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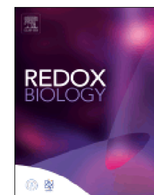

Brain ischemic preconditioning protects against ischemic injury and preserves the blood-brain barrier *via* oxidative signaling and Nrf2 activation

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ABSTRACT

Brain ischemic preconditioning (IPC) with mild ischemic episodes is well known to protect the brain against subsequent ischemic challenges. However, the underlying mechanisms are poorly understood. Here we demonstrate the critical role of the master redox transcription factor, nuclear factor (erythroid-derived 2)-like 2 (Nrf2), in IPC-mediated neuroprotection and blood-brain barrier (BBB) preservation. We report that IPC causes generation of endogenous lipid electrophiles, including 4-hydroxy-2-nonenal (4-HNE), which release Nrf2 from inhibition by Keap1 (*via* Keap1-C288) and inhibition by glycogen synthase kinase 3β (*via* GSK3β-C199). Nrf2 then induces expression of its target genes, including a new target, cadherin 5, a key component of adherens junctions of the BBB. These effects culminate in mitigation of BBB leakage and of neurological deficits after stroke. Collectively, these studies are the first to demonstrate that IPC protects the BBB against ischemic injury by generation of endogenous electrophiles and activation of the Nrf2 pathway through inhibition of Keap1- and GSK3β-dependent Nrf2 degradation.

1. Introduction

Stroke is a leading cause of long-term disability and death [10]. Although a number of preclinical studies suggest that sublethal, short-duration ischemic episodes induce ischemic tolerance against subsequent longer-duration ischemia [11,27,58], the clinical translation of ischemic preconditioning (IPC) has achieved mixed results [14,39]. One explanation for the lack of clinical translation may be the neurocentric approach to protection in most preclinical studies, which do not always ensure the preservation of non-neuronal cells, such as the components of the blood-brain barrier (BBB). BBB breakdown after stroke allows infiltration of inflammatory factors and immune cells, and produces detrimental clinical consequences, such as brain edema and hemorrhagic transformation [37]. The integrity of tight junctions (TJs) and adherens junctions (AJs) is critical for BBB integrity [17], and, therefore, both are potential targets of IPC-mediated protection. Although IPC has been reported to protect the BBB by enhancing angiogenesis and TJ expression and mitigating inflammation [36,52,70], the details of the molecular mechanisms remain unknown.

The classic view of IPC involves two temporal windows of tolerance—rapid and delayed preconditioning. Rapid tolerance unfolds within minutes of the preconditioning stimulus, whereas delayed tolerance emerges within hours to days [11,43] and confers more robust neuroprotection [58]. Recent evidence also supports the existence of a third window lasting as long as 8 weeks, which can be elicited by repetitive hypoxia, but not ischemia, perhaps by epigenetic regulation [60]. For IPC-mediated delayed tolerance, mild oxidative stress and *de novo* protein synthesis are required [5,40,54], as is upregulation of a panel of phase II enzymes [12,21,53], which possess electrophile response elements (EpRE) in their promoters [20]. EpRE is the binding site for nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a master switch that controls redox equilibrium [25,71]. Under normal conditions, Nrf2 is degraded by the proteasome shortly after synthesis by two independent routes—a canonical route mediated by Kelch-like ECH-associated protein 1 (Keap1) [26], and a non-canonical route mediated by glycogen synthase kinase 3β (GSK3β) [8,44].

The mechanism whereby IPC leads to Nrf2 activation is poorly understood. Among ten categories of Nrf2 inducers [21], the only

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endogenous inducers are the Michael reaction acceptors, also known as electrophiles. The predominant electrophiles in cellular systems are the end products of lipid peroxidation, including 4-hydroxy-nonenal (4-HNE) from omega-6 fatty acids and 4-hydroxy-hexenal (4-HHE) from omega-3 fatty acids [16,72]. These electrophiles react readily with Keap1 cysteine thiol groups and play an important role in Nrf2 signaling [32,45]. Given these collective observations, we hypothesized that IPC leads to the generation of a labile pool of lipid electrophiles, including 4-HNE and 4-HHE, which inhibit both Keap1 and GSK3β-dependent degradation of Nrf2, and result in the induction of Nrf2-dependent cytoprotective genes.

In the present study, we report that IPC confers brain protection against ischemic injury and preserves the BBB. We also demonstrate the key role of Nrf2 in IPC-mediated protection and the mechanism of activation of the Nrf2 pathway by lipid electrophiles. In both cellular and animal models, IPC reinforced the BBB along with Nrf2-dependent preservation of endothelial survival and junction proteins. Finally, at the molecular level, we identified critical residues in Keap1 and GSK3β for the inhibition of Nrf2 degradation by lipid electrophiles.

2. Materials and methods

2.1. Animals

All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) and carried out in accordance with Stroke Treatment and Academic Roundtable (STAIR) guidelines and the *National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals*. This manuscript was written in accordance with ARRIVE guidelines.

Adult male and female C57BL/6 wildtype and Nrf2 knockout (KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were housed in a 12:12 h light-dark cycle at 22–24 °C, with ad libitum access to rodent chow and water. Mice were randomly assigned to experimental groups with a lottery-drawing box. All outcome assessments, including evaluation of ischemic infarction, neurobehavioral performance, and gel analyses were performed by investigators blinded to group assignments. Animal numbers and mortality are listed in Supplementary Table 1.

2.2. Drug administration

For intracerebral ventricle (ICV) injections, mice were anesthetized with 1.5% isoflurane and placed in a stereotaxic frame (Kopf, Tujunga, CA). A midline incision was made over the skull and a burr hole was drilled in the skull. A Hamilton syringe was then lowered into the ventricular compartment with the following coordinates from Bregma: anteroposterior, 0.6 mm; lateral, 1.1 mm; and ventral, 2.2 mm. A total of 5 μL of drug or vehicle was injected. For N-acetylcysteine (NAC) injections, 5 μL of 50 mM NAC in saline was injected 1 h after IPC. For 4-HNE injections, 5 μL of 1.5, 3, 6, or 16 μg of 4-HNE in saline was injected, and cortical tissues were collected 24 h later. The injection lasted for 10 min and was controlled by an UltraMicroPump (World Precision Instruments, Sarasota, FL). The syringe was left in place for an additional 5 min to prevent diffusion up the needle track during withdrawal.

2.3. Middle cerebral artery occlusion (MCAO)

All mice underwent MCAO on the left side for 12 min to induce IPC [59] and 60 min to induce stroke [69,72]. This was followed by reperfusion for the indicated durations. The interval between IPC and stroke was 3 days. MCAO was performed with standard, previously published procedures [69,72]. In brief, mice were anesthetized with 1.5% isoflurane in a 30% O_2/70% N_2O mixture under spontaneous breathing, and rectal temperature was maintained at 37.0 ± 0.5 °C with a temperature-regulated heating pad throughout the surgery. Mean arterial blood pressure was monitored with a tail cuff. Using a surgical microscope, the left external, internal, and common carotid arteries were exposed through a midline neck incision. After coagulating and cutting of the branches of the external carotid arteries, an 8-0 monofilament nylon suture with a silicone coat was inserted in the lumen of the external carotid artery and advanced to the origin of the middle cerebral artery *via* the internal carotid artery.

As dictated by STAIR guidelines, the success of MCAO was confirmed by the measurement of regional cortical cerebral blood flow (rCBF), examination of neurological dysfunction, and the formation of the infarct, as described below. Neurological dysfunction was assessed in all mice once they recovered fully from anesthesia, and the measurements of rCBF and infarct volumes were performed in randomly selected mice, as described below. Mice were excluded from the study if their rCBF failed to fall below 40% of baseline or they showed no neurological deficits in stroke groups after recovery from anesthesia.

rCBF was monitored using Laser speckle contrast imaging, as described previously [51,57]. Briefly, a midline incision was made over the mouse skull, and a charge-coupled device camera (PeriCam PSI System; Perimed Inc., Ardmore, PA) was placed 10 cm above the head. The intact skull surface was illuminated by a laser diode to allow laser penetration through the brain in a diffuse manner. Two-dimensional microcirculation images were obtained 5 min before MCAO, 5 min after MCAO, and 10 min after the onset of reperfusion, including IPC intervals.

During anesthesia and surgery, physiological parameters, including core body temperature and blood pressure, were monitored (Supplementary Table 2). Rectal temperature was monitored and maintained in the normal range using a mouse rectal probe connected to a TCAT-2LV controller (Physitemp, Clifton, NJ). Blood pressure was measured using a mouse tail-cuff and a PowerLab system (ADInstruments, Colorado Springs, CO), as described previously [69,72].

2.4. Assessment of stroke outcome

Stroke outcome was assessed by neurological scoring, TTC staining, and behavioral tests

2.4.1. Neurological scoring and TTC staining

Neurobehavioral and histological studies were performed to evaluate ischemic outcomes by blinded observers. For the acute studies (48 h), neurologic dysfunction was scored at indicated time points after MCAO using the 5-point method [73], with 0 being the least dysfunction and 4 the worst dysfunction. Mice were then sacrificed and brains removed and sliced into 7 coronal sections each 1 mm thick. Sections were then stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) to determine infarct volumes [72,73]. Infarct volumes were measured blinded using NIH Image J software (Bethesda, MD).

2.4.2. Behavioral tests

To evaluate long-term sensorimotor functions, the Rotarod test (IITC Life Science Inc., Woodland Hills, CA) and adhesive removal test were performed up to 7 days after stroke [56,57,72]. The rotarod test evaluates sensorimotor coordination and balance. First, mice were trained for three days before surgery. After the mice were placed on the rods (diameter 3 cm), the rods began to rotate and accelerate to 25 rpm within 300 s. The maximal length of each trial was 300 s. Results are presented as the duration that the mice were able to remain on the rods. If the mice could remain on the rod for the entire 300 s, the rotations were stopped; if the mice fell from the rods before 300 s, the drop was recorded automatically by magnetic switches [57,72]. The adhesive removal test also evaluates sensorimotor function. Two adhesive paper patches (3 × 4 mm) were securely attached to the distal radial region of each forelimb. The time to remove each patch from the forelimbs was recorded [6]. Mice were trained once daily for 5 days before surgery.

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