Hypothermic Preconditioning Increases Survival of Purkinje Neurons in Rat Cerebellar Slices after an In Vitro Simulated Ischemia

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Background: A period of hypothermia before ischemia (hypothermic preconditioning) induces a delayed phase of ischemic tolerance in rat brain. However, whether hypothermic preconditioning induces an acute phase (within a few hours after the hypothermia) of ischemic tolerance remains unknown. This study was designed to determine the time window of the hypothermic preconditioning–induced acute phase of neuroprotection, which is useful information for situations during surgery with anticipated ischemic episodes, and its involved mechanisms.

Methods: Survival of Purkinje cells in rat cerebellar slices was evaluated after a 20-min oxygen-glucose deprivation (OGD, *in vitro* simulated ischemia) followed by a 4-h recovery. Mild hypothermia (33°C) for 20 min was applied at various times before the OGD.

Results: The hypothermia applied immediately to 3 h before the OGD equally effectively reduced OGD-induced Purkinje cell death/injury. Glibenclamide, a selective K_{ATP} channel blocker; 8-cyclopentyl-1,3-dipropylxanthine, a selective adenosine A_1 receptor antagonist; and farnesyl protein transferase inhibitor III, a selective inhibitor to reduce Ras farnesylation, abolished hypothermic preconditioning–induced neuroprotection when applied during the hypothermia. OGD increased the expression of high-mobility group I(Y) proteins, which are nuclear transcription factors to enhance the expression of putatively damaging proteins such as cyclooxygenase-2, in cerebellar slices. This increase was attenuated by hypothermic preconditioning.

Conclusions: Hypothermic preconditioning induces an acute phase of neuroprotection. This neuroprotection depends on activation of the signaling molecules, adenosine A_1 receptors, K_{ATP} channels, and Ras. Inhibition of putatively damaging proteins *via* the effects of hypothermic preconditioning on high-mobility group I(Y) expression may also be involved in hypothermic preconditioning–induced neuroprotection.

ISCHEMIC cell injury has been implicated in cell death after many types of surgery. Surgical procedures often involve temporary or permanent interruption of blood supply to cells or tissue and thus induce ischemic cell injury. In the central nervous system, in which the metabolic demands of neurons are extremely high, a brief ischemia can induce cell death of vulnerable populations of neurons.[1] This predisposition may explain some profound functional impairment after surgery. For example, late or persistent neurologic deficits occur in more than one quarter of patients after coronary artery bypass surgery.[2] Thus, it is important to search for effective and practical methods to reduce cerebral injury induced by anticipated periods of ischemia during surgery.

Ischemic preconditioning is a well-known phenomenon in which brief episodes of sublethal ischemia induce a robust protection against the deleterious effects of subsequent, prolonged, lethal ischemia. This phenomenon, initially discovered in the heart by Murry *et al.*[3] in 1986, has been demonstrated to occur in many organ systems,[4,5] including the brain.[6,7] Subsequently, preconditioning with many other stimuli such as hypoxia, toxins, and cytokines has been demonstrated to be neuroprotective.[8-10] However, most of these preconditioning stimuli are harmful by nature and can produce significant brain injury with only minor changes in their intensity or duration. Thus, it is not practical or safe to apply those preconditioning stimuli in clinical situations.

Intraischemic hypothermia[11-13] and postischemic hypothermia[14-17] have been shown to be neuroprotective and have been used frequently in clinical situations such as profound hypothermia during circulation arrest. Recently, Nishio *et al.*[18] demonstrated that a 20-min (actual hypothermic period ∼ 80 min) hypothermia (28.5°–31.5°C) administered 6–48 h but not 7 days before a focal brain ischemia induced neuroprotection in rats. This study suggests the effectiveness of hypothermia as a preconditioning stimulus. However, the temperatures used in this previous study were too low to apply safely in most clinical situations. In addition, the previous study mainly explored the delayed phase of hypothermic preconditioning–induced neuroprotection, which may not be practical to apply during surgery.

In this work, we designed experiments to study the hypothermic (33°C) preconditioning–induced acute phase (within 3 h) of neuroprotection. In addition, we evaluated the role of adenosine A_1 receptors, K_{ATP} channels, and p21^{ras} (Ras), signaling molecules that were found to be important in preconditioning-induced protection by other stimuli,[19-24] as well as the expression of a nuclear protein high-mobility group (HMG) I(Y) known to enhance the expression of many putatively damaging proteins and inflammatory mediators[25-28] in hypothermic preconditioning–induced neuroprotection.

We used Purkinje neurons in rat cerebellar slices as our

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study model. Although neurons in neocortex and hippocampus are sensitive to ischemia and have been used as models for studies involving ischemic or hypoxic injury, ischemia- or hypoxia-caused cerebellar injury and subsequent ataxia is a major and common disease, especially in the pediatric population, after ischemic or hypoxic episodes. Purkinje cells are big GABA-ergic neurons and interact with multiple other neurons in cerebellum.[29,30] The cells play an important role in motor coordination.[29,30] Purkinje cells are sensitive to *in vitro* simulated ischemia as demonstrated in this and our previous study.[31] In addition, the morphologic changes of Purkinje neurons are easy to recognize, resulting in high accuracy of the data on cell injury/death.

Materials and Methods

The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Virginia. All animal experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 80–23) revised in 1996. All efforts were made to minimize the number of animals used and their suffering.

Drugs

Unless specified below, all reagents were obtained from Sigma (St. Louis, MO). Glibenclamide, a selective K_{ATP} channel blocker, and 8-cyclopentyl-1,3-dipropylxanthine, a selective adenosine A_1 receptor antagonist, were first dissolved in ethanol and then diluted into the required concentrations with artificial cerebrospinal fluid (aCSF). The final ethanol concentrations in the aCSF used for incubation of cerebellar slices were less than 0.05%. A pilot study showed that 0.05% ethanol did not affect Purkinje cell survival compared with control slices in aCSF only under current experimental protocols. Farnesyl protein transferase inhibitor III (a potent and selective inhibitor of farnesyl transferase, which thus inhibits Ras farnesylation and processing) was aCSF soluble.

Preparation of Brain Slices

Similar to the methods reported previously,[31-33] cerebellar brain slices were prepared from 2- to 3-month-old, 200- to 250-g, male Sprague-Dawley rats (Hilltop, Scotdale, PA). Rats were anesthetized with halothane and then decapitated. The cerebellum was removed rapidly and placed in ice-cold aCSF bubbled with 5% CO_2 and 95% O_2. The aCSF contained 116 mM NaCl, 26.2 mM NaHCO$_3$, 5.4 mM KCl, 1.8 mM CaCl$_2$, 0.9 mM MgCl$_2$, 0.9 mM NaH$_2$PO$_4$, and 5.6 mM glucose, pH 7.4. The cerebellum was immediately hemisected and sectioned with a tissue slicer into 400-μm transverse slices in ice-cold cutting solution (260 mM sucrose, 26.2 mM NaHCO$_3$, 3 mM KCl, 1.2 mM NaH$_2$PO$_4$, 5 mM MgCl$_2$, and 9 mM glucose, pH 7.4) bubbled with 5% CO_2 and 95% O_2. After sectioning, slices were wrapped in filter paper and placed into a tissue holder (made of plastic, with small holes in it to allow free diffusion of gases and water; this holder also helps to avoid direct gas bubbling on slices). These slices were then immersed in circulating aCSF continuously bubbled with 5% CO_2 and 95% O_2 (oxygenated aCSF) at 37°C for at least 1 h for recovery of the synaptic function.[31-33]

In Vitro *Oxygen-Glucose Deprivation*

Ischemia was simulated *in vitro* by oxygen-glucose deprivation (OGD). As described previously,[31,33] cerebellar slices were transferred into a glass beaker containing glucose-free aCSF (also containing 1 mM dithionite, an oxygen absorbent) bubbled with 95% N_2 and 5% CO_2 (30-min bubbling before the placement of brain slices was allowed so as to reduce the oxygen content in the solution). Under these conditions, the P_{O_2} in the aCSF was lower than 0.1 mmHg as measured by a Clark oxygen electrode (Cameron Instrument Co., Port Aransas, TX). The beaker containing the slices was immersed in a water bath to keep the temperature of glucose-free aCSF in the beaker at 37° ± 0.2°C as monitored by a thermometer. After 20 min of OGD, slices were recovered in circulating oxygenated aCSF at 37°C for 4 h to allow cell injury and death that may not be evident immediately after the OGD episode to become apparent.

Hypothermic Preconditioning

Hypothermic preconditioning was performed by transferring cerebellar slices into a glass beaker containing oxygenated aCSF at 33°C. After 20 min of hypothermia, slices were placed in circulating oxygenated aCSF at 37°C for the indicated duration before they were subjected to the OGD insult.

Study Groups and Experimental Paradigms

In the first set of experiments as illustrated in figure 1, hypothermic preconditioning (33°C for 20 min) was performed immediately (0HI), 1 h (1HI), 2 h (2HI), or 3 h (3HI) before the OGD at 37°C for 20 min. Control experiments with the hypothermia applied at the corresponding time points but without the OGD were performed to evaluate the effects of hypothermia on cell survival (0HC, 1HC, 2HC, and 3HC groups). A time control experiment (C group) was also performed by keeping the slices in circulating oxygenated aCSF at 37°C for the whole experimental duration. Finally, the OGD-alone group (I group) was tested by subjecting the slices to the OGD at the time point corresponding to that in the other groups with the OGD insult.

In the second set of experiments, cerebellar slices were preconditioned with hypothermia (33°C for 20
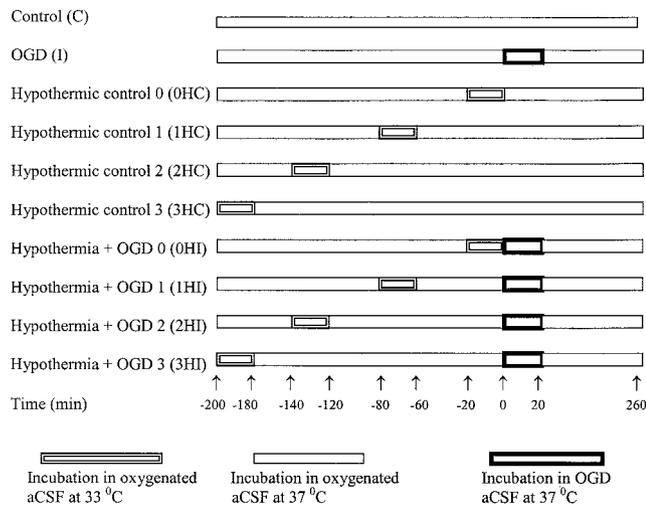
Fig. 1. Schematic diagram of the time course experiments on cerebellar slices. *In vitro* **ischemia was simulated by oxygen-glucose deprivation (OGD) for 20 min at 37°C. aCSF = artificial cerebrospinal fluid.**

min) 1 h before the OGD at 37°C for 20 min. In some experiments, 8-cyclopentyl-1,3-dipropylxanthine (100 nM), glibenclamide (0.3 µM), or farnesyl protein transferase inhibitor III (0.25 mM) was present during the period of hypothermia. Control experiments were performed by incubating the slices with these three drugs at the corresponding time point, and these slices were not exposed to the hypothermia or to both the hypothermia and the OGD. Similarly, time control and OGD-alone experiments were performed as described in the first set of experiments. Brain slices from the first and second sets of experiments were processed for morphologic evaluation of Purkinje cell survival.

In the third set of experiments, cerebellar slices were subjected to the hypothermia alone, OGD alone, hypothermia plus OGD, and time control with the same experimental protocols as described in the second set of experiments. These slices were used for Western blotting to detect the expression of HMG I(Y) proteins.

Morphologic Analysis

After incubation and recovery, cerebellar slices were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) overnight at 4°C. The slices then were paraffin embedded and sectioned into 5-µm thick sections. The sections were cut from an interior region of the brain slices (∼ 120 µm from the edge) to avoid areas that may have slicing trauma during slice preparation. Morphologic examination was performed under light microscopy after sections were stained with hematoxylin and eosin. The sections were examined by an observer blinded to the group assignment to determine the percentage of morphologically normal Purkinje cells (intact rate) and remaining (morphologically normal and injured) Purkinje cells (survival rate) in total counted

(morphologically normal, injured, and dead) Purkinje cells. Purkinje cells were recognized based on the morphology and location (between molecular and granular layers, fig. 2). Injured neurons were identified if the cells had one of the following presentations: cell swelling, vacuolization, or presence of shrunken and darkened nuclei.[34] Dead neurons were recognized by cell fragmentations. All Purkinje neurons present in one section were counted, and at least two sections were read for each experimental condition (total of 200–400 Purkinje cells) to compute the percentages.

Western Blot Analysis

Cerebellar slices were homogenized in 25 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 0.1% (vol/vol) 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 2 µM leupeptin, and 1 µM pepstatin A. The crude homogenates were centrifuged at 1,000g for 10 min at 4°C. Protein content in the supernatants was quantitated by the Lowry assay using a protein assay kit from Sigma. About 100 µg per lane of proteins was used for Western blotting as described previously.[35] Goat anti-HMG I(Y) polyclonal antibody was raised against a 19–amino acid peptide at the N-terminus of human HMG I(Y). This antibody cross-reacts with rat HMG I(Y) and is from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-β-actin polyclonal antibody was an affinity-purified antibody raised against the C-terminal actin fragment (11 amino acids) attached to multiple antigen peptide backbone and was acquired from Sigma. The volume of the protein bands was quantitated by an ImageQuant 5.0 Molecular Dynamics Densitometer (Molecular Dynamics, Sunnyvale, CA). The HMG I(Y) results were then normalized to those of β-actin to control for errors in protein sample loading and transferring during the Western blotting.

Statistical Analysis

Each experimental condition was repeated eight times with cerebellar slices from different rats. Statistical analysis for the cell survival/intact rates was performed by Kruskal–Wallis ANOVA for unpaired nonparametric data, followed by the Mann–Whitney rank sum test.
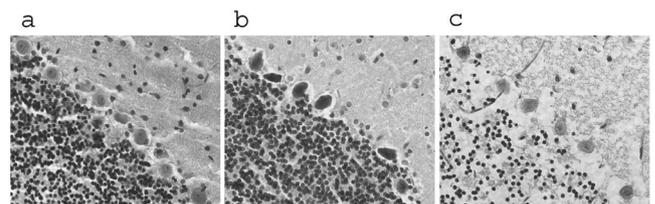

Fig. 2. Representative sections of cerebellar slices stained with hematoxylin and eosin. Time control (*a*), oxygen-glucose deprivation (OGD, 37°C for 20 min) (*b*), and hypothermic preconditioning (33°C for 20 min) plus OGD (*c*).
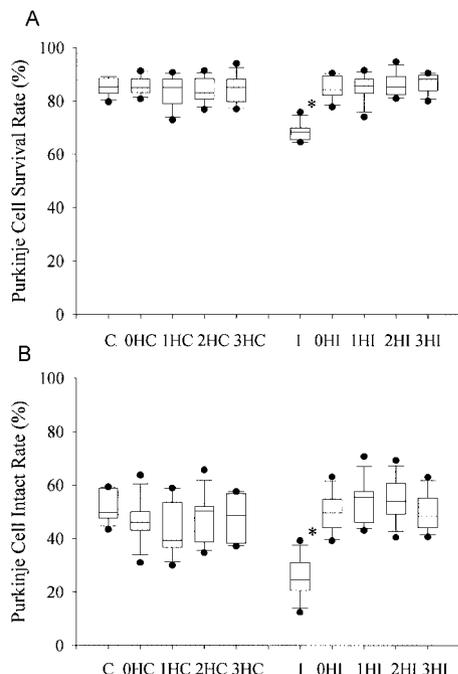
Fig. 3. Hypothermic preconditioning–induced acute phase of neuroprotection in rat cerebellar slices. Survival rate and morphologically intact rate of Purkinje cells are presented in *A* and *B*, respectively. *C*: Time control. I: oxygen-glucose deprivation (OGD) for 20 min at 37°C. 0HI, 1HI, 2HI, and 3HI: hypothermia (33°C for 20 min) applied immediately (0HI), 1 h (1HI), 2 h (2HI), and 3 h (3HI) before the OGD. 0HC, 1HC, 2HC, and 3HC: hypothermia applied at the corresponding time points to slices without being subjected to the OGD (see figure 1 for the experimental protocols). * *P* < 0.01 compared with C group. N = 7 to 8 for each experimental condition. ● = lowest and highest rates; between lines = 95% interval of the data; inside boxes = 25–75% interval, including the median of the data.

Statistical analysis for HMG I(Y) expression data was performed by unpaired *t* test. A *P* < 0.05 was accepted as significant.

Results

Acute Phase of Hypothermic Preconditioning-induced Neuroprotection

In vitro simulated ischemia (OGD) for 20 min followed by a 4-h recovery period reduced the percentages of remaining and morphologically normal Purkinje neurons in the cerebellar slices (figs. 2 and 3). This OGD-induced neuronal injury/death was abolished by a 20-min hypothermia (33°C) applied before the OGD (figures 2 and 3). The hypothermic preconditioning was similarly effective to prevent OGD-induced Purkinje cell injury/death when the hypothermia was applied from 3 h to immediately before the OGD (figure 3). Hypothermia alone did not alter neuronal injury/death rates under control conditions (fig. 3). These findings indicated that hypothermic preconditioning induced an acute phase of neuroprotection that developed immediately and persisted for at least 3 h.

Involvement of Adenosine A_1 Receptors, K_{ATP} Channels, and Ras in the Hypothermic Preconditioning–induced Neuroprotection

In the mechanistic studies, we chose to apply the hypothermic stimulus 1 h before the OGD. Consistent with the results presented previously, this preconditioning protocol also abolished OGD-induced Purkinje cell injury/death in this set of experiments (fig. 4). However, this hypothermic preconditioning–induced neuroprotection was abolished by 8-cyclopentyl-1,3-dipropylxanthine, glibenclamide, or farnesyl protein transferase inhibitor III, whereas these three drugs did not affect the neuronal injury/death rates under control and OGD-only conditions (fig. 4). These results suggest that adenosine A_1 receptors, K_{ATP} channels, and Ras play a role in hypothermic preconditioning–induced neuroprotection.
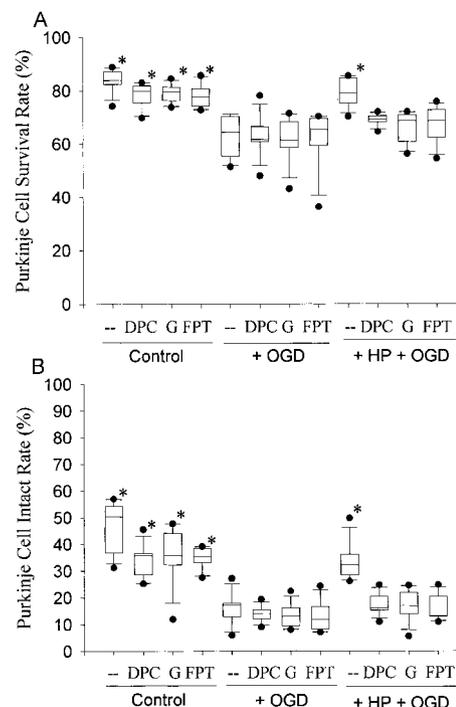

Fig. 4. The involvement of adenosine A_1 receptors, K_{ATP} channels, and $p21^{ras}$ (Ras) in the hypothermic preconditioning–induced acute phase of neuroprotection in rat cerebellar slices. Survival rate and morphologically intact rate of Purkinje cells are presented in *A* and *B*, respectively. Oxygen-glucose deprivation (OGD) was for 20 min at 37°C. Hypothermic preconditioning (HP, 33°C for 20 min) was applied 1 h before the OGD. 8-Cyclopentyl-1,3-dipropylxanthine (DPC), glibenclamide (G), or farnesyl protein transferase inhibitor III (FPT) was applied to slices for 20 min either during the hypothermia or during the corresponding period for slices that were not subjected to hypothermia. *Dashes* = no inhibitors were used. * *P* < 0.01 compared with OGD-only group. N = 6–8 for each experimental condition. ● = lowest and highest rates; between lines = 95% interval of the data; inside boxes = 25–75% interval, including the median of the data.
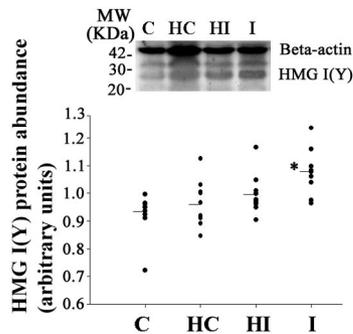
Fig. 5. Effects of hypothermic preconditioning on oxygen-glucose deprivation (OGD) –induced increase in expression of high-mobility group (HMG) I(Y) proteins. Rat cerebellar slices were pretreated with or without a 20-min hypothermia (33°C) 1 h before the OGD (20 min at 37°C), followed by a 4-h recovery before being used for Western blotting. C = control; HC = hypothermia control (slices only had the hypothermia without the subsequent OGD); HI = hypothermia plus OGD; I = OGD only. (*Top*) A representative Western blot. (*Bottom*) The graphic presentation of HMG I(Y) protein abundance quantified by integrating the volume of autoradiograms with slices from eight rats. Values were then normalized with those of β-actin. Each dot represents the value for a single rat; a horizontal bar indicates the mean value for each group. * $P < 0.05$ compared with control.

Attenuation of OGD-induced Increase in the Expression of HMG I(Y) Proteins by Hypothermic Preconditioning

A protein band with a mobility consistent with a molecular weight of ~ 25 kDa on sodium dodecyl sulfate–polyacrylamide gel electrophoresis was detected by Western blot analysis with an anti-HMG I(Y) antibody. Brain slices that had a 20-min OGD expressed a significantly higher level of HMG I(Y) proteins than controls. However, brain slices with hypothermic preconditioning and the OGD had a similar level of HMG I(Y) proteins to that in controls (fig. 5).

Discussion

The delayed phase of hypothermic preconditioning–induced neuroprotection has been investigated in two recent studies from the same group of investigators.[18,36] They gave adult rats a 20-min hypothermia (28.5°C or 31.5°C; the actual hypothermic period is about 80 min, because 30 min each was needed to cool and warm the animals to/from the target temperatures under halothane anesthesia) before brain ischemia. The ischemia was induced by clamping one middle cerebral artery and both carotid arteries for 1 h. The infarct size was measured 24 h after the ischemia. Animals with hypothermic preconditioning at 6, 24, and 48 h but not 7 days before the ischemia had significantly smaller cerebral infarct sizes.[18] In our study, we used a brief (20 min) and milder hypothermia (33°C) than that used in the previous studies and investigated the acute phase (0–3 h) of hypothermic preconditioning–induced neuroprotection.

These features in our study were designed to provide more relevant information for situations during surgery, because a short episode of hypothermia at 33°C is usually well tolerated by most patients and easily applied under anesthesia with minimal untoward effects on many organ systems, including the cardiovascular system. The time interval (0–3 h) between the preconditioning stimulus and an ischemic insult is also suitable for application in many clinical situations such as during carotid endarterectomy. In addition, the acute phase of ischemic preconditioning–induced cardioprotection has been shown to last for only 2 to 3 h.[3,5,37] Our results demonstrated that a 20-min hypothermia applied from 3 h to immediately before the OGD was equally effective in protecting Purkinje neurons from OGD-induced injury/death. These data suggest that inducing hypothermia even just before an anticipated ischemia will precondition neurons against ischemia.

Our findings may have important implications. Anticipated episodes of brain ischemia during surgery, especially cerebrovascular surgery, are common. Effective, practical, and safe methods to induce ischemic tolerance are needed to minimize ischemic brain injury in those patients. Our data suggest that hypothermic preconditioning may be used for this purpose. Hypothermia is usually noninvasive and has a substantial safety margin with respect to neuronal or cardiac injury. This is especially true when only mild hypothermia is needed for inducing neuroprotection as demonstrated in our study. Mild hypothermia has fewer and less severe side effects than the moderate to severe hypothermia used in the previous studies by Nishio *et al.*[18] and Yunoki *et al.*[36] and is easy to achieve during surgery. Taken together, using hypothermia as a preconditioning stimulus for inducing neuroprotection is worth further evaluation for potential clinical use.

Our study has limitations. We used brain tissues from rats. It is not appropriate to extrapolate these data to human patients. It is also not appropriate to extrapolate our *in vitro* results to *in vivo* conditions. In addition, we evaluated Purkinje cell injury/death 4 h after the OGD, because neuronal death in brain slices is manifest within 4 to 5 h after OGD, hypoxia, or overstimulation of glutamate receptors.[31-33,38] However, delayed cell death that can occur even 14 days after brain ischemia has been reported in rats.[39-41] Thus, our study may have underestimated the neuronal death induced by OGD, and it is not known whether hypothermic preconditioning–induced neuroprotection observed at 4 h after OGD will persist at later time points. Interestingly, a recent study has shown that the effects of the hypothermic preconditioning–induced delayed phase of neuroprotection were increased with the increase in the duration (from 20 to 180 min tested in the study) of the hypothermia (33°C) or in the depth (from 31.5° to 25.5°C tested in the study) of hypothermia.[36] Further studies are

needed to determine whether this association exists in the acute phase of hypothermic preconditioning–induced neuroprotection. Lastly, all slices (including control slices) in our study had a brief episode of hypothermia during the preparation of slices. Thus, our results suggest that preconditioning with two episodes of hypothermia may make Purkinje cells more resistant to OGD than that with only one episode of hypothermia. Nevertheless, our study still revealed an interesting phenomenon whose mechanistic studies may suggest new interventions for ischemic brain injury. In this regard, our *in vitro* model does provide some advantages over *in vivo* models. For example, it is easier to apply pharmacologic manipulations for mechanistic studies in *in vitro* models than in *in vivo* models. Temperature changes of the brain slices can be quickly achieved by transferring them to a beaker with the desired temperature. Moreover, our *in vitro* model also eliminates the interference of systemic variables such as blood pressure and cerebral blood flow existing in an *in vivo* model. Consequently, our results also suggest that hypothermic preconditioning–induced neuroprotection was caused, at least in part, by cellular elements intrinsic to the neuropil.

What are the mechanisms for hypothermic preconditioning–induced neuroprotection? Nishio *et al.*[18] suggested that protein synthesis is required for the development of the hypothermic preconditioning–induced delayed phase of neuroprotection. Similar mechanisms were proposed for the development of the delayed phase of ischemic preconditioning–induced neuroprotection and cardioprotection.[6,7,19,37] Multiple mediators, including adenosine receptors and protein kinase C in the heart and Ras in the brain, have been proposed to be involved in inducing protein synthesis after ischemic preconditioning.[19,21,42–45] However, the acute phase of ischemic preconditioning–induced neuroprotection and cardioprotection may not require protein synthesis because of the rapid onset of protection (within minutes after the preconditioning stimuli). It is generally accepted that activation of cell signaling molecules such as adenosine receptors, protein kinase C, and K_{ATP} channels is important for the development of the acute phase of ischemic or anesthetic preconditioning–induced cardioprotection.[19,20,22–24] Similarly, our results suggest that adenosine A_1 receptors and K_{ATP} channels play an important role in the acute phase of hypothermic preconditioning–induced neuroprotection. In addition, our results suggest that Ras activation may be necessary in this neuroprotection (the implication of adenosine A_1 receptors, K_{ATP} channels, and Ras in the hypothermic preconditioning–induced neuroprotection was based on

the results using one inhibitor for each of these signaling molecules, and the inhibitory effects of these inhibitors on their respective target molecules were not confirmed biochemically or electrophysiologically in our study). A previous study showed that *N*-methyl-D-aspartate receptor–initiated and Ras-dependent activation of mitogen-activated protein kinases was critical in the development of the delayed phase of OGD preconditioning–induced neuroprotection.[21] In a recent study from our laboratory, we showed that Ras was also important for the acute phase of OGD preconditioning–induced neuroprotection.[31] However, Ras and K_{ATP} channel activation was not demonstrated to be involved in isoflurane preconditioning–induced neuroprotection.[31] Thus, our results presented here suggest that hypothermic preconditioning–induced neuroprotection involves signaling molecules that are important for ischemic preconditioning–induced neuroprotection and are different from those for isoflurane preconditioning–induced neuroprotection. The exact relation between adenosine receptors, K_{ATP} channels, and Ras in cellular signaling pathways to mediate hypothermic preconditioning–induced neuroprotection is not known from our study. However, a potential link between adenosine receptors, K_{ATP} channels, and Ras may be proposed, because it has been reported that activation of adenosine receptors can lead to opening of K_{ATP} channels, which can induce production of oxygen free radicals to activate Ras.[20,23,24,46,47]

The development of brain injury after ischemia involves the synthesis of multiple putatively damaging proteins such as inducible nitric oxide synthase, cyclooxygenase-2, and cytokines.[48] Thus, inhibition of the synthesis of these proteins may reduce ischemic brain injury. HMG I(Y), an important nuclear transcription protein, has been demonstrated to enhance the expression of inducible nitric oxide synthase, cyclooxygenase-2, and cytokines.[25–28] Therefore, the expression of HMG I(Y) proteins may be increased after ischemia. This phenomenon was demonstrated in a previous study.# Our current study confirmed this finding and further showed that hypothermic preconditioning attenuated the OGD-induced HMG I(Y) increase. Thus, inhibition of HMG I(Y) expression and, therefore, a decrease in the expression of putatively damaging proteins after ischemia is an attractive mechanism for hypothermic preconditioning–induced neuroprotection. However, it is not known from our current study whether hypothermic preconditioning–induced inhibition of HMG I(Y) protein expression plays a role in the development of the acute phase or delayed phase or both phases of hypothermic preconditioning–induced neuroprotection.

In conclusion, we showed that hypothermic preconditioning induced an acute phase of neuroprotection in rat cerebellar slices. Activation of adenosine A_1 receptors, K_{ATP} channels, and Ras may be involved in this neuroprotection. In addition, our data suggest that hypothermic

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Abe H, Tsuchida H, Shiratsuka H, Hidaka K, Ueda Y: Molecular mechanisms involved in the neuroprotective effect of mild hypothermia in the gerbil hippocampus following transient forebrain ischemia (abstract). Available at: www.asa-abstracts.com. Go to Abstract Archive, 2001 Meeting Abstracts, p 789. Accessed June 5, 2003.

preconditioning attenuates OGD-induced increase in HMG I(Y) expression, a potential mechanism for hypothermic preconditioning–induced neuroprotection.

References

1. Smith ML, Auer RN, Siesjo BK: The density and distribution of ischemic brain injury in the rat following 2-10 min of forebrain ischemia. Acta Neuropathol (Berl) 1984; 64:319–32

2. McKhann GM, Goldsborough MA, Borowicz LMJ, Selnes OA, Mellits ED, Enger C, Quaskey SA, Baumgartner WA, Cameron DE, Stuart RS, Gardner TJ: Cognitive outcome after coronary artery bypass: A one-year prospective study. Ann Thorac Surg 1997; 63:510–5

3. Murry CE, Jennings RB, Reimer KA: Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74:1124–36

4. Raeburn CD, Cleveland JC Jr, Zimmerman MA, Harken AH: Organ preconditioning. Arch Surg 2001; 136:1263–6

5. Kloner RA, Jennings RB: Consequences of brief ischemia: Stunning, preconditioning, and their clinical implications: Part 2. Circulation 2001; 104:3158–67

6. Chen J, Simon R: Ischemic tolerance in the brain. Neurology 1997; 48: 306–11

7. Barone FC, White RF, Spera PA, Ellison J, Currie RW, Wang X, Feuerstein GZ: Ischemic preconditioning and brain tolerance: Temporal histological and functional outcomes, protein synthesis requirement, and interleukin-1 receptor antagonist and early gene expression. Stroke 1998; 29:1937–50

8. Gidday JM, Fizgibbons JC, Shah AR, Park TS: Neuroprotection from ischemic brain injury by hypoxic preconditioning in the neonatal rat. Neurosci Lett 1994; 168:221–4

9. Tasaki K, Ruetzler CA, Ohtsuki T, Martin D, Nawashiro H, Hallenbeck JM: Lipopolysaccharide pre-treatment induces resistance against subsequent focal cerebral ischemic damage in spontaneously hypertensive rats. Brain Res 1997; 748:267–70

10. Nawashiro H, Tasaki K, Ruetzler CA, Hallenbeck JM: TNF-alpha pretreatment induces protective effects against focal cerebral ischemia in mice. J Cereb Blood Flow Metab 1997; 17:483–90

11. Berntman L, Welsh FA, Harp JR: Cerebral protective effect of low-grade hypothermia. ANESTHESIOLOGY 1981; 55:495–8

12. Tharion J, Johnson DC, Celermajer JM, Hawker RM, Cartmill TB, Overton JH: Profound hypothermia with circulatory arrest: Nine years' clinical experience. J Thorac Cardiovasc Surg 1982; 84:66–72

13. Busto R, Dietrich WD, Globus MY, Valdes I, Scheinberg P, Ginsberg MD: Small differences in intraischemic brain temperature critically determine the extent of ischemic neuronal injury. J Cereb Blood Flow Metab 1987; 7:729–38

14. Marion DW, Leonov Y, Ginsberg M, Katz LM, Kochanek PM, Lechleuthner A, Nemoto EM, Obrist W, Safar P, Sterz F, Tisherman SA, white RJ, Xio F, Zar H: Resuscitative hypothermia. Crit Care Med 1996; 24(suppl):S81–9

15. Colbourne F, Sutherland G, Corett D: Postischemic hypothermia: A critical appraisal with implications for clinical treatment. Mol Neurobiol 1997; 14:171–201

16. Ko H, Zeller M, Gabriel S, Graulich J, Heinemann U, Obladen M: Mild postischemic hypothermia is neuroprotective in the immature rat neocortex slice. Brain Res 2001; 894:297–300

17. Colbourne F, Li H, Buchan AM: Indefatigable CA1 sector neuroprotection with mild hypothermia induced 6 hours after severe forebrain ischemia in rats. J Cereb Blood Flow Metab 1999; 19:742–9

18. Nishio S, Yunoki M, Chen ZF, Anzivino MJ, Lee KS: Ischemic tolerance in the rat neocortex following hypothermic preconditioning. J Neurosurg 2000; 93:845–51

19. Cohen MV, Baines CP, Downey JM: Ischemic preconditioning: From adenosine receptor to K_{ATP} channel. Annu Rev Physiol 2000; 62:79–109

20. Nandagopal K, Dawson TM, Dawson VL: Critical role for nitric oxide signaling in cardiac and neuronal ischemic preconditioning and tolerance. J Pharmacol Exp Ther 2001; 297:474–8

21. Gonzalez-Zulueta M, Feldman AB, Klesse LJ, Kalb RG, Dillman JF, Parada LF, Dawson TM, Dawson VL: Requirement for nitric oxide activation of p21(ras)/extracellular regulated kinase in neuronal ischemic preconditioning. Proc Natl Acad Sci USA 2000; 97:436–41

22. Perez-Pinzon MA, Mumford PL, Rosenthal M, Sick TJ: Anoxic preconditioning in hippocampal slices: Role of adenosine. Neuroscience 1996; 75:687–94

23. Toller WG, Kersten JR, Gross ER, Pagel PS, Warltier DC: Isoflurane preconditions myocardium against infarction *via* activation of inhibitory guanine nucleotide binding proteins. ANESTHESIOLOGY 2000; 92:1400–7

24. Zaugg M, Lucchinetti E, Spahn DR, Pasch T, Schaub M: Volatile anesthetics mimic cardiac preconditioning by priming the activation of mitochondrial K_{ATP} channels *via* multiple signaling pathways. ANESTHESIOLOGY 2002; 97:4–14

25. Perrella MA, Pellacani A, Wiesel P, Chin MT, Foster LC, Ibanez M, Hsieh CM, Reeves R, Yet SF, Lee ME: High mobility group-I(Y) protein facilitates nuclear factor-kappaB binding and transactivation of the inducible nitric-oxide synthase promoter/enhancer. J Biol Chem 1999; 274:9045–52

26. Thanos D, Maniatis T: The high mobility group protein HMG I(Y) is required for NF-kappa B-dependent virus induction of the human IFN-beta gene. Cell 1992; 71:777–89

27. Thanos D, Maniatis T: Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome. Cell 1995; 83:1091–100

28. Pellacani A, Wiesel P, Razavi S, Vasilj V, Feinberg MW, Chin MT, Reeves R, Perrella MA: Down-regulation of high mobility group-I(Y) protein contributes to the inhibition of nitric-oxide synthase 2 by transforming growth factor-beta1. J Biol Chem 2001; 276:1653–9

29. Altman J: Postnatal development of the cerebellar cortex in the rat. II: Phases in the maturation of Purkinje cells and of the molecular layer. J Comp Neurol 1972; 145:399–465

30. Noback CR, Strominger NL, Demarest RJ: Cerebellum, The Human Nervous System: Introduction and Review. Edited by Noback CR, Strominger NL, Demarest RJ. Malvern, Lea & Febiger, 1991, pp 281–96

31. Zheng S, Zuo Z: Isoflurane preconditioning reduces Purkinje cell death in an in vitro model of rat cerebellar ischemia. Neuroscience 2003; 118:99–106

32. Li J, Zheng S, Zuo Z: Isoflurane decreases AMPA-induced dark cell degeneration and edematous damage of Purkinje neurons in the rat cerebellar slices. Brain Res 2002; 958:399–404

33. Popovic R, Liniger R, Bickler PE: Anesthetics and mild hypothermia similarly prevent hippocampal neuron death in an *in vitro* model of cerebral ischemia. ANESTHESIOLOGY 2000; 92:1343–9

34. Bak IJ, Misgeld U, Weiler M, Morgan E: The preservation of nerve cells in rat neostriatal slices maintained in vitro: A morphological study. Brain Res 1980; 197:341–53

35. Zuo Z, Johns RA: Inhalational anesthetics up-regulate constitutive and lipopolysaccharide-induced inducible nitric oxide synthase expression and activity. Mol Pharmacol 1997; 52:606–12

36. Yunoki M, Nishio S, Ukita N, Anzivino MJ, Lee KS: Characteristics of hypothermic preconditioning influencing the induction of delayed ischemic tolerance. J Neurosurg 2002; 97:650–7

37. Baxter GF: Ischaemic preconditioning of myocardium. Ann Med 1997; 29:345–52

38. Bickler P, Hansen B: Hypoxia-tolerant neonatal CA1 neurons: Relationship of survival to evoked glutamate release and glutamate receptor-mediated calcium changes in hippocampal slices. Dev Brain Res 1998; 106:57–69

39. Kawakami S: Glial and neuronal localization of ionotropic glutamate receptor subunit-immunoreactivities in the median eminence of female rats: GluR2/3 and GluR6/7 colocalize with vimentin, not with glial fibrillary acidic protein (GFAP). Brain Res 2000; 858:198–204

40. Du C, Hu R, Csernansky CA, Hsu CY, Choi D: Very delayed infarction after mild focal cerebral ischemia: A role for apoptosis? J Cereb Blood Flow Metab 1996; 16:195–201

41. Li Y, Chopp M, Jiang N, Yao F, Zaloga C: Temporal profile of in situ DNA fragmentation after transient middle cerebral artery occlusion in the rat. J Cereb Blood Flow Metab 1995; 15:389–97

42. Nawa H, Takei N: BDNF as an anterophin: A novel neurotrophic relationship between brain neurons. Trends Neurosci 2001; 24:683–4

43. Bolli R: The late phase of preconditioning. Circ Res 2000; 87:972–83

44. Raval AP, Dave KR, Mochly-Rosen D, Sick TJ, Perez-Pinzon MA: Epsilon PKC is required for the induction of tolerance by ischemic and NMDA-mediated preconditioning in the organotypic hippocampal slice. J Neurosci 2003; 23: 384–91

45. Heurteaux C, Lauritzen I, Widmann C, Lazdunski M: Essential role of adenosine, adenosine A1 receptors, and ATP-sensitive K^+ channels in cerebral ischemic preconditioning. Proc Natl Acad Sci USA 1995; 92:4666–70

46. McPherson BC, Yao Z: Signal transduction of opioid-induced cardioprotection in ischemia-reperfusion. ANESTHESIOLOGY 2001; 94:1082–8

47. McPherson BC, Yao Z: Morphine mimics preconditioning via free radical signals and mitochondrial K(ATP) channels in myocytes. Circulation 2001; 103: 290–5

48. Lipton P: Ischemic cell death in brain neurons. Physiol Rev 1999; 79:1431–568