60 μm for a–l (high magnification photos). (M) Relative analysis as percent in the mean number of CV-positive cells in the SP of the CA1 area (n = 7 in each group; *P < 0.05, vs. sham group; #P < 0.05, vs. corresponding ischemia group). The bars indicate the mean ± SEM.

Figure 1 Cresyl violet (CV) staining of the gerbil hippocampus in the sham (A, a), IPC + sham (B, b), ischemia (C, c, E, e, G, g, I, i, K, and k), and IPC+ ischemia (D, d, F, f, H, h, J, j, L, and l) groups.
and this phenomenon is called “ischemic tolerance” (Saad et al., 2015).

Several mechanisms, which explain the neuroprotective effects of IPC, have been published (Lee et al., 2014, 2015a, b, 2016; Kim et al., 2015; Park et al., 2016). However, to the best of our knowledge, there are no reports on calcium binding proteins in IPC-mediated ischemic brains. We, therefore, investigated the effects of IPC on CB immunoreactivity in the hippocampal CA1 pyramidal neurons following subsequent ischemia/reperfusion injury in gerbils.

Materials and Methods

Experimental animals

As previously described (Kim et al., 2015), male gerbils weighing 65–75 g and aged 6 months, were divided into four groups: (1) sham group (both common carotid arteries were exposed but not occluded); (2) ischemia group (5-minute transient ischemia); (3) IPC + sham group (IPC 2-minute transient ischemia) and no ischemia); and (4) IPC + ischemia group (IPC followed by ischemia). Gerbils (n = 7 at each point time in each group) were recovered 1, 2, 3, 4 and 5 days after ischemia. Procedures for animal handling and care complied with the guidelines that follow the current international laws and policies (Guide for the Care and Use of Laboratory Animals, The National Academies Press, 8th Ed., 2011), and all the experimental protocols of this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), Kangwon National University (approval No. KW-160802-1).

Induction of IPC and transient ischemia

As previously described (Park et al., 2016), in brief, the gerbils were anesthetized with a mixture of 2.5% isoflurane (Baxtor, Deerfield, IL, USA) in 33% oxygen and 67% nitrous oxide. Bilateral common carotid arteries were occluded for 2 minutes for IPC followed by 5-minute ischemia with 1 day interval. Body (rectal) temperature was controlled under normothermia (37 ± 0.5°C) during the surgery with a rectal temperature probe (TR-100; Fine Science Tools, Foster City, CA, USA).

Tissue processing

Tissue preparation for histology was carried out according to our published method (Kim et al., 2014). In brief, the gerbils were perfused transcardially with 4% paraformaldehyde. The brains were serially sectioned into 30-μm coronal sections using a cryostat (Leica, Wetzlar, Germany).

 Cresyl violet (CV) staining

In order to observe the distribution of pyramidal cells in the stratum pyramidale of hippocampal CA1 region, CV staining was done as previously described (Park et al., 2016). Briefly, 1% cresyl violet acetate (Sigma, St. Louis, MO, USA) and 0.28% glacial acetic acid were used for CV staining.

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

To examine neuronal death after ischemia, F-J B histofluo-

rescence staining was carried out according to our published procedure (Park et al., 2016). In short, the brain tissues were immersed in a 0.06 % potassium permanganate solution and stained with 0.0004% F-J B (Histochem, Jefferson, AR, USA) solution. The stained brain tissues were observed using an epi-fluorescent microscope (Carl Zeiss, Oberkochen, Germany) with blue (450–490 nm) excitation light and a barrier filter.

Immunohistochemistry for CB

CB immunoreactivity was determined according to our published method (Bae et al., 2015). Briefly, the brain tissues were incubated with diluted rabbit anti-CB antibody (1:500; Abcam, Cambridge, MA, USA) overnight at 4°C, then exposed to biotinylated goat anti-rabbit antibody (1:250; Vector Laboratories, Burlingame, CA, USA) for 2 hours at room temperature and streptavidin peroxidase complex (1:200; Vector Laboratories) and finally visualized with 3,3′-diaminobenzidine tetrachloride (Sigma).

Data analysis

NeuN- and F-J B-positive cells were counted according to our published procedure (Bae et al., 2015). Fifteen brain tissue sections were chosen in each animal with 120 µm interval. NeuN- and F-J B-positive cells in 200 × 200 µm2 at the center of the CA1 stratum pyramidale were counted using an AxioM1 light microscope (Carl Zeiss) equipped with a digital camera (Axiocam, Carl Zeiss) interleaved with a PC monitor. Cell counts were analyzed as a percent, with the sham group and ischemia group (5 days) designated as 100%. To quantitatively analyze CB immunoreactivity, in brief, according to our method (Lee et al., 2016), images were calibrated into an array of 512 × 512 pixels corresponding to a tissue area of 140 × 140 µm2 (40× original magnification). The mean CB immunoreactivity was determined in hippocampal CA1 pyramidal neurons by a 0–255 gray scale system in ImageJ (National Institutes of Health, MD, USA). The background density was subtracted, and the relative immunoreactivity (RI) of image file was calibrated as % using Adobe Photoshop version 8.0, and the RI was analyzed using NIH Image 1.59 software (National Institutes of Health). RI was calibrated as %, with sham group designated as 100%.

Statistical analysis

All data are expressed as the mean ± SEM of the means across the groups and were statistically analyzed using SPSS 18.0 (SPSS, Chicago, IL, USA). Analysis of variance (ANOVA) with a post hoc Bonferroni’s multiple comparison test was performed to present differences among experimental groups. Statistical significance was considered at P < 0.05.

Results

CV-positive cells

CV-positive cells were obviously observed in the stratum pyramidale, which are called pyramidal neurons, in the gerbil hippocampus proper (CA1–3 area) of the sham group
In the ischemic gerbils, the morphology of CV-positive pyramidal neurons in the hippocampus proper was not changed until 3 days after ischemia/reperfusion (Figure 1C, 1c, 1E, 1e, 1G, 1g and 1M); however, CV-positive cells were rarely detected in the stratum pyramidale of the CA1 area, not CA2 and CA3 area from 4 days after ischemia/reperfusion (Figure 1I, 1i, 1K, 1k and 1M).

The morphology of CV-positive cells in the hippocampus proper of gerbils of the IPC + sham group was not different from that in the sham group (Figure 1B, 1b and 1M). Furthermore, the morphology of CV-positive cells in the IPC + ischemia group was not different from that in the IPC + sham group (Figure 1D, 1d, 1F, 1f, 1H, 1h and 1M); in particular, CV-positive CA1 pyramidal neurons were well preserved 4 and 5 days after ischemia/reperfusion (Figure 1J, 1j, 1L, 1l and 1M).

F-J B-positive cells

F-J B-positive cells, which are dead cells, were not observed in the CA1 area of the ischemia group until 3 days post-ischemia (Figure 2A, 2B, 2E, 2F and 2M). However, many F-J B-positive cells were observed in the pyramidal layer of the CA1 area at 4 and 5 days post-ischemia (Figure 2I, 2j and 2M). F-J B-positive cells were not observed in the CA1 area of the IPC + sham group (Figure 2C and 2M). In the IPC + ischemia group, F-J B-positive cells were not observed until 3 days post-ischemia (Figure 2D, 2G, 2H and 2M), and 4 and 5 days after ischemia/reperfusion, only a few F-J B-positive cells were observed in the pyramidal layer (Figure 2K, 2L and 2M).

CB immunoreactivity

In the sham group, CB immunoreactivity was detected in the pyramidal neurons of the CA1–3 area (Figure 3A and 3a). In the ischemia group, CB immunoreactivity was not significantly altered in pyramidal neurons at 1 day post-ischemia (Figure 3B, 3b and 3m); however, CB immunoreactivity was significantly decreased only in the CA1 pyramidal neurons 2 days after ischemia/reperfusion (Figure 3E, 3e and 3m), and CB immunoreactivity in the CA1 pyramidal cells was hardly detected from 3 days after ischemia/reperfusion (Figure 3F, 3I, 3J, 3F, 3I, 3J and 3m). In the IPC + sham group, CB immunoreactivity in pyramidal neurons of the CA1–3 area was not different from that in the sham group (Figure 3C, 3c and 3m). In the IPC + ischemia group, CB immunoreactivity in all pyramidal cells was steadily maintained until 5 days post-ischemia (Figure 3D, 3G, 3H, 3K, 3L, 3d, 3g, 3h, 3k, 3l and 3m).

Discussion

Transient global cerebral ischemia selectively kills neurons in the hippocampus, namely, CA1 pyramidal neurons slowly die a few days after transient global cerebral ischemia (Kirino, 1982; Kirino and Sano, 1984; Kirino, 2000). In the present study, we found a striking loss of pyramidal neurons in the pyramidal layer of the CA1 area, which are named CA1 pyramidal cells, from 4 days after 5-minute transient ischemia via CV staining and F-J B histofluorescence staining. This finding is consistent with previous reports using gerbils that were subjected to 5-minute transient cerebral ischemia (Kim et al., 2014; Yan et al., 2014).

Previous studies demonstrated that IPC, which was induced by non-fatal brief transient ischemia, generated ischemic tolerance and protected neuronal damage/death following subsequent fatal transient ischemia (Kirino et al., 1996; Lehotsky et al., 2009). We investigated the neuroprotective effect of IPC against 5-minute transient cerebral ischemia in the CA1 area using CV staining and F-J B histofluorescence staining, and our finding was similar to that in previous studies (Lee et al., 2014; Kim et al., 2015). Brief (2-minute) transient ischemia in the brain does not cause the death of CA1 pyramidal neurons, and however, protects CA1 pyramidal neurons after a subsequent longer time (5-minute) of transient cerebral ischemia in gerbils. Previous studies have demonstrated that IPC mediates neuroprotection through attenuating ubiquitin aggregation (Lee et al., 2014), reducing oxidative damage (Lee et al., 2015a; Park et al., 2016), increasing the level of anti-inflammatory cytokines (Kim et al., 2015) and inhibiting Na(+)/H(+) exchanger 1 expression (Lee et al., 2015b). However, to the best of our knowledge, there are no studies correlating CB immunoreactivity with IPC-mediated neuroprotection following subsequent transient ischemic insults.

Calcium ions conduct important physiological functions that activate and regulate the fast transport of substances in axons, membrane excitability in neurons, and neurotransmitter synthesis and release (Heizmann and Braun, 1992). However, massive neuronal degeneration takes place in several brain diseases and the expression of calcium binding proteins changes during the course of the diseases (Heizmann and Braun, 1992). In ischemic brain injury, calcium ions are overloaded and lead to the activation of biochemical processes, enzymatic breakdowns of proteins, lipids and nucleic acids, mitochondrial malfunction, energy failure, and finally the destruction of neurons (Lee et al., 1999; Li et al., 2011). In addition, Sadowski et al. (2002) reported that, in a rat model of cardiac arrest, CB immunoreactivity disappeared completely in CA1 pyramidal neurons 3 days after cardiac arrest. In our present research, CB immunoreactivity in the pyramidal cells of the CA1 area began to be significantly decreased from 2 days and hardly detected 5 days after transient ischemia. We, in addition, reported that CA1 pyramidal cells of the young gerbils showed more resistance to transient cerebral ischemia than those in the adult gerbils and CB expression in the CA1 pyramidal cells was longer maintained in the young gerbils than in the adult gerbils (Lee et al., 2013b).

In this study, we found that CB expression was consistently maintained in the IPC + ischemia group, which shows IPC-mediated protection of the CA1 pyramidal cells against a fatal subsequent transient ischemia. It seems that the maintenance of CB expression in the IPC + ischemia group might be related with the protection of IPC against a fatal subsequent cerebral ischemia. It is well known that calcium
binding proteins including CB have a greater capacity of intercellular calcium ion buffering that would be more resistant to some brain disorders (Heizmann and Braun, 1992). However, studies regarding CB-mediated neuroprotection in cerebral ischemic condition have been reported by some researchers. Yenari et al. (2011) injected viral vector-mediated CB into the striatum of the rat and found the attenuation of neuronal damage/death in the striatum following focal cerebral ischemia (Yenari et al., 2001). Freimann et al. (2010) overexpressed CB in the striatum and cerebral cortex in the mouse using an adeno-associated viral vector for long time and found neuroprotective effects after focal cerebral ischemia induced by the occlusion of the middle cerebral artery (Freimann et al., 2010). Sung et al. (2012) reported that ginkgo biloba extract prevented the reduction of parvalbumin, a kind of calcium binding protein, in cerebrocortical neuronal cells of the rat after transient focal cerebral ischemia. Koh (2013) reported that nicotinamide restored the reduction of parvalbumin in the rat cerebral cortex induced by transient focal cerebral ischemia.

In brief, our results showed that IPC protected pyramidal neurons of the hippocampal CA1 area against subsequent fatal transient ischemia and restored the reduction of CB expression in the pyramidal neurons in the hippocampal CA1 area after subsequent fatal transient ischemia. These results indicate that CB in the brain plays important roles in the neuroprotection of neurons against brain damage including ischemic insults.

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Author contributions: All authors were responsible for design, implementation and evaluation of the study and approved the final version of this paper.

Conflicts of interest: None declared.

Research ethics: Procedures for animal handling and care complied with the guidelines that follow the current international laws and policies (Guide for the Care and Use of Laboratory Animals, The National Academies Press, 8th Ed., 2011), and all the experimental protocols of this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), Kangwon National University (approval no. KW-160802-1).

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CB immunoreactivity was easily detected in the stratum pyramidale (SP) of the sham group; however, CB immunoreactivity was gradually decreased in the SP (asterisks) from 2 days (d) post-ischemia. CB-D28k immunoreactivity in the SP was well preserved in the IPC + sham and IPC + ischemia groups. Scale bar: 200 μm valid for A–L.

High magnification of CB immunohistochemistry in the CA1 area of the sham (a), IPC + sham (c), ischemia (b, e, f, i, and j) and IPC + ischemia (d, g, h, k, and l) groups.

CB immunoreactivity was hardly detected in the SP (asterisks) from 3 d post-ischemia. CB immunoreactivity in the SP of the IPC + ischemia group was well preserved after ischemia/reperfusion. SO: Stratum oriens; SR: stratum radiatum; IPC: ischemia preconditioning. Scale bar: 50 μm valid for a–l.

(m) Relative immunoreactivity (RI) as percent values of CB immunoreactivity in the SP (n = 7 in each group; *P < 0.05, vs. sham group; #P < 0.05, vs. corresponding ischemia group; †P < 0.05, vs. respective pre-time point group). The bars indicate the mean ± SEM.

Figure 3 Low magnification of calbindin-D28k (CB) immunohistochemistry in the hippocampus of the sham (A), IPC + sham (C), ischemia (B, E, F, I, and J), and IPC + ischemia (D, G, H, K, and L) groups.
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