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Mechanisms and Prospects of Ischemic Tolerance Induced by Cerebral Preconditioning

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In the brain, brief episodes of ischemia induce tolerance against a subsequent severe episode of ischemia. This phenomenon of endogenous neuroprotection is known as preconditioning-induced ischemic tolerance. The purpose of this review is to summarize the current state of knowledge about mechanisms and potential applications of cerebral preconditioning and ischemic tolerance. Articles related to the terms *ischemic preconditioning* and *ischemic tolerance* were systematically searched via MEDLINE/PubMed, and articles published in English related to the nervous system were selected and analyzed. The past two decades have provided interesting insights into the molecular mechanisms of this neuroprotective phenomenon. Although both rapid and delayed types of tolerance have been documented in experimental settings, the delayed type has been found to be more prominent in the case of neuronal ischemic tolerance. Many intracellular signaling pathways have been implicated regarding ischemic preconditioning. Most of these are associated with membrane receptors, kinase cascades, and transcription factors. Moreover, ischemic tolerance can be induced by exposing animals or cells to diverse types of endogenous and exogenous stimuli that are not necessarily hypoxic or ischemic in nature. These cross-tolerances raise the hope that, in the future, it will be possible to pharmacologically activate or mimic ischemic tolerance in the human brain. Another promising approach is remote preconditioning in which preconditioning of one organ or system leads to the protection of a different (remote) organ that is difficult to target, such as the brain. The preconditioning strategy and related interventions can confer neuroprotection in experimental ischemia, and, thus, have promise for practical applications in cases of vascular neurosurgery and endo-vascular therapy.

Keywords: Cerebral ischemia; Preconditioning; Ischemic tolerance; Pharmacological preconditioning; Remote preconditioning

CEREBRAL ISCHEMIA/STROKE

The term *ischemia* (Greek *iskhein* to keep back + *haema* or *hema* blood) means a restriction in blood supply to a bodily organ or tissues. Interruption of blood flow to the brain or part(s) of the brain is known as cerebral ischemia or stroke. A stroke is a medical emergency, which can cause permanent neurological damage, complications, and death. More than 2,400 years ago, the father of medicine, Hippocrates, recognized and described stroke as the sudden onset of paralysis. In the past, stroke was referred to as cerebrovascular accident, but the term *stroke* is now preferred.

Stroke causes 9% of all deaths around the world, is the third most common cause of death after ischemic heart disease and cancer, and may soon become the leading cause of death worldwide. It is the leading cause of adult disability, because 76% of people in the United States and Europe survive their stroke [1].

The central goal of therapy in acute ischemic stroke is to preserve the area of oligemia in the ischemic penumbra. The area of oligemia can be preserved by limiting the severity of ischemic injury (i.e., neuronal protection) or by reducing the duration of ischemia (i.e., restoring blood flow to the compromised area). The ischemic cascade offers many points at which such interventions could be attempted. However, it has become increasingly clear that therapeutic interventions targeting only part of the complex network of mediators that contribute to ischemic

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brain damage produce only subtle effects on the outcome of stroke in clinical trials [2,3]. A solution to this problem could be derived from an understanding of the mechanisms by which the cell adapts to ischemic stress [4-7].

ISCHEMIC PRECONDITIONING AND ISCHEMIC TOLERANCE

Ischemic stress induces both harmful and protective responses, and the balance between these two responses determines cellular fate. If the stress is sublethal (i.e., below the threshold of damage), protective mechanisms prevail. When another stress is reapplied at the peak of the stress-controlling period, cells show better tolerance. This phenomenon is known as stress adaptation or preconditioning, in which sublethal stress induces an adaptive response to subsequent lethal stress. In fact, this observation has long been expressed as "Adaptation to perturbations is the basis for homeostasis" (Cannon), "the general adaptation syndrome" (Selye), "The dose makes the poison" (Paracelsus) or "Poisons are stimulants in small doses" (Arndt-Schultz). This cellular response can be observed in a wide variety of species from bacteria to mammalian cells [8]. The terms *preconditioning* and *tolerance* were first introduced by Janoff [9] in a study of the shock model. Generally, preconditioning can be defined as "presenting a stressful but non-damaging stimulus to cells to induce an endogenous adaptive response which would help cells to tolerate subsequent severe stresses." A condition of transiently increased resistance to ischemic stress as a result of the activation of endogenous protective mechanisms by preconditioning is known as ischemic tolerance [10]. This novel phenomenon has been described in a variety of organ systems, including the brain, heart, liver, intestine, lung, skeletal muscle, kidney and bladder [11-14]. Although it is generally assumed that the preconditioning phenomenon was first described in the heart in the 1980s [11,15] and not until 1990 in the brain, in fact, Dahl and Balfour [16] first described this phenomenon in 1964 in relation to brain ischemia, 20 years before the classic cardiology experiments were published. Several reports in the late 1980s again drew attention to ischemic tolerance in the brain [17-18]. Since then, this phenomenon has been confirmed in many animal models of global [19] and focal ischemia [20], in *in vitro* brain slice preparations [21], in cultured primary neurons [22], and in human beings in the form of short episodes of ischemia without infarction, known as a transient ischemic attack (TIA) [23].

STIMULI THAT EVOKE NEURONAL ISCHEMIC TOLERANCE

Even though short episodes of cerebral ischemia or cerebral hypoxia were initially considered as prototypical preconditioning stimuli, subsequent studies have shown that ischemic tolerance can be induced by exposing animals or cells to diverse types of endogenous and exogenous stimuli that are not necessarily hypoxic or ischemic in nature [5]. These stimuli include spreading depression, hyperoxia, oxidative stress, prolonged hypoperfusion, hypothermia, and hyperthermia. Therefore, one stressor can promote 'cross-tolerance' to another. The diversity of stimuli capable of inducing an ischemia-resistant phenotype in the brain indicates that the signaling pathways activated by these different triggers converge downstream on some common, fundamental mechanisms that ultimately account for the protection. Many exogenously delivered chemical preconditioning agents (e.g., inflammatory cytokines [24], anesthetics [25], and metabolic inhibitors [26]) can also induce ischemic tolerance, which raises the hope that, it will be possible in the future to pharmacologically activate these distal pathways in the human brain. Moreover, physical exercise [27] and skeletal muscle [28] preconditioning-induced neuroprotection against cerebral ischemia promise another attractive strategy, remote preconditioning, to protect organs that are highly susceptible to damage but that are difficult to target, such as the brain. However, the underlying complex molecular mechanisms of ischemic tolerance are still not well known.

MECHANISM OF ISCHEMIC TOLERANCE

Two temporally distinct types of ischemic tolerance are afforded by sublethal pretreatment: early and delayed tolerance, the mechanisms of which may differ. In the early (or rapid) type, the trigger induces tolerance within minutes and is transient; in the delayed type, tolerance takes hours to days to become apparent and lasts for days to weeks. Although both types of ischemic tolerance have been found in the brain and in the heart, the time course of ischemic tolerance in the brain usually follows the delayed pattern; however, in the heart, the tolerance induced by ischemic preconditioning usually follows the rapid pattern, also known as classic preconditioning, which represents a marked difference between the heart and the brain. In general, it is widely accepted that early acquisition of tolerance is independent of protein synthesis, mediated by posttranslational modification,

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and the effective duration is brief. Conversely, the general agreement is that delayed induction of ischemic tolerance requires new protein synthesis and is sustained for a few days to weeks.

To induce tolerance by means of ischemic episodes, three factors should be considered: 1) the duration, 2) the time interval, and 3) the number of episodes. First, the preconditioning stimuli must be severe enough to initiate a response, but not so severe as to cause permanent damage. Second, some interval of time must exist between sublethal and lethal stress. Third, the number of short ischemic episodes should be considered for sufficient stimulation of the protective response against a lethal ischemic insult.

Ischemia is an unspecific injury that causes disturbances in a multitude of cellular processes [29]. Therefore, induction of tolerance is most likely the result of several mechanisms and molecular pathways. In general, the process of tolerance induction can be divided into the following elements: sensors of the stress signal, transducers of the stimulus, and effectors of the tolerance [30]. First, the preconditioning stimulus must be recognized by cellular sensors so that the cells can be prepared for upcoming stress. Neurotransmitter and cytokine receptors, ion channels and redox-sensitive enzymes generally work as molecular sensor of stress stimuli. These sensors activate enzymes, such as kinase protein Ras, Raf, mitogen-activated protein kinase (MAPK) kinase (MEK), extracellular regulated kinase (ERK), Akt, and protein kinase C, and signaling molecules, such as nitric oxide (NO), diacylglycerol, inositol triphosphate, Ca^{2+}, and ceramide, which transduce the signal and initiate an adaptive response. Finally, effectors of the preconditioning response confer tolerance to cells or tissues through anti-excitotoxicity, anti-apoptosis, anti-inflammation, protection of mitochondria and increased anti-oxidant mechanisms [30-32]. The following is an overview of various components and the potential mechanisms that may responsible for ischemic tolerance.

The Membrane Receptors

Membrane stabilization to prevent toxic intracellular Ca^{2+} levels could provide a first line of protection, because the intracellular accumulation of Ca^{2+} through overactivation of Ca^{2+} channels is the key trigger in neuronal excitotoxicity and death [33]. As in cardiac preconditioning, adenosine, adenosine A1 receptors, and adenosine triphosphate (ATP)-sensitive K^+ channels may play a role in neuronal preconditioning and early ischemic tolerance [34]. This hypothesis was supported by the fact that adenosine uptake inhibition could potentiate ischemic tolerance [35] and that adenosine receptor antagonists were shown to block the ischemic tolerance phenomenon [36]. The proposed mechanism is as follows: a change in ATP-sensitive K^+ channels hyperpolarizes the neuronal cell membrane and thereby protects the neuron from detrimental depolarization.

Glutamate has long been known to kill neurons through an *N*-methyl-D-aspartate (NMDA) receptor-mediated mechanism. In contrast, preconditioning of neurons with subtoxic concentrations of NMDA protect neurons against subsequent glutamate-mediated excitotoxicity and in vitro ischemia [37]. One mechanism of NMDA mediated neuroprotection involves a rapid adaptation of the voltage-dependent calcium flux. Another mechanism involves the activation of NMDA receptors, which leads to the rapid release of brain-derived neurotrophic factor (BDNF) [38]. BDNF binds to and activates its cognate receptor, receptor tyrosine kinase B. Exactly how the neurons mediate neuroprotection by activation of the receptors is just beginning to be understood.

The α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor (AMPAR) activates the opening of an ionotropic channel, which permits the entry of intracellular sodium and the exit of potassium to the extracellular medium. Depending on the electrical charge originating from the amino acids that form the channel pore, passage of the calcium ion is also permitted. Ischemic preconditioning induces a small, transient down-regulation of AMPA receptor Ca^{2+} ion gatekeeper subunit GluR2 mRNA expression and greatly attenuates subsequent ischemia-induced GluR2 mRNA and protein down-regulation, and neuronal death [39]. The suppression of GluR2 down-regulation was proposed as a mediator of ischemic tolerance in carbonic anhydrase (CA)1 hippocampal neurons. However, this hypothesis was challenged by the finding that GluR2 reduction also occurs in neurons without subsequent neuronal death [40]. Therefore, further studies are needed to clarify the role of AMPAR subunit changes in tolerance induction.

Pharmacological stimulation of the γ-aminobutyric acid (GABA)-ergic system, a major inhibitory neurotransmitter system, has been shown to protect vulnerable neurons against ischemic damage [41]. In 2003, Sommer et al. [42] showed that ischemic tolerance in the preconditioned gerbil hippocampus is associated with increased ligand binding to inhibitory GABAA receptors between 30 minutes and 48 hours of recirculation. A relative shift between excitatory and inhibitory neurotransmission may promote post-ischemic survival of CA1 neurons. The conclusion that functional suppression of excitatory neuro-

transmission by the GABA pathway contributes to cellular survival during ischemia appears justified.

The delta opioid receptor was shown to be involved in both rapid and delayed hypoxic preconditioning-induced ischemic tolerance in cultured rat cortical neurons [43]. It was shown that the hypoxic preconditioning-induced ischemic tolerance effect could be blocked by a specific delta opioid receptor antagonist, which suggests the involvement of these receptors; however, the molecular mechanism is not well understood.

Signal Transduction

How cells behave and answer to outside stress stimulation has been considered another important factor in ischemic tolerance induction. Second messenger molecules and related protein kinase-cascades are probably the most widely studied components of the transmission of stress and survival signal.

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is believed to play a vital role in mediating survival signals in a wide range of neuronal cell types. The serine-threonine kinase Akt, also known as protein kinase B, is mainly activated in a PI3K manner by a variety of stimuli, including growth factors, traumatic brain injury [44], and ischemia [45]. Phosphorylation of Akt results in the full activation of its kinase activity and exerts a cell survival role via the phosphorylation of its many downstream targets, such as B-cell lymphoma 2 (Bcl-2) associated death protein, glycogen synthase kinase-3, pro-caspases-9, cyclic adenosine monophosphate (cAMP) response element-binding protein, and forkhead transcription factors. Numerous studies suggest that increased Akt activity induced by preconditioning is involved in ischemic tolerance. For example, Nakajima et al. [46] found that preconditioning prevents ischemia-induced neuronal death through persistent Akt activation in the penumbra region of the rat brain. Wick et al. [47] demonstrated that neuroprotection by hypoxic preconditioning requires sequential activation of vascular endothelial growth factor (VEGF) receptor and Akt. Neurons incubated under hypoxic conditions showed increased levels of VEGF, VEGF receptor-2 (VEGFR-2), phosphorylated Akt, and ERK1. Incubation with a neutralizing anti-VEGF and anti-VEGFR-2 antibody, the PI3K inhibitor wortmannin, or antisense-Akt, reversed the resistance acquired by hypoxic preconditioning.

Another type of signaling pathway, MAPK family is, also, thought to play an important role in the cellular adaptation to various stimuli, including ischemia. The MAPK family of proline-directed serine-threonine kinases consists of ERK1/2, c-Jun N-terminal kinase (JNK), and p38 MAPK, which have been extensively studied regarding the ischemia/reperfusion related cell death and survival paradigm. It is generally suggested that ERK1/2 plays a positive role in cellular survival, growth, and differentiation; however, ERK1/2 has been reported to play a negative role in both *in vivo* and *in vitro* cerebral ischemia models [48,49]. Tauskela et al. [50] showed that MAPK did not change after in vitro ischemia, and pharmacological inhibition of MAPK by PD98059 did not block preconditioning-induced neuroprotection in cultured rat cortical neurons. These findings suggest the lack of involvement of MAPK in ischemic tolerance. In contrast, Gonzalez-Zulueta et al. [51] demonstrated the involvement of the $p21^{ras}$/ERK1/2 signaling pathway in ischemic tolerance induced by oxygen-glucose deprivation (OGD) preconditioning in matured primary cultured cortical neurons. They showed that blocking of MEK activity during the preconditioning paradigm by using an MEK inhibitor U0126 suppressed the induction of ischemic tolerance. JNK is activated by many stress stimuli and plays a key role in ischemic cell death. Zhang et al. [52] reported in a previous study that JNK3 is implicated in ischemic tolerance in vivo. They showed that JNK was negatively regulated by preconditioning-induced active Akt. Interestingly, p38 MAPK was reported to play a positive role in ischemic tolerance [53] and a negative role in the hypoxic preconditioning of cortical neurons [43]. The role of MAPK cascades in neuronal death and survival seems to be complicated and might result from differences in experimental models, such as the type of cells, the age of neurons, the magnitude and timing of insults, and the type of insult [50,54].

Protein kinase C (PKC) represents a family of second messenger dependent serine/threonine kinases that are stimulated by Ca^{2+} and/or phospholipid. At least 10 isoforms of PKC are known and are classified on the basis of their second messenger requirements. PKC signaling has been shown to be differentially involved in preconditioning. Current data suggest that ischemic preconditioning enhances the down-regulation of cell signaling mediated by PKC gamma and Ca^{2+}/calmodulin-dependent protein kinases (CaMK) II, which enhances the normalization of calcium homeostasis [55]. On the other hand, preconditioning appears to specifically activate PKC delta and epsilon [56].

Although NO production during ischemic stress is toxic, it may also be linked to ischemic tolerance [57]. Gidday et al. [58] reported that NO production by endothelial nitric oxide synthase (NOS) is important in the induction of ischemic tolerance in a

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newborn rat hypoxic preconditioning model. Moreover, it was also shown that neuronal NOS (nNOS) mediated NO was involved in anoxic preconditioning in a hippocampal slice model [59]. Production of NO depends on NMDA receptor activation [37], which activates nNOS. Increased NO can activate the Raf/MEK/ERK cascade and can induce new protein synthesis [60]. In cell cultivation models, a major loss of neuroprotection is observed when NOS inhibitor and NMDA receptor antagonists are added during a preconditioning paradigm [37,51]. Similarly, inducible NOS (iNOS) is known to exacerbate ischemic damage in the brain [61]; however, iNOS induction has also been implicated in tolerance induction pathways initiated by anesthetic preconditioning in the brain [25].

Inflammatory Cytokines and Ceramide

Inflammatory cytokines, particularly tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6, have been implicated in the mechanism involved in ischemic tolerance [62-64]. IL-1 receptor antagonists can block tolerance induced by brief priming ischemia [32]. Cytoprotective preconditioning with TNF-α induces manganese superoxide dismutase (MnSOD). Preclinical stroke models and primary cultures of cortical neurons, cortical astrocytes and microvessel endothelial cells show that TNF-α and its downstream mediator ceramide are involved in tolerance signaling [62,65,66]. The activity of TNF-α at its membrane receptor leads to the generation of ceramide and to the release of nuclear factor-κB (NF-κB) from the inhibitor of the inhibitor (I)-κB complex. The translocation of NF-κB leads to the gene transcription of a variety of pro- and anti-apoptotic molecules [31]. Although ceramide has long been considered to induce cellular apoptosis, the exogenous application of subtoxic concentrations of ceramide can induce neuronal ischemic tolerance and act as a neuroprotective agent against lethal OGD stress [65,66].

Transcription Factors

Transcription factors are the link between kinase cascades and gene expression. Some of the most studied transcription factors include NF-κB, cAMP response element-binding protein, and the activator-protein 1 (AP-1) family.

NF-κB is a dimeric transcription factor consisting of heterodimers or homodimers of Rel proteins and is bound in the cytoplasm to inhibitory proteins of the I-κB family. NF-κB is activated by various signals, such as cytokines, neurotrophic factors, neurotransmitters, oxidative stress and intracellular elevation of Ca^{2+}. NF-κB plays a pivotal role in the induction of neuroprotective genes (e.g., MnSOD and Bcl-2) expressions, which are known to be related to tolerance induction [31]. It has been reported that the expression of NF-κB increases in three different paradigms of ischemic tolerance induced by sublethal ischemia, epilepsy and polyunsaturated fatty acids [67]. Pretreatment with NF-κB inhibitor or κB decoy DNA blocked NF-κB activity and eventually suppressed the neuroprotective effect of preconditioning. The AP-1 factor is a dimeric complex consisting of the Fos and Jun families, which were originally known as protooncogenes. The activation of AP-1 occurs through phosphorylation of its components by JNK and p38 kinase cascades. A complex pattern of Fos and Jun protein expression seems to underlie tolerance induction and delayed neuronal death [68,69]. The available literatures indicates that the AP-1 transcription factor family plays a crucial role in both neuroprotection and neurodegeneration [70].

CLINICAL PROSPECTS AND FUTURE CHALLENGES

The preconditioning phenomenon has been successful as an experimental procedure for identifying the mechanisms responsible for brain protection and regeneration. Important examples of strategies to modulate these mechanisms include erythropoietin, activators of mitochondrial K_{ATP} channels, and volatile anesthetics. The phenomenon of ischemic tolerance has not only been found in cells, organs and animal experimental models; some clinical observational data indicate that this phenomenon may occur naturally in the human brain in the form of short episodes of ischemia without infarction, known as TIA. In a retrospective clinical study, Weih et al. [71]. evaluated 148 stroke patients with and without antecedent TIA and found that TIA before stroke is associated with significantly less severe stroke on admission and improved outcomes on follow-up. Another retrospective study, which included more than 2000 patients, confirmed these results 1 year later [72]. In support of these findings, studies using magnetic resonance imaging and neuroradiological analysis showed that ischemic stroke patients with prodromal TIA have significantly smaller ischemic lesions after stroke than those patients without TIA [73,74]. These observations suggest that endogenous preconditioning triggered by TIA is present in the human brain.

Induction of ischemic tolerance in the brain has been suggested to be a promising clinical strategy for preparing the brain for

situations of possible ischemia, such as cardiac or brain surgery and in patients with a high risk of stroke. However, because of ethical and safety concerns associated with ischemic preconditioning, researchers are trying to identify a safer preconditioning stimulus that would be both practical and effective or a biological agent that can mimic preconditioning pharmacologically. Many candidate pharmacological regulators of the stress response and inducers of ischemic tolerance have been proposed [75-79], one of which is erythropoietin. Erythropoietin is approved for the treatment of anemia and seems safe and effective for critically ill patients who are anemic and have experienced trauma [80]. The iron chelator desferrioxamine is clinically approved for various indications, including thalassemia and other iron-overload syndromes. Various inhalational anesthetics used in human beings (e.g., sevoflurane) induce tolerance against brain ischemia and act as brain protectants after ischemia in preclinical experiments [81-83]. These compounds are safe and effective at eliciting early preconditioning in patients undergoing coronary artery bypass graft surgery in randomized controlled trials [84]. These drugs also elicit delayed preconditioning in human beings [85,86].

Another promising approach is remote preconditioning in which preconditioning of one organ or system leads to protection of a different (remote) organ. The prototypical approach for remote preconditioning is the initiation of short ischemic insult(s) to a limb to protect organs such as the heart [87,88] and the brain [89,90]. Remote preconditioning might indicate a crosstalk between the brain and the rest of the body in response to stress through the peripheral nervous system or paracrine signal. Randomized clinical trials have already shown the efficacy of this strategy for the heart [91,92]. Remote preconditioning is a particularly attractive strategy for protecting organs that are highly susceptible to damage but that are difficult to target, such as the brain.

Although many researchers are actively characterizing the signaling mechanisms of ischemic preconditioning in the nervous system, our knowledge of cerebral ischemic tolerance is still in its infancy and insufficient to be able to translate the laboratory results into application. Many issues need to be resolved to avoid disappointing results from the clinical application of ischemic preconditioning, e.g., whether the tolerant state can be maintained long term and provide chronic neuroprotection. Thus far, it appears that ischemic tolerance persists for approximately 1 week in the brain. Are there side effects from the long-term expression of ischemic preconditioning-induced genes? The threshold for tolerance induction and for cell injury needs to be determined. Pharmacological substances used for tolerance induction need to be safe.

In conclusion, the past two decades have provided interesting insights into the mechanisms and potential applications of ischemic tolerance in the brain. Current knowledge suggests that the preconditioning strategy and related interventions, such as remote preconditioning and pharmacological preconditioning, can protect neurons and improve neuronal survival after critical ischemia, and, thus, have promise for practical application in cases of vascular neurosurgery and endo-vascular therapy and possibly in the management of brain trauma. As knowledge in this field advances, the unresolved issues concerning the preconditioning cascade will likely be resolved and will lead to pharmacological strategies for protecting the brain from ischemic injury, traumatic brain injury, and other neurodegenerative disorders.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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