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HYPOTHERMIC PRECONDITIONING REDUCES PURKINJE CELL DEATH POSSIBLY BY PREVENTING THE OVER-EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE IN RAT CEREBELLAR SLICES AFTER AN *IN VITRO* SIMULATED ISCHEMIA

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Abstract—We showed that hypothermic preconditioning (HPC) increased survival of Purkinje neurons in rat cerebellar slices after oxygen–glucose deprivation (OGD). HPC also reduced the OGD-increased expression of high mobility group I (Y) proteins, a transcription factor that can enhance inducible nitric oxide synthase (iNOS) expression. iNOS is a putatively damaging protein that contributes to ischemic brain injury. Heat shock proteins (HSPs) can be induced by various stimuli to protect cells. We hypothesize that HPC induces neuroprotection by reducing the expression of putatively damaging proteins such as iNOS and/or by increasing the expression of putatively protective proteins such as HSPs. Cerebellar slices were prepared from adult male Sprague–Dawley rats and incubated in circulating artificial cerebrospinal fluid. OGD was for 20 min at 37 °C and was followed by a 5-h recovery at 37 °C before slices were used for morphological, immunohistochemical and Western analyses. HPC was performed by incubating slices at 33 °C for 20 min at 1 h before the OGD. HPC and aminoguanidine, an iNOS inhibitor, prevented OGD-induced Purkinje cell death/injury. OGD increased the expression of iNOS and nitrosylated proteins. These increases were abolished by aminoguanidine and HPC. Interestingly, the expression of HSP70 was increased by OGD but not by HPC. Our results suggest that an increased iNOS expression contributes to the pathophysiology of OGD-induced Purkinje neuronal death in our model. Our results also suggest the involvement of inhibiting the expression of the putatively damaging iNOS proteins in the HPC-induced neuroprotection. HSP70 may not contribute to the HPC-induced neuroprotection. **© 2006 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: neuroprotection, oxygen–glucose deprivation, heat shock protein, hypothermia, inducible nitric oxide synthase, preconditioning.

Recently, we demonstrated that a 20-min hypothermia (33 °C) administered immediately before to 3 h before oxygen–glucose deprivation (OGD, *in vitro* simulated ischemia) effectively reduced OGD-induced Purkinje cell death/injury in rat cerebellar slices (Yuan et al., 2004). This phenomenon is called hypothermic preconditioning (HPC). We also showed that OGD increased the expression of high mobility group (HMG) I(Y) proteins and that this increase was attenuated by HPC (Yuan et al., 2004).

It has been proposed that the development of ischemic brain injury involves synthesis of multiple putatively damaging proteins such as inducible nitric oxide synthase (iNOS) (Lipton, 1999). iNOS spontaneously produces nitric oxide (NO) (de Vera et al., 1995). NO and its products such as peroxynitrite can oxidize essential molecules including proteins and lipids to change their functions (Beckman, 1996; Beckman and Koppenol, 1996). This process can ultimately result in cell death (Moncada et al., 1991; Gross and Wolin, 1995). HMG I(Y), a nuclear transcription protein, has been demonstrated to be one of the important inducers for iNOS expression (Perrella et al., 1999). Thus, inhibition of HMG I(Y) expression and the subsequent reduction of iNOS expression and NO generation after ischemia are an attractive mechanism for HPC-induced neuroprotection.

Heat shock proteins (HSPs) play a role in maintaining cellular homeostatic functions such as protein folding and protein degradation (Sreedhar et al., 2004). They are also essential in the cellular response to stresses and can be induced by various stimuli such as heat, cold and oxygen deprivation (Moseley, 1998). An increased level of HSPs has been demonstrated to be a protective mechanism (Brown et al., 1989; Moseley, 1998; Giffard and Yenari, 2004). Accordingly, HSP is another potential mechanism for HPC to induce neuroprotection.

Thus, we hypothesize that HPC-induced neuroprotection is mediated by inhibiting the expression of iNOS and/or by enhancing the expression of HSP. To test this hypothesis, we studied the survival of Purkinje neurons in rat cerebellar slices. Application of OGD to brain slices, such as forebrain slices and hippocampal slices, has been used as an *in vitro* global ischemia model (Moro et al., 1998; Bickler et al., 2005). The cerebellar slice model was

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Abbreviations: aCSF, artificial cerebrospinal fluid; AG, aminoguanidine; AgC, aminoguanidine (200 μM) applied at the periods corresponding to the oxygen–glucose deprivation and the recovery but without subjecting the slices to the oxygen–glucose deprivation; AgI, aminoguanidine applied to slices during the oxygen–glucose deprivation period; AgIR, aminoguanidine applied to slices during both the oxygen–glucose deprivation period and the reoxygenation periods; HC, hypothermic control; HMG, high mobility group; HPC, hypothermic preconditioning; HSC70, heat shock cognate protein 70; HSPs, heat shock proteins; iNOS, inducible nitric oxide synthase; MCAO, middle cerebral arterial occlusion; NO, nitric oxide; OGD, oxygen–glucose deprivation; PBS, phosphate-buffered saline.

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used in our previous studies (Li et al., 2002; Zheng and Zuo, 2003; Yuan et al., 2004) and has been well accepted. Although neurons in neocortex and hippocampus are often used in studies involving ischemic or hypoxic injury due to their sensitivity to ischemia (Moro et al., 1998; Bickler et al., 2005), Purkinje cells are very sensitive to *in vitro* simulated ischemia as demonstrated in our previous studies (Li et al., 2002; Zheng and Zuo, 2003; Yuan et al., 2004). ischemia- or hypoxia-caused cerebellar injury and subsequent ataxia are a major and common disease especially in the pediatric population. Purkinje cells are big GABAergic neurons and interact with multiple other neurons in cerebellum (Altman, 1972; Noback et al., 1991). These cells play an important role in motor co-ordination (Altman, 1972; Noback et al., 1991). In addition, the morphological changes of Purkinje neurons are easy to recognize, resulting in high accuracy of the data on cell injury/death.

EXPERIMENTAL PROCEDURES

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.

Unless specified below, all reagents were obtained from Sigma (St. Louis, MO, USA).

Preparation of brain slices

Similar to the methods reported before (Li et al., 2002; Zheng and Zuo, 2003; Yuan et al., 2004), cerebellar brain slices were prepared from 2- to 3-month-old, 200- to 250-g, male Sprague–Dawley rats (Hilltop, Scotdale, PA, USA). Rats were anesthetized with halothane and then decapitated. The cerebellum was removed rapidly and placed in ice-cold artificial cerebrospinal fluid (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_2PO_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_2PO_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 tissue paper bag 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 (Popovic et al., 2000; Li et al., 2002; Zheng and Zuo, 2003; Yuan et al., 2004).

In vitro OGD

ischemia was simulated *in vitro* by OGD. As described previously (Popovic et al., 2000; Zheng and Zuo, 2003; Yuan et al., 2004), cerebellar slices were transferred into a glass beaker containing glucose-free aCSF (also containing 1 mM dithionite, an oxygen absorbent) bubbled with 5% CO_2 and 95% N_2 (30-min bubbling before the placement of brain slices was allowed to reduce the oxygen content in the solution). Under these conditions, the PO_2 in the aCSF was lower than 0.1 mmHg as measured by a Clark oxygen electrode (Cameron Instrument Co., Port Aransas, TX, USA). The beaker containing the slices was immersed in a waterbath to keep the temperature of glucose-free aCSF in the beaker at 37$\pm$0.2 °C as monitored by a thermometer. After 20 min of OGD, slices were recovered in circulating oxygenated aCSF at 37 °C for 5 h to allow cell injury and death that may not be evident immediately after the OGD episode to become apparent.

HPC

HPC 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 1 h before they were subjected to the OGD insult. This preconditioning protocol was shown to induce neuroprotection in our time-course study reported before (Yuan et al., 2004).

Study groups and experimental paradigms

In the first set of experiments, cerebellar slices were preconditioned with hypothermia (33 °C for 20 min) 1 h before the OGD at 37 °C for 20 min (HI group). In some experiments, aminoguanidine (AG, 200 μM; aCSF soluble), an iNOS inhibitor (Wolff and Lubeskie, 1995), was present during the period of OGD only (AgI group) or during both the OGD and the recovery/reoxygenation periods (AgIR group). We chose to use 200 μM AG because this concentration of AG effectively inhibited iNOS activity in the lipopolysaccharide-activated microglial cultures from rats (Han et al., 2002). The application of AG during two different periods was designed to determine whether a short and early application of AG was effective to reduce OGD-induced neuronal injury. Drug control experiment was performed by incubating the slices with AG at the corresponding time periods of OGD and reoxygenation but without being exposed to the OGD (AgC group). Hypothermic control experiment (HC group) with the hypothermia applied at the corresponding time period but without the OGD was also performed as described in our previous study (Yuan et al., 2004). Time control experiment (C group) was performed by keeping the slices in circulating oxygenated aCSF at 37 °C for the whole experimental duration without being exposed to hypothermia, AG or the OGD. The OGD alone experiment (I group) was done by subjecting the slices to the OGD only. After 5 h of recovery/reoxygenation period, brain slices from above experiments were processed for morphological evaluation of Purkinje cell survival.

In the second set of experiments, cerebellar slices were subjected to various experimental protocols as described in the first set of experiments. These slices were harvested at 5 h after the OGD for Western blotting and immunohistochemical staining to detect the expression of iNOS, proteins containing nitrotyrosine (nitrosylated proteins), HSP70 and heat shock cognate protein 70 (HSC70).

Morphological analysis

After a 5-h reoxygenation period, cerebellar slices were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) for 4 h at room temperature and then were cryoprotected in 30% sucrose in PBS overnight at 4 °C. The slices then were paraffin embedded and sectioned into 4 μm thick sections. The sections were cut from an interior region of the brain slices ($\sim$120 μm from the surface) to avoid areas that may have slicing trauma during slice preparation. Morphological 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. Both the intact rate and the survival rate of Purkinje cells were determined to complement each other. Purkinje cells were recog-

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