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Review

The neuroprotective mechanism of brain ischemic preconditioning

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Brain ischemia is one of the most common causes of death and the leading cause of adult disability in the world. Brain ischemic preconditioning (BIP) refers to a transient, sublethal ischemia which results in tolerance to later, otherwise lethal, cerebral ischemia. Many attempts have been made to understand the molecular and cellular mechanisms underlying the neuroprotection offered by ischemic preconditioning. Many studies have shown that neuroprotective mechanisms may involve a series of molecular regulatory pathways including activation of the *N*-methyl-*D*-aspartate (NMDA) and adenosine receptors; activation of intracellular signaling pathways such as mitogen activated protein kinases (MAPK) and other protein kinases; upregulation of Bcl-2 and heat shock proteins (HSPs); and activation of the ubiquitin-proteasome pathway and the autophagic-lysosomal pathway. A better understanding of the processes that lead to cell death after stroke as well as of the endogenous neuroprotective mechanisms by which BIP protects against brain ischemic insults could help to develop new therapeutic strategies for this devastating neurological disease. The purpose of the present review is to summarize the neuroprotective mechanisms of BIP and to discuss the possibility of mimicking ischemic preconditioning as a new strategy for preventive treatment of ischemia.

Keywords: brain ischemia; brain ischemic preconditioning; NMDA receptors; mitogen-activated protein kinases; heat shock proteins; reactive oxygen species

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Introduction

Generally speaking, any stimulus capable of causing injury to a tissue or organ can, when applied close to (but below) the threshold of damage, activate endogenous protective mechanisms and thus potentially lessen the impact of a subsequent, more severe attack. A sub-threshold ischemic insult applied to an organ, for example, activates cellular pathways that can help to reduce damage caused by subsequent severe ischemic episodes — a phenomenon known as ischemic preconditioning (IP) or ischemic tolerance (IT)[1]. This observation suggests that ischemic preconditioning is an adaptive reaction to a potentially noxious stimulus, such as ischemia, hypoxia, hypoglycemia, or inflammation. Although tolerance to ischemic insults can also result from cortical spreading depression[2], sleep deprivation[3], dietary restriction[4], metabolic inhibition and oxygen free radicals[5], and both hyperthermia and hypothermia[1], ischemic preconditioning has been more aggressively studied.

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Ischemic preconditioning was first identified in the heart but was subsequently found to occur in the brain as well, where it is known as BIP. The brain is one of the most sensitive organs to injury. It is well accepted that, at least in mammals, the brain is approximately 3% of the body mass but receives approximately 15% of the total cardiac output and consumes 20% of the body's oxygen. A constant flow of blood to the brain is essential for delivering oxygen and glucose to neurons. If this flow is disrupted for even a short period of time, the result is cell damage or death. Neurons are rarely replaced once they have died, so the damage to affected regions may be permanent[6]. It has been found that there are two threshold values for cerebral blood flow. Reduction in blood flow below the first threshold results in electrical failure within neurons, and further reduction in blood flow below the second threshold leads to the failure of metabolism and ion pumps. Cells with lower perfusion than the second threshold are designated to die. Between the two thresholds, cells are electrically silent but maintain a low level of metabolic activity and can be stable for hours. If normal blood flow is restored within a reasonable amount of time, they may recover with no apparent damage. Longer periods of ischemia, however, will result in their

death. So these cells have a variable fate, and constitute what is known as the ischemic penumbra[1]. It is the cells within the penumbra that receive the most benefit from ischemic preconditioning, as their chance for survival is increased through mechanisms evoked by BIP.

In recent years, the mechanisms of BIP have been systematically studied and several molecular regulatory pathways participating in preconditioning have been discovered. This opens a window to uncover endogenous neuroprotective mechanisms and, potentially, a window of opportunity to utilize these mechanisms in the clinic to treat patients with stroke and other CNS disorders. This review surveys the current understanding of cerebral preconditioning and the neuroprotective mechanisms evoked by BIP.

Excitatory/inhibitory neurotransmitters and neuroprotection

Glutamate and NMDA receptors

Glutamate is the major excitatory neurotransmitter in the mammalian brain and a key mediator of intercellular communication, neuronal plasticity, development, and differentiation of neurons. Under normal physiological conditions, the extracellular concentration of glutamate is maintained in the micromolar range and is responsible for initiation of postsynaptic excitatory signaling through distinct ionotropic and metabotropic glutamate receptors. The ionotropic glutamate receptors include the *N*-methyl-*D*-aspartic acid (NMDA) receptor, the 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) proprionate (AMPA) receptor, and the kainate receptor subtypes[7]. Glutamate has long been known for its capability to kill neurons through the NMDA receptor-mediated mechanism, and NMDA receptor antagonists have been shown to be neuroprotective in animal models of several neurological disorders. Unfortunately, many NMDA receptor antagonists have undesirable side effects in humans that make them unsuitable for therapeutic use.

Paradoxically, subtoxic concentrations of NMDA protect neurons against glutamate-mediated excitotoxicity[8]. Bond *et al* reported that the non-competitive NMDA receptor antagonist MK-801 and the competitive NMDA receptor antagonist LY202157 all produced significant reductions in the induced ischemic tolerance, suggesting that mild NMDA receptor activation is involved in the neuroprotective mechanism of BIP[9]. Consistent with these findings, exposure of cortical cell cultures to low levels of glutamate or NMDA to induce NMDA receptor activation has a preconditioning effect[10].

One underlying mechanism involved in the NMDA receptor-mediated BIP is the rapid adaptation of the voltage-dependent calcium flux. Shimazaki[11] showed that the hippocampus of gerbils that did not acquire tolerance showed a high elevation of calcium flux, while tolerant gerbils were better able to regulate calcium and kept calcium below the critical level for initiating neuronal death. Since hypoxia/ischemia-induced neurodegeneration can be triggered by cytoplasmic calcium overload, the NMDA receptor mediates rapid calcium adaption in preconditioning that may alleviate the cell damage caused by calcium overload.

The other mechanism of NMDA receptor neuroprotection involves the activation of NMDA receptors leading to the rapid release of brain-derived neurotrophic factor (BDNF). BDNF then binds to and activates its cognate tyrosine kinase B (TrkB) receptors. Both NMDA and TrkB receptors activate nuclear factor kappaB (NF-κB), a transcription factor which is expressed in neurons. NF-κB is activated in response to a variety of stress- and injury-related stimuli including exposure to cytokines such as tumor necrosis factor-α (TNF-α), and excitotoxic and oxidative insults. Although NF-κB is an important inflammatory factor often involved in neuronal injury, it may play a part in the anti-death actions of TNF-α in cultured hippocampal neurons exposed to metabolic and oxidative insults. Mattson *et al*[12] reported that activation of NF-κB protected hippocampal neurons against oxidative stress-induced apoptosis, suggesting that NF-κB is involved in protecting neurons under certain conditions.

Other key mediators which have been involved in synaptic NMDA receptor-dependent neuroprotection include cyclic AMP responsive element binding protein (CERB), phosphatidylinositol 3 (PI3)-kinase, Akt and glycogen synthase kinase 3 beta (GSK3β). These mediators can be induced only by low doses of NMDA via the action potential-dependent route[13, 14]. CREB is a transcription factor that is activated in response to intracellular calcium elevation regulated by NMDA receptor activation[15]. Phosphorylation of CREB is rapidly and robustly enhanced in the penumbral region of preconditioned rats[16], and thus is implicated as an important player in the preconditioning process mediated by NMDA receptor.

GABA and GABA receptors

Gamma-aminobutyric acid (GABA) is a well-known inhibitory neurotransmitter in the brain. Recently, Dave *et al* found that BIP promoted a robust release of GABA in rats after lethal ischemia[17, 18]. They also observed that the activity of glutamate decarboxylase (the rate-limiting enzyme in GABA synthesis in the brain) was higher in the BIP group compared with controls and ischemic groups. They further tested the hypothesis that $GABA_B$ receptor activation was also neuroprotective during ischemia or early reperfusion by using an *in vitro* model (organotypic hippocampal slice culture). They found that administration of the $GABA_B$ agonist baclofen during ischemia and the first hour of reperfusion provided significant neuroprotection. They concluded that increased GABA release in preconditioned rats after ischemia might be one of the factors responsible for BIP neuroprotection and that $GABA_B$ receptors may be the GABA receptors activated after BIP. In addition, Sommer's group has shown that ischemic tolerance in the preconditioned gerbil hippocampus is associated with increased ligand binding to inhibitory $GABA_A$ receptors between 30 min and 48 h of recirculation[19]. GABAergic disinhibition has been attributed to lesion progression because neuronal hyperexcitability associated with a sustained downregulation of $GABA_A$ receptors was found in peri-infarct regions. Preconditioning can cause increased GABA release or increased GABA recep-

tor expression. The changes in GABAergic transmission, both pre- and postsynaptic, are likely to contribute to a shift of the glutamate/GABA balance toward inhibition in the preconditioning brain[20].

Adenosine and K^+ channels

In 1995, Heurteaux *et al* demonstrated the essential role of adenosine, adenosine A1 receptors, and ATP-sensitive K^+ channels in BIP. Adenosine is an endogenous neuroprotectant that can inhibit the release of excitatory amino acids. When ischemia occurs, adenosine can increase conspicuously. Adenosine inhibits synaptic transmission, decreases K^+-stimulated glutamate release, and inhibits presynaptic calcium fluxes via adenosine A1 receptors. The response to calcium influx is also important for the development of protection against ischemia, because calcium influx has been linked to the production of reactive oxygen species and the initiation of a number of signaling cascades leading to cell damage[21]. Many reports have shown that preconditioning-induced neuroprotection is dependent on adenosine A1 receptors. In rats, ischemic preconditioning increased adenosine A1 receptor immunoreactivity in the hippocampal CA1 region at days 1, 3, and 7 after preconditioning induction, within the window of ischemic tolerance[22]. Some unspecific and specific adenosine A1 receptor antagonists abolished the ischemic tolerance. Moreover, Liu *et al* also demonstrated that isoflurane-induced tolerance against focal cerebral ischemia in the rat middle cerebral artery occlusion (MCAO) model was attenuated by adenosine A1 receptor antagonists[23].

Activation of the ATP-sensitive potassium channels (K^+_{ATP} channels) also has a role in BIP. Blockade of the K^+_{ATP} channels abolished preconditioning and the protection afforded by adenosine and R-PIA (an adenosine A1 receptor agonist). By contrast, a K^+_{ATP} channel opener (RP-52891, aprikalim) induced ischemic tolerance. Meanwhile, recent evidence showed that transient infusion of the K^+_{ATP} channel antagonist sulfonylurea tolbutamide prior to ischemia could block BIP protection after forebrain ischemia, whereas pinacidil, a K^+_{ATP} channel agonist, can emulate BIP in hippocampal slices[24]. Although the precise K^+_{ATP} channels involved in BIP remain undefined, two K^+_{ATP} channels have been described recently. One of these channels resides in the mitochondrial inner membranes; the other resides in the plasma membranes. The mitochondrial K^+_{ATP} (mK^+_{ATP}) has been suggested to be the key channel involved in ischemic preconditioning, because the mK^+_{ATP} blocker 5-hydorxydecanoate (5-HD) prevented BIP-induced neuroprotection. It has been hypothesized that opening the mK^+_{ATP} channels may depolarize mitochondrial membrane potential and promote an increase in the electron transport chain rate and thus increase ATP production[25].

Opioid receptors

There are three types of opioid receptor: δ, κ, μ opioid receptors. The activation of opioid receptors is neuroprotective when the body encounters ischemia, hypoxia and cold. Zhang and collaborators showed that the δ opioid receptors were involved in hypoxia preconditioning in cultured rat cortical neurons[26]. They observed that δ opioid receptor activation protected cortical neurons from hypoxia injury, whereas the δ opioid receptor antagonist naloxone blocked such protection. In addition, Zhao *et al* found that morphine (an agonist for δ, κ, μ opioid receptors) and Tan-67 (a selective δ receptor agonist) induced a delayed preconditioning effect both *in vivo* and *in vitro*. The morphine preconditioning-induced neuroprotection was inhibited by β-funaltrexamine, a μ-opioid receptor antagonist, but not by 7-benzylidenenaltrexone, a δ-receptor antagonist, or norbinaltorphimine, a κ-receptor antagonist. The Tan-67 preconditioning-induced neuroprotection was inhibited by 7-benzylidenenaltrexone. These results suggest that the delayed phase of morphine preconditioning may involve μ opioid receptors and δ opioid receptors. Morphine and Tan-67 may activate a shared intracellular signaling pathway to induce the delayed preconditioning effects in the brain[27].

Inflammatory cytokines

Inflammatory cytokines are known to have an important role in acute stroke. Cytokines such as interleukin-1 (IL-1), and TNF-α are important mediators of the inflammatory reactions seen in cerebral ischemia. The importance of the cytokine system in the setting of ischemia was emphasized by Kariko *et al*[28]. TNF-α is one of the pro-inflammatory cytokines and is expressed in the ischemic brain. Nawashiro *et al*[29] studied the effects of pretreatment with TNF administered intracisternally in mice that were subjected to MCAO 48 h later. A significant reduction in infarct size was noted in mice pretreated with TNF at the dose of 0.5 microgram/mouse. Immunohistochemical analysis of brains subjected to 24 h of MCAO revealed a significant decrease in CD11b immunoreactivity after TNF pretreatment compared with the MCAO control. Therefore, TNF induces significant protection against ischemic brain injury and is likely to be involved in the signaling pathways that regulate ischemic tolerance. Liu *et al*[30] demonstrated that preconditioning of rat cortical neurons with mild hypoxia protected them from hypoxia and oxygen and glucose deprivation (OGD) injury 24 h later (50% protection). Interestingly, TNF-α pretreatment could be substituted for hypoxic preconditioning (HP). HP was attenuated by TNF-α-neutralizing antibody. Ohtsuki *et al*[31] explained the role of IL-1 in the induction of tolerance to global ischemia in Mongolian gerbils. Arterial IL-1α and IL-1β became elevated between 1 and 3 days after a 2-min ischemic exposure. Recombinant human IL-1 receptor antagonist (IL-1ra) ip blocked ischemic tolerance induction by 2-min preconditioning ischemia. The possible mechanisms of IL-1 action include release of arachidonic acid, enhancement of NMDA activation and stimulation of nitric oxide synthase [32].

Intracellular survival signals and neuroprotection

MAPK, Akt/PKB, and PKC signal pathways

In ischemic conditions, rapid changes occur in the activity of many different signaling paths, involving diverse protein kinase families. Alterations in the expression or activity of

MAPK, Akt/protein kinase B (PKB), and protein kinase C (PKC) suggest that multiple kinases participate in the response to ischemia and reperfusion. MAPK-mediated signaling participates in the transduction of cellular responses from the extracellular environment to the nucleus and other intracellular targets, through sequential phosphorylation. The activation of specific components of MAPK cascades involves conserved three kinase modules consisting of MAPK, MAPK kinase (MEK), and MAPK kinase kinase (MEKK). It is now becoming evident that MAPK signaling plays a significant part in cerebral ischemia[33, 34]. Neuronal apoptosis and cerebral ischemia both induce the robust activation of MAPK cascades. However, Zheng *et al* also demonstrated that preconditioning-induced neuroprotection against ischemia was mediated by activation of MAPK signaling pathway including extracellular signal-regulated kinases (ERK), c-Jun N-terminal protein kinases and p38[35, 36]. MAPKs are activated by phosphorylation on both threonine and tyrosine residues and subsequently phosphorylate intracellular enzymes and transcription factors. Generally speaking, ERK promotes cell survival and proliferation, whereas c-Jun N-terminal protein kinases induce apoptosis. Therefore, the roles of MAPK cascades in neuronal death and survival seem to be complex and can be altered by the types of cells and the magnitude and timing of insults.

Akt/PKB is a serine/threonine kinase primarily involved in cellular survival pathways by inhibiting apoptotic processes. Akt can also induce protein synthesis pathways, and is therefore a key signaling protein in the cellular pathways that lead to skeletal muscle hypertrophy and general tissue growth. Since it can block apoptosis, thereby promoting cell survival, Akt has been implicated as a major factor in tumorigenesis in many types of cancer. Akt can be activated by phosphatidylinositol 3-kinase (PI-3K), so the PI-3K antagonists wortmannin and LY294002 may induce apoptotic signals by inhibiting Akt activation[14, 37]. Recent evidence demonstrates the involvement of the activation of Akt in ischemic tolerance. Nakajima *et al* showed that Akt was activated in both non-preconditioned and preconditioned animals 1 h after ischemia, but the activation was long-lasting in the preconditioned rats. They also demonstrated that the preconditioning treatment inhibited the augmentation of neuronal apoptosis in the penumbral region to prevent the spread of infarction, and that the preconditioning-induced neuroprotection was due to the persistent activation of Akt in the penumbra[37]. Wick *et al* demonstrated that neuroprotection by hypoxic preconditioning required sequential activation of vascular endothelial growth factor (VEGF) receptor and Akt[38]. Neurons incubated at 5% O_2 for 9 h showed increased levels of the VEGF, VEGF receptor-2 (VEGFR-2), phosphorylated Akt/PKB, and ERK1. Incubation with a neutralizing anti-VEGF and anti-VEGFR-2 antibody, wortmannin, or antisense-Akt, reversed the resistance acquired by hypoxic preconditioning. Moreover, inhibition of VEGFR-2 blocked the activation of Akt/PKB, whereas pretreatment with recombinant VEGF also resulted in a hypoxia-resistant phenotype in the absence of hypoxic preconditioning. These data suggested a sequential requirement for VEGF/VEGFR-2 activation and Akt/PKB phosphorylation for neuronal survival mediated by hypoxic preconditioning, and further implicated Akt/PKB as a major mediator in VEGF-dependent neuroprotection.

The PKC family of serine/threonine kinases consists of 10 different isozymes. In the brain and spinal cord, PKCα, PKCβ1, PKCβ2, PKCγ, PKCε, PKCδ, PKCη, PKCθ, and PKCξ mRNA and protein are present and demonstrate unique tissue, cellular, and subcellular localizations. Previous reports suggest that ischemic preconditioning enhances downregulation of cell signaling mediated by PKCγ, normalization of calcium homeostasis, and activation of PKCδ and ε[39]. Recent data demonstrate the key role of the PKCε signaling pathway in the context of ischemic preconditioning. Ischemic preconditioning promoted significant increases in the levels of synaptosomal PKCε in rat hippocampus. Activation of PKCε increased synaptosomal mitochondrial respiration and phosphorylation of mitochondrial respiratory chain proteins[40]. Delivery of a PKCε inhibitory peptide abated NMDA-induced preconditioning in cell culture and isolated hippocampal slice models[41]. Correspondingly, delivery of a PKCε-specific activator peptide reduced damage, as measured with lactate dehydrogenase (LDH) release, when administered before OGD in pure neuronal and mixed neuronal/astrocyte cultures[42]. The molecular basis of PKCε-induced protection is unclear. One mechanism implicates adenosine and mK^+_{ATP}. In ischemic preconditioning, increases in adenosine levels (in addition to opioids) initiate a series of intracellular signaling events via G-protein coupled receptor signaling, leading to activation of phospholipases, production of diacylglycerol (DAG), calcium influx, and PKC activation[43]. Multiple studies have now demonstrated that adenosine administration protects neuronal cells against ischemic-type injury via PKCε. In addition, PKCε activity at the mitochondria may also contribute to regulation of mK^+_{ATP} channels, important for preserving mitochondrial membrane potential, maintaining energy and reducing calcium influx during metabolic challenge[44].

Bcl-2 protein

The protein Bcl-2 is an anti-apoptotic protein that resides in the outer mitochondrial membranes and the membranes of the endoplasmic reticulum. Overexpression of Bcl-2 is known to block the release of cytochrome *c*, which contributes to a signaling pathway leading to apoptosis. Kato *et al*[45] showed that two minutes of bilateral carotid artery occlusion in gerbils produced an increase in Bcl-2 at 30 h and peaked at 96 h, suggesting that the expression of Bcl-2 is involved in ischemic preconditioning. In addition to this, in the study of Shimizu *et al*[46], ischemic preconditioning for 20 min with transient focal ischemia produced ischemic tolerance (attenuated infarction volume). The results of Western blot analysis from tolerant caudate-putamen demonstrated an increase in Bcl-2 expression 3–7 days after preconditioning. Immunocytochemical examination also found that Bcl-2 was expressed in cells with both neuronal and non-neuronal morphology in striatum. Bcl-2 antisense oligodeoxynucleotides (ODNs) treatment

reduced expression of Bcl-2 in the striatum and blocked the induction of tolerance by preconditioning ischemia. Therefore, Bcl-2 appears to be a major determinant in this model of induced tolerance to focal ischemia. The transcription factors driving the induction of Bcl-2 during tolerance include CREB, which is the Bcl-2 promoter containing a cAMP-response-element (CRE). As mentioned above, CREB is regulated by NMDA receptor activation, and phosphorylated robustly in the penumbral region of the preconditioned rats[47]. Moreover, multiple protein kinases can activate transcription via the CRE, by phosphorylation of CREB, further implicating the interrelation of these different molecular regulatory pathways involved in ischemic preconditioning.

Heat shock proteins (HSPs)

Although most protein synthesis is inhibited after cerebral ischemia, the remaining active protein synthesis may have an important role in maintaining cell viability. HSPs are molecular chaperones and among the proteins synthesized during ischemia. HSPs are expressed both constitutively (cognate proteins) and under stressful conditions (as inducible forms). In addition to heat shock, a variety of stressful situations including environmental (ultraviolet radiation or heavy metals), pathological (infections or malignancies), or physiological (growth factors or cell differentiation) stimuli induce a marked increase in HSPs synthesis, known as the stress response[48]. Inducible HSPs are thought to assist the maintenance of cellular integrity and viability by preventing protein denaturation and improper polypeptide aggregation during exposure to physiochemical insults[49].

HSP70 is the most abundant HSP found in cells. It is expressed constitutively and is only mildly inducible. Many researchers have shown the extensive links between HSP70 overexpression and tolerance in ischemic brain injury. McLaughlin *et al* have reported an increase in HSP70 expression in an *in vivo* model of preconditioning[50]. Furthermore, HSP70 messenger RNA (mRNA) was compared in the cases of a 15-min ischemia 2 days after sham treatment and a 15-min ischemia 2 days after 10-min preconditioning by Sakurai *et al*[51]. They reported that HSP70 mRNA in the motor neurons was strong at 8 h after preconditioning with 10-min ischemia, mild at 1 or 2 days, and not observed at 7 days after preconditioning. Chen *et al*[52] reported that thermal preconditioning with 44℃ body temperature protected cerebellar granule neurons of rats by modulating HSP70 expression. HSP70 mRNA was detected after thermal preconditioning at 30, 60, and 90 min and increased gradually with time, whereas HSP70 antisense oligodeoxynucleotides inhibited the protective effects of thermal preconditioning against apoptosis[14, 52]. All these results implicate that HSP70 is an important player in the preconditioning process.

The underlying mechanisms of HSP70-mediated BIP are not only related to its important functions in protein refolding and transport. Emerging evidence suggests that the HSP70 family is also capable of binding and sequestering activated caspases, such as Apaf and AIF[53]. Overexpression of HSP70 inhibits the activation of NF-κB, which is persistently activated during ischemia and appears to promote apoptotic cell death. On the contrary, the deletion of the HSP70 gene notably increases cytochrome *c* release into the cytoplasm and subsequent caspase-3 activation, thereby exacerbating apoptosis and increasing infarction volume after focal cerebral ischemia[54]. Furthermore, HSP70 expression is regulated by transcription factors, the activity of which is increased by ERK phosphorylation[55], a process which has been implicated in preconditioning.

The ubiquitin–proteasome pathway

There are two protein degradation systems in mammalian cells, the autophagy/lysosomal pathway and the ubiquitin-proteasomal pathway. The ubiquitin-proteasomal pathway has been studied in cerebral ischemia and ischemic preconditioning. In rat forebrain ischemia models, transient cerebral ischemia followed by reperfusion leads to delayed selective neuronal death in hippocampal CA1 pyramidal neurons. Under electron microscopy (EM), visible protein aggregates progressively accumulate in some CA1 neurons and accumulation of the aggregates seem to occur primarily in neurons destined to undergo delayed neuronal death after brain ischemia. Further evidence[56, 57] showed that the endoplasmic reticulum (ER), mitochondria and cytoplasm all respond to the accumulation of unfolded proteins by compartment-specific signaling pathways to participate in neuronal injury, whereas ubiquintin-proteasome as well as beneficial chaperones function to prevent protein aggregation and assist in protein folding[58, 59]. Furthermore, by utilizing a rat transient cerebral ischemic preconditioning model, Liu *et al* found that ischemic preconditioning significantly reduced protein aggregation in CA1 neurons after ischemia. Biochemical analyses revealed that ischemic preconditioning decreased accumulation of ubiquitin-conjugated proteins (ubi-proteins) and reduced free ubiquitin depletion after brain ischemia. Ischemic preconditioning also reduced redistribution of heat shock cognate protein 70 and Hdj1 (HSP40) from cytosolic fraction to protein aggregate-containing fraction after brain ischemia[60].

Indeed, the proteasome comprises multiple protein subunits and degrades cytosolic proteins as well as misfolded proteins that fail to pass protein quality control in the ER[61]. Misfolded proteins in the ER are recognized by ER-specific E3 ligases that mediate polyubiquitination of the misfolded protein on the cytosolic side of the ER and are subsequently degraded by the proteasome[62]. Proteasome degradation of ubi-proteins is strictly ATP-dependent. Ubi-proteins serve as signals to activate heat shock transcription factors to induce expression of molecular chaperones[63], which then shield hydrophobic surfaces of proteins in non-native states, thereby blocking their aggregation. Major cellular chaperones are ATPases and assist protein folding through numerous cycles of binding and release of unfolded protein substrates by hydrolysis of ATP. Therefore, ubiquitin–proteasome and molecular chaperons may be concerned with the neuroprotection of ischemic preconditioning and may together prevent protein aggregation in lethal ischemia.

Hypoxia-inducible factor (HIF)

Arthur *et al* developed two *in vitro* models of ischemia/reperfusion: OGD, in which neuronal cell death was predominantly by necrosis (necrotic model) and programmed cell death (PCD model). Hypoxic preconditioning 24 h prior to OGD significantly reduced cell death from 83% to 22% in the necrotic model and 68% to 11% in the PCD model[64]. In this IPC model, the activity of the antioxidant enzymes glutathione peroxidase, glutathione reductase, and Mn superoxide dismutase were significantly increased, whereas superoxide and hydrogen peroxide concentrations following OGD were significantly lower in the IPC group. Furthermore, cytochrome *c* release from mitochondria to the cytosol was suppressed in the ischemia tolerance-induced hippocampal CA1 region[65] Recent reports suggested that one mechanism of preconditioning probably involves hypoxia-inducible factor-1alpha (HIF-1alpha)[66, 67]. HIF-1alpha is a transcription factor that binds with a second protein (HIF-1beta) in the nucleus to promote elements in hypoxia-responsive target genes during hypoxia. This binding causes upregulation of HIF target genes, including VEGF, erythropoietin, iNOS, glucose transporter-1, glycolytic enzymes, and many other genes that protect the brain against ischemia 24 h later. Hypoxia preconditioning can be mimicked by iron chelators like desferrioxamine and transition metals like cobalt chloride that inhibit prolyl hydroxylases, increase HIF-1alpha levels in the brain, and produce protection of the brain against combined hypoxia-ischemia 24 h later.

Nitric oxide/reactive oxygen species and neuroprotection

Nitric oxide

Gidday and colleagues demonstrated in 1999 that NO is involved in the development of hypoxic and ischemic tolerance[68]. They showed that NO production and activity were critical to the induction of ischemic tolerance and that endothelial nitric oxide synthase (eNOS), not neuronal nitric oxide synthase (nNOS) or inducible nitric oxide synthase (iNOS), was the isoform responsible for producing ischemic tolerance. In the study by Sunghee Cho and his colleagues, both inhibiting nitric oxide synthase (NOS) and scavenging NO during preconditioning significantly attenuated the induced ischemic tolerance, however, neither eNOS nor nNOS knockout mice demonstrated altered ischemic preconditioning[14, 69, 70]. Meanwhile, preconditioning by volatile anesthetics also appears to involve the NO pathway. Kapinya and coworkers showed that tolerance against ischemic neuronal injury in rats can be induced by volatile anesthetics and that this effect is dependent on iNOS[14, 71].

The exact mechanisms responsible for NO-induced induction of ischemic tolerance are not clear. Up-regulation of eNOS appears to be triggered via the PI3-K pathway, and both eNOS and PI3-K contribute to ischemic tolerance in the CA1 neurons of gerbil hippocampus[72]. Furthermore, NO appears to activate Ras and then the ERK cascade, which is also known as the microtubule-associated or MAPK pathway[14]. Ras is a G protein, a regulatory GTP hydrolase that cycles between two conformations — an activated or inactivated form. Ras is activated by mitogenic signals as well as autocatalytically through a feedback mechanism. Ras activates a number of pathways, but the most important seems to be MAPK, which transmits signals downstream to other protein kinases and gene regulatory proteins. Gonzalez-Zulueta and collaborators reported the essential role of NO activation in neuronal ischemic preconditioning[73], and also found that preconditioning induced Ras activation in an NMDA receptor- and NO-dependent, but cGMP-independent manner. They also demonstrated that Ras activity was necessary and sufficient for ischemic tolerance induction in neurons. Pharmacological inhibition of Ras by farnesyl protein transferase inhibitor III, as well as a dominant Ras negative mutant, blocked preconditioning, whereas a constitutively active form of Ras promoted the induction of ischemic tolerance against lethal insults. These studies suggest that Ras might be the downstream factor of NO-mediated neuroprotection in ischemic preconditioning[14].

Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are traditionally recognized as important intracellular signals implicated in myocardial ischemia/reperfusion injury[74, 75]. Mitochondria are the main source of ROS, and ROS are mainly regarded as toxic byproducts of metabolism with the potential to cause damage to lipids, proteins, and DNA[76]. It has been demonstrated that endogenous free radicals (the ROS product) can cause cellular calcium loading with inhibition of the sarcoplasmic reticulum calcium ATPase and inhibition of the sodium potassium ATPase, leading to sodium mediated calcium gain. Meanwhile, oxygen radicals are also responsible for lipid peroxidation, resulting in cell membrane breakdown and cell swelling[77]. Although mammalian cells express endogenous free radical scavenging enzymes[74], such as superoxide dismutase (which reduces $O_2^{-}\cdot$ to H_2O_2), catalase, and glutathione peroxidase (which reduces H_2O_2 to H_2O), these antioxidative defenses are overwhelmed after ischemia and reperfusion.

However, recent studies[5, 78] indicate that oxygen free radicals also have an important role in triggering the signal transduction pathways in ischemic preconditioning. Some oxygen free radicals such as hydroxyl radicals ($\cdot OH$) may be involved in ischemic preconditioning induction[79]. The relationship between oxygen free radicals and ischemic preconditioning was first suggested by Murry and colleagues[80], who showed that administration of oxygen radical scavengers during the first reperfusion period could block the beneficial effect of IP on infarct size in dogs. They therefore proposed that the generation of a low amount of oxygen free radicals during a short ischemic episode is not sufficient to cause cell necrosis, but enough to modify cellular activity and induce preconditioning effects. Therefore, low levels of oxygen radicals and oxidants are normally formed in cells and play an important part in cellular homeostasis[77]

Autophagy/lysosomal pathway and neuroprotection

Recently, the roles of autophagy and the lysosomal pathway in

cerebral ischemia have attracted intensive attention. Studies have reported the activation of autophagy following ischemic insults, but the contribution of autophagy to neuronal death/survival is still under debate[81–85]. In an earlier study, we found profound activation of autophagy and lysosomes after permanent middle cerebral artery occlusion (pMCAO) and an autophagic mechanism may contribute to ischemic neuronal injury[86].

However, autophagy is a double-edged sword[87]. Although massive autophagy is associated with cell death through excessive self-digestion and degradation of cellular constitutions, activation of autophagy can also protect the neurons by degrading unfolded proteins and damaged organelles. A study carried out in primary cultured cardiomyocytes[88] showed that inhibition of autophagy with 3-methyladenine (3-MA, an autophagy inhibitor) during anoxia-reoxygenation caused an increase in the number of necrotic cells and a decrease of the living cell population. Thus, the process of autophagy during anoxia-reoxygenation was proposed to provide some protective effects. In addition, a recent *in vitro* study in PC12 cells also showed that autophagy was associated with neuroprotective effects induced by IPC[89]. Inhibition of autophagy, especially during reperfusion or lethal oxygen-glucose deprivation periods, ameliorated the neuroprotective effects of IPC, and also attenuated HSP70 upregulation induced by IPC. Furthermore, our recent observations showed that autophagy was also induced in a rat ischemic preconditioning model. Pretreatment with 3-MA before the onset of ischemic preconditioning nearly completely suppressed the neuroprotective effects of preconditioning. These results strongly supported the hypothesis that induction of autophagy in ischemic preconditioning may play a part in protecting against the sequential lethal cerebral ischemia (Sheng *et al*, manuscript submitted).

Potential clinical applications of preconditioning

Since the first description of ischemic preconditioning, it has been demonstrated experimentally that endotoxin, tumor necrosis factor-α, and metabolic inhibitor 3-nitro-propionic acid can mimic ischemic preconditioning and induce BIP in the brain. Unfortunately, the clinical use of these substances is unacceptable because of their toxicity or other side effects.

However, preconditioning promises practical usefulness for vascular neurosurgery, cardiovascular surgery and possibly also in the management of brain tumors and trauma. Chan and his team applied ischemic preconditioning for cerebral aneurysm surgery[90]. They evaluated the effects of ischemic preconditioning produced by 2 min proximal temporary artery occlusion on brain tissue gases and acidity during clipping of cerebral aneurysms. Twelve patients with aneurismal subarachnoid hemorrhage were recruited. In patients assigned to the preconditioning group, the proximal artery was occluded initially for 2 min and was allowed to reperfuse for 30 min. All patients underwent cerebral artery occlusion for clipping of the aneurysm. Baseline brain tissue gases and pH were similar between groups. However, following artery occlusion, the declines in oxygen tension and pH were significantly slower in the preconditioning group compared with the routine care group. These results suggested that a brief occlusion of the proximal artery may be a simple maneuver for brain protection during complex cerebrovascular surgery. Moreover, hyperbaric oxygen (O_2) is also able to mimic ischemic preconditioning and induce neuroprotection against ischemic injury in animal brains and spinal cords. In addition, some easily applied methods of preconditioning such as the application of isoflurane can also be introduced conveniently into the practice for operation of neurosurgery.

On the other hand, certain pharmacologic agents have been shown to be as effective as ischemic preconditioning in achieving their protective effects on cerebral ischemia, and are described as pharmacological preconditioning[91]. Those agents that affect key proteins involved in the protective signaling pathways can potentially be used as tolerance-producing drugs. Recently, research from our laboratory[92, 93] showed that pretreatment with prostaglandin A1 (PGA1) or PGE1 and lithium significantly reduced infarct volume, neurological deficits and brain edema in MCAO rats, and their combination (PGA1+lithium, PGE1+lithium) exert greater neuroprotection in the MCAO model. Importantly, all these drugs significantly enhanced the expression of HSP70 in the ischemic striatum, which is widely accepted to be involved in ischemic preconditioning. Moreover, lithium also activates autophagy activity[94]. Actually, the selective autophagy activator rapamycin can mimic the biological effects of preconditioning (Sheng *et al*, manuscript submitted). We thus speculate that drugs inducing HSPs and activating autophagy may be used for pharmacological preconditioning. The possible cumulative neuroprotection by pharmacological preconditioning should be studied systematically.

Conclusion

Current knowledge suggests that the concept of preconditioning can help to improve neuronal survival after temporary critical ischemia. Although the mechanisms behind the formation of protection are still largely unknown, significant progress has been made toward identifying some of the major molecules involved (Figure 1). Molecules and proteins that are important for the development of ischemic tolerance in the brain are potential targets for the development of new treatments for ischemia. One strategy to identify such targets is to look at factors that have already been determined to be important for tolerance in other tissues. As mentioned previously, ischemic tolerance was first identified in the heart and has been studied more extensively in the heart than in the brain. It is likely that some of the processes essential for the development of tolerance are the same in both tissues. Newly identified targets in the heart should therefore be considered as being potentially important in the brain, and vice versa. Another strategy to identify new targets is to look at molecules and proteins that interact with currently known targets. If one component of a particular pathway is known to be important for the development of tolerance, the other components of that

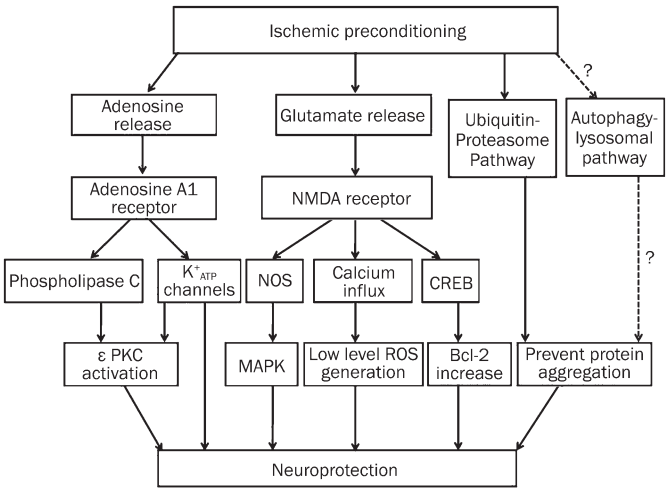

Figure 1. Simplified scheme depicting some basic signaling pathways involved in the neuroprotection of brain ischemic preconditioning. Triggering pathways include activation of the NMDA and adenosine A1 receptors which in turn are involved in activating some intracellular signaling pathways such as mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), bcl-2, heat shock proteins (HSPs), ubiquitin–proteasome pathway and autophagic-lysosomal pathway. NMDA receptor: *N*-methyl-*D*-aspartate receptor; NOS: nitric oxide synthase; CREB: cyclic AMP responsive element binding protein; ROS: reactive oxygen species.

pathway might be important as well. Of course, there may be novel factors involved in tolerance that cannot be detected by these strategies and that will require broader studies. Once the processes involved in preconditioning are more fully understood, the potential benefits for prevention and treatment of brain damage due to ischemia are substantial.

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