Neurobiology of Disease 35 (2009) 264–269

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Heme oxygenase 1 is associated with ischemic preconditioning-induced protection against brain ischemia☆

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ARTICLE INFO

Article history:
Received 25 February 2009
Revised 12 May 2009
Accepted 15 May 2009
Available online 22 May 2009

Keywords:
Heme oxygenase
Ischemic preconditioning
Middle cerebral artery occlusion
Neuroprotection
Permanent middle cerebral artery occlusion

ABSTRACT

Ischemic preconditioning (IPC) protects brain against ischemic injury by activating specific mechanisms. Our goal was to determine if the inducible heme oxygenase 1 (HO1) is required for such protection. IPC before transient or permanent ischemia reduced cortical infarct volumes by 57.4% and 33.9%, respectively at 48 h in wildtype adult mice. Interestingly, IPC failed to protect the HO1 gene deleted mice against permanent ischemic brain injury. IPC also resulted in a significant increase in HO1 protein levels in the brain and correlated with reduced neurological deficits after permanent and transient brain ischemia. Our study demonstrates that neuroprotective effects of IPC are at least partially mediated via HO1. Elucidating the physiological/cellular role by which HO1 is protective against brain ischemia may aid the development of selective drugs to treat stroke and its associated neurological disorders.

Introduction

Endogenous protective mechanisms against various injuries have developed throughout evolution in living organisms. If such an injury is tolerable, it triggers cellular responses that precondition the body against more severe stimuli. Preconditioning stimuli are diverse in nature and, therefore, can activate various pathways, altered gene translations, and synthesis of proteins, resulting in a stronger phenotype. Ischemic preconditioning (IPC) is a phenomenon in which brief ischemic episodes result in resistance of an organ to later severe ischemic insult(s). In clinical practice, brief ischemic episodes are known as transient ischemic attacks (TIAs) and have been studied for over two decades (Murry et al., 1986; Wegener et al., 2004). In human brain, TIAs induce tolerance by raising the threshold of tissue vulnerability (Wegener et al., 2004), a response that is critical for neuroprotection and its underlying molecular mechanisms. Despite promising results obtained from animal studies and an abundance of experimental neuroprotective compounds, clinical practice still lacks effective methods of stroke therapy. Investigating the underlying mechanisms activated by TIAs, could lead to novel targets for treating or preventing stroke.

Studies of IPC have revealed that tolerance induced in the brain can be early (minutes to hours) or late (hours to days) and that various protective molecular pathways are activated at different time points after IPC (Steiger and Hanggi, 2007). In particular, expression of the heat shock protein heme oxygenase 1 (HO1) is activated 12 to 24 h after IPC and remains up-regulated for up to 7 days in newborn rat brains (Bergeron et al., 1997). HO1 is an inducible enzyme that degrades the heme molecule to biliverdin, carbon monoxide (CO), and iron; its expression and activity can be increased by various preconditioning stimuli in the brain and in the whole body. Many investigators have suggested HO1 to be potentially responsible for preconditioning-induced protection *in vivo* and *in vitro*. For example, HO1 was reported to be involved in long-term tolerance after hypoxic preconditioning in the retina of mice (Zhu et al., 2007). Also, cardioprotection in diabetic rats provided by pharmacologic preconditioning was found to be at least partially mediated by HO1 (Thirunavukkarasu et al., 2007). Similarly, HO1 plays a role in the protection of cultured spinal neurons exposed to hyperbaric preconditioning (Li et al., 2007). HO1 up-regulation after lipopolysacharide preconditioning was shown to protect intestinal and lung tissue (Tamion et al., 2001); and myocardial cells induce HO1 expression after ischemic preconditioning, resulting in protection against apoptotic-like cell death and oxidative stress (Jancso et al., 2007). Activation of HO1 by

Abbreviations: IPC, ischemic preconditioning; MCA, middle cerebral artery; *t*MCAO, transient middle cerebral artery occlusion; *p*MCAO, permanent distal middle cerebral artery occlusion; HO1, heme oxygenase 1; TIA, transient ischemic attack; WT, wildtype; $HO1^{-/-}$ mice, heme oxygenase 1 knockout mice; CO, carbon monoxide; BCCAO, bilateral common carotid artery occlusion; CBF, cerebral blood flow; NDS, neurological deficit score; TTC, 1% triphenyltetrazolium chloride.

☆ Some of the data were previously presented at the 2006 Annual Meeting of the Society for Neuroscience.

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doi:10.1016/j.nbd.2009.05.010

various stimuli is a common protective phenomenon for different organ systems after IPC, but mechanisms of HO1 activation and signaling that are unique to the brain still need to be differentiated.

The importance of HO1 in protection against excitotoxicity in the brain (Ahmad et al., 2006) and in prevention of cell death (Ferris et al., 1999) has been previously shown. However, the question of whether HO1 alone is sufficient to protect the brain against ischemia still remains to be fully addressed. The primary function of HO1 enzyme is to degrade the heme molecule to biliverdin, iron, and CO, but the antioxidant and intrinsic properties of these end-products and HO1 intracellular signaling can be equally important factors for neuroprotection. To determine whether IPC-induced HO1 contributes to the neuroprotection, we subjected wildtype (WT) and HO1 knockout ($HO1^{-/-}$) mice to transient and permanent focal ischemia models.

Experimental procedures

Animals

WT C57BL/6 mice (Charles River Laboratories, Wilmington, MA), and $HO1^{-/-}$ mice (bred in our laboratory) were used at 8–10 weeks of age (20–25 g). Animals were housed at 22 ± 1 °C with a 12 h:12 h light/dark cycle; water and food were available *ad libitum*. All protocols were approved by the Johns Hopkins University Institutional Animal Care and Use Committee and were carried out in accordance with the guidelines of the National Institutes of Health.

Experimental design

Mice were exposed to anesthesia preconditioning alone by halothane or intraperitoneal injection of 250 mg/kg Avertin (2, 2-tribromoethanol; Sigma Co, St. Louis, MO) or IPC by bilateral common carotid artery occlusion (BCCAO) (Cho et al., 2005) before being subjected to either transient or permanent middle cerebral artery (MCA) occlusion (*t*MCAO and *p*MCAO, respectively). For the BCCAO, the mice were anesthetized before both common carotid arteries were exposed and ligated with 6-0 silk sutures three times for 1 min each. Between each ligation, the arteries were reopened for 5 min. Cerebral blood flow (CBF) was reduced by 90 ± 2% (mean ± SEM) during each occlusion and was restored to baseline during the 5-min reperfusions. Animals for which the CBF did not decrease by at least 80% were excluded from the study. All mice were allowed to recover in their cages for 24 h after preconditioning. A separate cohort of mice ($n = 6$) was used to determine whether IPC by BCCAO alone induces brain infarction.

Transient focal ischemia (tMCAO)

Mice were anesthetized with 1.5% isoflurane in 25% oxygen-enriched air. Transient focal ischemia was induced by MCAO via the intraluminal suture technique (Shah et al., 2006; Zeynalov et al., 2006). A reduction in blood flow over the ipsilateral parietal cortex of 87–90% was used as confirmation of successful occlusion. Blood flow was monitored with a laser-Doppler flowmeter (Moor Instruments Ltd, England), and body temperatures were maintained at 37.0 ± 0.5 °C in all groups during the experiment. The filament was removed from the lumen 90 min after occlusion to allow the blood to return to the ischemic region of the brain. All mice were allowed to recover from anesthesia before being returned to their cages.

Assessment of neurological deficit score (NDS) after tMCAO

During the 24 h recovery period after IPC, and after 48 h of *t*MCAO, mice were tested for neurological deficits based on the following scoring system: 0 = normal motor function, 1 = flexion of torso and of contralateral forelimb on tail lift, 2 = circling to the contralateral side but normal posture at rest, 3 = leaning to contralateral side at rest, and 4 = no spontaneous motor activity. Mice that had an NDS ≤1 after completion of the *t*MCAO procedure and recovery from anesthesia were excluded from the study.

Permanent focal ischemia (pMCAO)

For the *p*MCAO model, the distal portion of the MCA was occluded according to a method described by Majid et al. (2000). Mice were anesthetized with halothane, and a 1.0-cm vertical skin incision was made between the right eye and ear. The temporal muscle was moved aside, and the underlying temporal bone exposed. With the aid of a surgical microscope, a 2.0-mm burr hole was drilled just over the area of the MCA so that it was visible through the temporal bone. The main trunk of the distal MCA was occluded directly with a bipolar coagulator; complete interruption of blood flow at the occlusion site was confirmed by severance of the MCA. Core body temperature was maintained at 37.0 ± 0.5 °C during and after the procedure, first with a heating blanket that was attached to the temperature probe for automatic temperature regulation, and then with a temperature-regulated incubator in which the mice recovered from the surgery. A successful occlusion was confirmed by placing the laser-Doppler probe above the temporal ridge to establish that blood flow into the region was terminated. Animals that did not circle toward the paretic side after the onset of ischemia and those that developed subarachnoid hemorrhage were not included in the study.

Assessment of NDS after pMCAO

To evaluate the neurological deficits caused by IPC or *p*MCAO, a 28-point score pattern was used (Wang et al., 2006). Forty-eight hours after the *p*MCAO procedure, an experimenter blinded to genotype scored all mice for neurological deficits. The tests included body symmetry, gait, climbing, circling behavior, front limb symmetry, compulsory circling, and whisker response. Each test was graded from 0 to 4, establishing a maximum deficit score of 28. Immediately after the evaluation, the mice were sacrificed for infarct volume analysis.

Histological analysis

All animals were euthanized at 48 h after *t*MCAO or *p*MCAO. A separate cohort of animals ($n = 6$) was euthanized 24 h after IPC to determine whether the repeated BCCAO alone causes cerebral injury. Brains were harvested, sliced into five 2-mm thick coronal sections, mounted onto slides, and stained with 1% triphenyltetrazolium chloride (TTC, Sigma Co). Infarct areas of all slides were traced with the Image Analysis software (SigmaScan pro 5, Systat, Inc., Point Richmond, CA). The infarct area of the ipsilateral hemisphere of each brain was integrated into cubic millimeters and translated into a percentage of the contralateral hemisphere of the same brain with correction for swelling.

Western blot analysis

Cortices of the preconditioned WT mice were dissected out and homogenized for Western blot analysis. Protein concentrations were determined by BCA kit (Pierce, Rockford, IL). Equivalent amounts of protein per sample were migrated through SDS-PAGE on 10% gels. After electrophoretic transfer of the proteins to a nitrocellulose membrane, the membrane was blocked for 1 h at 22 °C with 5% dried milk and then exposed to primary antibodies overnight at 4 °C and secondary antibodies in 5% dried milk for 1 h at 22 °C. HO antibodies were purchased from Stressgen (Ann Arbor, MI), and