Reprogramming the Response to Stroke by Preconditioning

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Stroke causes neuronal injury and death because of deprivation of oxygen and nutrients that are essential for cell survival. In addition, inflammatory mediators are released that trigger a cascade of responses that exacerbate injury. Although these injurious pathways are well defined, it has been a major challenge to identify ways to mitigate these pathways to reduce ischemic damage.

One approach to the management of stroke injury under development in the research laboratory involves tapping into powerful endogenous mechanisms of protection through a process known as preconditioning. Preconditioning is a well-defined phenomenon whereby a small dose of an otherwise-harmful stimulus confers tolerance to a subsequent injurious event. Preconditioning stimuli that provide significant protection against ischemic brain injury include exposure to brief ischemia, small seizures, immune activation, exposure to hypo- and hyperthermia, and inhalation of volatile anesthetics. Although distinct, these preconditioning stimuli initiate a cascade of endogenous neuroprotective pathways that produce tolerance to ischemic injury.

Preconditioning with the immune activators Toll-like receptor (TLR) ligands has shown exceptional efficacy in the induction of ischemic tolerance. Systemic administration of ligands for TLR2, TLR4, TLR7, or TLR9 before focal cerebral ischemia profoundly reduces ischemic injury in rodent models of stroke. TLR preconditioning has also been shown to be effective in a neonatal ischemia model, demonstrating significant cerebral protection against hypothermic circulatory arrest in neonatal pigs. In addition, the TLR9 ligand has shown significant efficacy in a clinically relevant nonhuman primate model of experimental stroke.

Many TLR ligands have been approved for clinical use in other indications, making them ideal candidates for translation of pharmacological preconditioning from the laboratory to the clinic. Preconditioning is being developed as a prophylactic treatment for cerebral ischemia and is being investigated at a basic-science level to determine the endogenous molecular mechanisms that govern neuroprotection. As a prophylactic therapy, preconditioning would be directed toward patients who undergo cardiac or cerebrovascular surgery. These patients are at high risk of experiencing perioperative cerebral ischemic lesions, many of which occur within 48 hours after surgery. Such lesions are the likely cause of increased cognitive decline, morbidity, and mortality. Additional research into the mechanisms that underlie preconditioning offers promise in identifying protective pathways that could be used in the setting of acute stroke.

Preconditioning induces a transient window of protection that requires gene activation and new protein synthesis. If stroke occurs during this window after preconditioning, the response is dramatically reprogrammed to produce new signaling cascades that promote protection and resist injury. Preconditioning can be viewed in 3 sequential phases: a priming phase that sets up protection, a refractive phase that exists for 1 to 7 days when the system is resistant to injury, and a neuroprotective phase that is characterized by a reprogrammed response to stroke that reduces injury. In this review, we will discuss these 3 phases of preconditioning with specific focus on pharmacological preconditioning using TLR ligands. We will also describe promising results that suggest that preconditioning can be translated into substantial benefit for patients at high risk of cerebral ischemia.

**Priming Phase**

Typically, a low dose of TLR ligand is administered systemically to induce preconditioning. TLRs are expressed on nearly all cell types including circulating leukocytes, endothelial cells, and central nervous system cells. Because of the numerous potential cellular targets, it is unclear how the initial TLR signal is perceived and translated to reduce brain injury ultimately in response to cerebral ischemia. Within a few hours of TLR preconditioning, inflammatory serum cytokines are increased including tumor necrosis factor (TNF), interleukin-1 (IL-1), and IL-6. In addition, systemic administration of TLR ligands induces acute inflammatory gene expression changes in the brain, including the induction of genes associated with TLR signaling cascades.

A key role for inflammation in TLR preconditioning was first shown by the fact that inhibition of TNF abrogated TLR4-mediated neuroprotection and that mice deficient in TNF failed to be protected against ischemic injury after preconditioning with ligands for either TLR4 or TLR9. Although systemic administration of the TLR4 ligand, lipopolysaccharide, or the TLR9 ligand, cytosine-phosphate-guanine–rich oligodeoxynucleotide (CpG), induces TNF in the serum, there is compelling evidence that TNF induction in the brain, and not the circulation, correlates with the protective effect. Specifically,
the systemic induction of TNF can be bypassed by preconditioning with CpG using an intranasal route of administration, which induced cerebral TNF mRNA and caused a robust reduction in infarct size with no detectable TNF in plasma before stroke.22 The importance of cerebral TNF is supported by studies using direct TNF administration into the brain, whereby Nawashiro et al23 showed that intracisternal administration of TNF 48 hours before cerebral ischemia significantly reduced injury. In contrast, systemic administration of TNF failed to reduce the injury size. Thus, a low-level inflammatory response in the central nervous system may be critical to the priming phase of preconditioning.

Systemic preconditioning with a TLR ligand induces clear responses in both the systemic circulation and the brain parenchyma; however, the critical site-of-action of TLR stimulation is not readily apparent. The potential contribution of both the systemic and brain responses to TLR preconditioning-induced neuroprotection was highlighted in a study using TLR9-deficient reciprocal bone marrow chimeric mice lacking TLR9 on either hematopoietic cells (leukocytes) or radiation-resistant cells of nonhematopoietic origin (ie, endothelial cells, neurons, astrocytes, and microglia). Systemic preconditioning with the TLR9 ligand, CpG, failed to protect against cerebral ischemia in either chimeric strain. This indicates that TLR signaling is required in both the systemic circulation and the central nervous system to induce neuroprotection.22

**Refractive Phase**

After the priming phase, the system becomes refractive to subsequent injury for an extended period of time. This window of protection or refractive phase takes time (=1 day) to develop and can last up to 7 days.1 The precise endogenous neuroprotective mechanisms that are engaged during this refractive phase are unclear. Importantly, the genomic response to ischemic injury produced during the refractive phase is reprogrammed compared with that induced in naive animals,24–26 demonstrating that during the refractive phase the cellular processes that perceive and respond to injury have been restructured.

Although the mechanistic composition of the refractive phase induced by TLR preconditioning has not been well studied, insights can be gained from work on ischemic preconditioning. Ischemic preconditioning is a well-studied preconditioning paradigm that uses brief cerebral ischemia to provide protection against subsequent stroke. Interestingly, ischemic preconditioning-induced neuroprotection is partially mediated through endogenous activation of TLR4, as protection is attenuated in TLR4-deficient mice.27 Thus, mechanisms engaged during ischemic preconditioning may directly relate to pharmacological TLR-mediated protection.

There are multiple processes that may be altered during the refractive phase to ultimately engage the reprogrammed response to stroke. Extensive genomic analysis of TLR and ischemic preconditioning suggests that brain gene expression changes do not contribute to the refractive phase as the majority of genes have returned to baseline levels by 72 hours post-treatment.7,25,26 Additional potential modulators of the refractive phase include microRNAs, epigenetics, and protein changes. MicroRNAs and epigenetic alterations are master transcriptomic regulators that have widespread effects on entire genomic programs and could orchestrate the reprogrammed response to ischemic injury. Autophagy, a mechanism that adapts cells to survive during reduced energy availability, may also play a role in altering the brains response to injury.

MicroRNAs are short sequences of nucleotides that modulate gene expression post-transcriptionally. Individual microRNAs can target hundreds of genes, and thus, gene expression is affected greatly by the expression of a single microRNA. Several studies using microRNA microarrays have revealed robust regulation of microRNAs after ischemic preconditioning.28–30 Members of the miR-200 family of microRNAs were found to be upregulated in the brain following ischemic preconditioning. One of the gene targets of the miR-200 family is prolyl hydroxylase 2, a protein that modifies hypoxia-inducible factor 1α causing it to be degraded during periods of normoxia.29 Lee et al29 showed that transfection of a neuronal cell line with microRNA-200 family members decreased prolyl hydroxylase 2 with a concomitant increase in hypoxia-inducible factor 1α and reduced cell death in response to oxygen glucose deprivation. These results highlight the potential of microRNA expression during the refractive phase to alter the brain’s response to ischemia resulting in a reprogrammed genomic profile and neuroprotection.

Epigenetic changes occur at chromosomal level through chromatin remodeling and histone modification. Such changes alter the response of entire gene programs and are typically long lasting. An investigation into neuroprotection induced by ischemic preconditioning revealed that several gene repressor proteins were upregulated after ischemic preconditioning including histone 2A and polycomb group protein SCMH (sex comb on midleg homolog 1),31 both of which are associated with chromatin remodeling. Interestingly, overexpression of SCMH induced ischemic tolerance without the need for preconditioning in an in vitro model of ischemia.31 Consistent with this finding, knockdown of SCMH in vivo reversed the preconditioning-induced neuroprotection against ischemic injury. Epigenetic alterations such as these could play a role in defining the refractive window because they take time to develop and result in long-term changes of genomic responses.

Autophagy is another intriguing process that may play a role in the refractive phase. Autophagy is an evolutionarily conserved mechanism that degrades and recycles damaged cellular constituents to adapt to stressful conditions and maintain energy homeostasis. Recently, the induction of autophagy has been associated with the neuroprotective effect of ischemic preconditioning.32,33 Sheng et al33 identified an increase in autophagosomes and proteins associated with autophagy in the brain 24 hours after ischemic preconditioning. Importantly, inhibition of autophagy during ischemic preconditioning abolished the protective effect, whereas exogenous induction of autophagy 24 hours before ischemic challenge was sufficient to induce tolerance without ischemic preconditioning.33 An active autophagic response at the time of the ischemic challenge may alleviate endoplasmic reticulum...
stress by degrading toxic protein aggregates and providing an emergency source of energy and basic constituents for the synthesis of new proteins required for cell survival.

**Neuroprotective Response**

Preconditioning alters the brain’s environment causing the subsequent ischemic challenge to be perceived differently, which creates an altered or reprogrammed response to stroke that reduces injury. Cerebral ischemia induces new gene regulation in the brain that participates in the injurious response. Genomic studies using microarrays have shown that preconditioning suppresses damaging genes during ischemic injury and induces unique genes that are not evident normally in the response to ischemia in nonpreconditioned animals. This reprogrammed response forms the basis for endogenous neuroprotection and provides insight into the molecular mechanisms that make the brain resistant to ischemic injury.

Transcriptional studies of 2 TLR preconditioning paradigms (lipopolysaccharide and CpG) and ischemic preconditioning in mouse cortical tissue revealed that ≈12% of the genomic response 3 hours after ischemia was distinct from that induced in nonpreconditioned mice. These genomic changes were not evident before stroke and were manifested only after injury, indicating that preconditioning altered the endogenous response to stroke. At 24 hours poststroke, comparison of the genes induced in the reprogrammed response to injury in the 3 preconditioning paradigms revealed a set of 13 regulated genes common to all 3 preconditioning paradigms that are not evident in stroke alone. Analysis of the promoter regions of these 13 genes identified a significant over-representation of transcriptional regulatory elements associated with type I interferon regulatory factors (IRFs), suggesting that there is a shared reprogrammed response that is likely mediated through IRF-induced gene transcription. Although the importance of this IRF-mediated response to neuroprotection has not been fully established, mice deficient in IRF3 or IRF7 failed to be protected by preconditioning with either CpG or lipopolysaccharide, suggesting that IRF3 and IRF7 are key mediators of TLR-induced protection. Additionally, ischemic preconditioning of IRF3- and IRF7-deficient mice demonstrated attenuation of the protective response in ischemic preconditioning; the partial effect is likely attributable to the partial dependence of ischemic preconditioning on TLR4 activation. Taken together, this suggests that IRF3 and IRF7 are key mediators of a conserved neuroprotective mechanism induced via TLR preconditioning.

The brain is not the only site of preconditioning-induced reprogramming that influences stroke injury. It is well known that infiltration of inflammatory cells into the brain parenchyma and the breakdown of the blood–brain barrier both contribute to cerebral ischemic injury in response to stroke. Thus, altering or reprogramming the response of these cellular components to stroke may also play an important role in mitigating injury. TLR preconditioning is most often initiated systemically, thus circulating immune cells and endothelial cells are exposed to the preconditioning agent. TLR-induced reprogramming of leukocytes has been well documented and demonstrates that activation in response to a secondary stimulus is altered, resulting in decreased inflammatory cytokine production and increased anti-inflammatory/antiviral responses. Redirecting the stroke-induced systemic cytokine storm away from proinflammatory mediators and toward an anti-inflammatory response could contribute to reducing cerebral ischemic injury.

Endothelial cells, key components of the blood–brain barrier, can also be reprogrammed via pretreatment with TLR ligands. Endothelial cells respond to lipopolysaccharide by increasing expression of endothelial-leukocyte adhesion molecule (E-selectin), intracellular adhesion molecule 1, vascular cell adhesion molecule 1, and IL-6 with a resultant increase in leukocyte adhesion. However, preconditioning of endothelial cells with lipopolysaccharide produces a reprogrammed response to a secondary lipopolysaccharide exposure characterized by reduced expression of E-selectin, vascular cell adhesion molecule 1, and IL-6; suppressed leukocyte adhesion; and increased expression of the antioxidant manganese superoxide dismutase. In addition, it has been shown that systemic lipopolysaccharide preconditioning suppressed E-selectin and leukocyte-endothelial cell adhesion molecule 3 (P-selectin) expression and suppressed leukocyte infiltration into the brain in response to central nervous system inflammation induced by an intracerebral ventricular injection of IL-1β. In human trials, induction of TLR tolerance reduced the vascular reactivity and cytokine storm associated with endotoxemia, which demonstrates that TLR preconditioning can reprogram systemic inflammatory responses in humans. Thus, TLR preconditioning-induced reprogramming of leukocytes and endothelial responses may contribute to the protective phenotype through suppression of leukocyte activation and trafficking across the blood–brain barrier in response to stroke. In addition, reprogramming of leukocytes to suppress proinflammatory mediators and enhance anti-inflammatory cytokines may also mitigate stroke injury.

**Preconditioning Phases: Summary**

The phases of preconditioning define the development of reprogramming and the resultant neuroprotective response to ischemia (Figure). Understanding the cellular and molecular mechanisms that orchestrate each of these phases has the potential to identify therapeutic targets for the prophylactic and acute treatment of ischemic injury. In addition, the evidence strongly implicates multiple cell types and pathways that mediate priming, refraction, and reprogramming, indicating that preconditioning may reveal an endogenous systems-wide approach to reducing injury.

**Translation to Clinical Application**

Clinical translation of preconditioning to protect patients at risk of cerebral ischemia has made significant progress in recent years. Multiple clinical trials have examined the benefit of remote ischemic preconditioning (RIPC) in which protection results from a mild regiment of ischemia and reperfusion at a site remote from the target organ. The most common model of RIPC involves repeated brief periods of limb ischemia induced using an inflation cuff. Although the majority of clinical trials using RIPC have been centered on protecting
against ischemic cardiac disease, promising results have also been obtained for neurological injury.42-44 These clinical trials using RIPC demonstrate the potential of preconditioning as a prophylactic treatment to reduce neurological damage associated with cerebral ischemic injury. Although RIPC is relatively noninvasive, it is cumbersome and difficult to standardize from patient to patient. Administration of a pharmacological agent, such as a TLR ligand, via injection would provide a simple, quick, and accurate means of preconditioning for patients at risk of cerebral ischemia. Preclinical studies using a TLR9 agonist in a nonhuman primate model of stroke show promising results for moving this pharmacological preconditioning agent to phase I clinical trials. In these studies, rhesus macaques were preconditioned with a single injection of a TLR9 agonist 3 days before surgically induced stroke. TLR9 preconditioning significantly reduced infarct volume (by >40%)14 and neurological deficits associated with the injury (unpublished observation). This study strongly supports the therapeutic value of pharmacological TLR preconditioning to reduce the extent of ischemic injury and enhance recovery.

Conclusions

More than 7.5 million patients in the United States undergo a cardiovascular or cerebrovascular procedure annually, putting them at risk of brain ischemia from embolic events associated with these medical procedures. Targeting the bodies own endogenous system of protection through preconditioning could offer a relatively noninvasive and financially feasible mechanism for protecting these patients against potential debilitating ischemic complications. Furthermore, only 1 acute stroke therapy exists, tissue plasminogen activator, and its use is extremely limited because of its narrow window of effectiveness and high risk of side effects. Thus, unlocking the mechanisms of endogenous neuroprotection induced by preconditioning will provide insight into novel pathways that could be exploited acutely to treat and protect the brain of patients with stroke.

Figure. Model of the phases of preconditioning-induced reprogramming. Preconditioning engages endogenous mechanisms of protection that are developed through 3 phases of molecular alterations. The priming phase initiates the response to the preconditioning stimuli. Induction of tumor necrosis factor (TNF) in the brain may be a key component of the priming phase. The subsequent refractive phase defines the period in which the response to an ischemic challenge is reprogrammed. Potential mediators of this phase are microRNA (miRNA), epigenetic factors, and autophagy-related changes in metabolism. The reprogrammed response to the injury results in the neuroprotective phase, through sustaining cellular homeostasis, suppression of cellular infiltration in response to ischemia, and induction of interferon regulatory factor (IRF)–mediated gene transcription.

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