Preconditioning Reprograms the Response to Ischemic Injury and Primes the Emergence of Unique Endogenous Neuroprotective Phenotypes

A Speculative Synthesis

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Abstract—Ischemic tolerance in the brain, in which sub-threshold insults increase resistance to subsequent injurious ischemia, is a powerful adaptive defense that involves an endogenous program of neuroprotection. Emerging evidence from genomic studies suggests diverse stimuli that trigger preconditioning achieve neuroprotection through a common process which depends on a fundamental reprogramming of the response to injury. Such reprogramming of the genomic response to injury leads to the induction of novel neuroprotective pathways not ordinarily found in the setting of ischemia. Genomic studies also indicate that the nature of the preconditioning stimulus (e.g., brief ischemia or endotoxin [lipopolysaccharide]) dictates the phenotype of neuroprotection, a phenotype that parallels protective adaptations also found in certain physiological conditions where the preconditioning stimulus exists at levels that can induce injury. The idea that preconditioning leads to a fundamental reprogramming event that confers neuroprotection is a novel and important concept in the field of ischemic tolerance. Moreover, the view that distinct preconditioning stimuli confer neuroprotection via effectors that differ according to the nature of the preconditioning stimulus offers promise that multiple, nonoverlapping pathways may be discovered as novel neuroprotective therapies. (Stroke. 2007;38[part 2]:680-685.)

Key Words: genomics ■ neuroprotectants ■ preconditioning ■ Toll-like receptors ■ stroke

Ischemic tolerance in the brain, in which exposure to brief, noninjurious ischemia increases resistance to subsequent injurious ischemia, is a powerful adaptive defense that involves an endogenous program of neuroprotection.\(^1\)\(^-\)\(^3\) Tolerance to ischemic brain injury can be induced by several distinct preconditioning stimuli: noninjurious ischemia, cortical spreading depression, brief episodes of seizure, exposure to anesthetic inhalants, and low doses of endotoxin (lipopolysaccharide [LPS]).\(^4\)\(^-\)\(^9\) The existence of multiple, diverse preconditioning stimuli results in the well-known phenomenon of “cross-tolerance” wherein a tolerance-inducing stimulus of one kind provides protection against an entirely different type of injury such as in the case of exposure to a brief epileptic seizure protects the brain from subsequent ischemic injury.\(^10\) Although the molecular mechanisms that underlie preconditioning are not well-understood, it is generally accepted that preconditioning requires small doses of an otherwise harmful stimulus to induce protection against subsequent injurious challenge.\(^3\)

Several biologic features are considered hallmarks of delayed preconditioning and these features are independent of the nature of the preconditioning stimulus. Delayed tolerance develops slowly over time, protection against subsequent injury is only evident 24 to 48 hours after the preconditioning event. Neuroprotection peaks at 3 days and diminishes over the course of 1 week. This temporal profile distinguishes delayed tolerance from rapid tolerance, which is a protective phenotype that develops within minutes and lasts only a few hours. Delayed tolerance is also characterized by a dependence on new gene expression and de novo protein synthesis after preconditioning.\(^11\)

Evidence is emerging that diverse stimuli that trigger preconditioning may share a common process, genomic reprogramming, that confers neuroprotection via equally diverse effector mechanisms.\(^12\)\(^-\)\(^18\) This neuroprotective process sets into motion a complex cascade of signaling events, which leads to the synthesis of new proteins that fundamentally change the molecular and genetic response to subsequent injury, a process we previously referred to as genomic reprogramming.\(^19\)\(^,\)\(^20\) Our proposed model of genomic reprogramming has evolved further since its initial description in The Lancet.\(^19\) The original proposal was based on studies performed on brains preconditioned with a brief period of ischemia. Inclusion of new insights derived from studies performed in the setting of cross-tolerance using endotoxin (LPS) preconditioning against ischemic injury has expanded
our understanding of genomic reprogramming and added new features that define further the endogenous molecular properties of neuroprotection induced by preconditioning.21

**Genomic Reprogramming Revealed by a New Gene Signature That Occurs in Response to Ischemia**

Ischemic tolerance reflects a fundamental change in the genomic response to injury that shifts the outcome from cell death to cell survival.19 This change in gene expression in response to ischemia was first evinced by gene expression profiles of animals preconditioned by exposure to a brief period of ischemia. Preconditioned brains not only lacked the robust transcriptional upregulation that nonpreconditioned brains display in response to injurious ischemia but also actively suppressed genes that control metabolism, cell cycle regulation, and ion channel activity, indicating a complete reprogramming of the genomic response to ischemia. These features appear to mimic specific adaptive neuroprotective strategies seen in hibernation and other hypoxia-tolerant states. Thus, we postulated that preconditioning elicits endogenous genetic adaptations that confer tolerance to the injurious effects of oxygen deprivation similar to those found in hibernating and anoxia-tolerant species.

The finding that exposure to a brief ischemic event (preconditioning) causes a complex reprogramming of the cellular responses to subsequent ischemia may reflect a fundamental regulatory mechanism for governing host responses to harmful stimuli. This concept was evaluated further using low-dose administration of LPS as a preconditioning stimulus to protect against injurious ischemia. LPS preconditioning in the brain shares several hallmark characteristics with ischemic preconditioning in the brain,22 such as the delayed induction of tolerance after preconditioning and a dependency on de novo protein synthesis.22,23 Unique aspects include the systemic route of LPS administration and the induction of systemic changes such as attenuated cellular activation of monocyte/macrophage populations in peripheral blood. These aspects of “systemic preconditioning” may contribute to the protective effect. It is important to note that similar to ischemic preconditioning wherein brief preconditioning exposure to ischemia does not induce brain damage, a preconditioning dose of LPS does not cause brain injury. Ischemic tolerance conferred by LPS likely occurs via stimulation through Toll-like receptor 4 (TLR4), one of a family of pathogen-sensing cellular receptors.24 TLRs are pattern recognition receptors thought to be involved in the recognition and response to foreign pathogens.24 A common pathway in all the TLRs is the ability to induce nuclear factor-κB, which subsequently leads to the transcription of various cytokines, chemokines, and cell surface molecules.25

Microarray analyses provide a genetic snapshot of the brain’s response to ischemia in the presence or absence of prior preconditioning. Genomic profiles of early (3 hours) responses to brain ischemia from animals preconditioned with brief ischemia or low-dose LPS have similar patterns (Figure 1). Brains exposed to ischemic preconditioning before injurious ischemia display an early genomic response (3 hours after stroke) in which >60% of the genes regulated are unique to these animals and not observed in animals exposed to stroke alone. Similarly, brains from mice preconditioned with LPS before stroke display a genomic response where >60% of the genes regulated within 3 hours of stroke are found only in animals given LPS preconditioning before exposure to stroke. At later times (24 hours after stroke) the genomic profiles in each of the 2 preconditioning paradigms share even fewer genes in common with brains exposed to stroke alone. These findings suggest that preconditioning stimuli have in common the ability to alter the brain’s transcriptional response to subsequent injury, which in turn, drives preconditioning-specific signatures of ischemic neuroprotection.

**The Nature of the Preconditioning Stimulus Determines the Phenotype of Neuroprotection**

Whereas the pattern of gene regulation is similar between the 2 preconditioning stimuli (ie, preconditioning plus stroke
comprises a large set of unique genes not seen in brains from animals given stroke alone) a substantial subset of the genes regulated are unique to each preconditioning stimulus. This could suggest that certain genes and pathways used to achieve neuroprotection may be defined by the specific nature of the preconditioning stimulus. Analyses of the identity and function of those genes regulated in each of the 2 paradigms supports this idea.

The genomic response to ischemia in the setting of prior preconditioning with brief ischemia produces a phenotype of neuroprotection tailored to protect against injury from low-oxygen states such as those encountered in conditions of hibernation. In particular, suppression of gene expression is a dominant outcome in animals preconditioned with brief ischemia before exposure to injurious ischemia. The specific genes that are suppressed fall into two major categories: metabolism and channels/transport (Figure 2). These 2 functional categories contain genes whose activities are energy-related and, thus, suppression of their activities would lead to energy conservation, an important adaptation for successful existence during periods of low oxygen. Suppressed metabolic activity (metabolic arrest) and decreased ion transport activities via “channel arrest” are features found in animals that hibernate or exist without oxygen for extended times.

Thus, preconditioning with a brief period of ischemia (low oxygen and blood flow) leads to ischemic tolerance that appears to be accompanied by neuroprotective strategies that mimic evolutionarily conserved responses seen in anoxia-tolerant species.

Analyses of the genomic response to LPS preconditioning provides additional evidence that the nature of the preconditioning stimulus determines the phenotype of neuroprotection found in the tolerant brain. Specifically, LPS, a known stimulus of innate inflammation, produces a tolerant state in the brain via effectors of inflammation that are neuroprotective and through processes and mediators that counteract pro-inflammatory processes. As such, the genomic response to LPS preconditioned brains is characterized by a robust cytokine response, the majority of which involve genes associated with neuroprotective type I interferons (Figure 3).

In addition, preconditioned brains display a marked absence of deleterious inflammatory mediators (interleukin-6, macrophage inflammatory protein 1α, Tnf receptor-associated factor 6) ordinarily seen in response to stroke injury. Thus, as in the setting of ischemic preconditioning in which protection against ischemic injury occurs through mediators of anoxia tolerance, LPS preconditioning induces protection against ischemia through regulation of pro- and anti-inflammatory pathways.

The results from these 2 studies have led us to posit that the reprogrammed phenotype is determined by the nature of the preconditioning stimulus. Such an idea is consistent with our current microarray results but should be tested further. Other factors may contribute to the diverse genomic profile found between the 2 preconditioning paradigms. The extent of injury encountered between ischemic and LPS preconditioning is not identical, which may underlie some of the differences observed in the gene profiles. This is a common problem encountered in such studies, which we tried to minimize by using the maximal effective dose of the preconditioning stimulus in both studies. Importantly, both studies exhibit little evidence of injury at the earliest time point (3 hours) studied after stroke—a time when substantial diversity already exists in the genomic response of the 2 preconditioning stimuli. It should be noted that we used different versions of gene chips in each of the studies and although the chips shared ~80% of the genes, it is possible that incomplete overlap could contribute to differences seen between the preconditioning profiles. Thus, although our findings support the dominance of a unique preconditioning-specific genomic profile, a finding that has been noted in cardiac preconditioning as well, it is conceivable that a subset of genes important in neuroprotection exists that are shared between the 2 preconditioning stimuli.

Collectively, these observations have led us to hypothesize that preconditioning reprograms the response to injury and that the reprogrammed neuroprotective phenotype is specifically tailored by the nature of preconditioning stimulus. If
correct, such findings would underscore the substantial adaptive benefit of physiological responses that are shaped by previous signals and thereby governed by the context in which they are generated.

Preconditioning and Acute Neuroprotective Therapy Display Contrasting Genomic Responses to Ischemic Injury

Preconditioning can be viewed as a protective response to anticipated danger or injury, which could be best served by reprogramming the injury response from one of cell death to a response favoring cell survival. In contrast, acute neuroprotective therapy for ischemic injury may work more efficiently by means of suppressing or limiting deleterious effectors. Gene expression patterns support these 2 outcomes when comparisons are made between brains preconditioned before ischemia and brains taken from animals given a neuroprotectant (osteopontin [OPN]) at the time of ischemia. Such comparisons demonstrate that, as described earlier, in preconditioned animals >50% of the regulated genes identified 24 hours after stroke were unique to the setting of preconditioning and not observed in mice given a stroke only. However, in the setting of OPN therapy for neuroprotection, none of the regulated genes at 24 hours was unique to this condition (Figure 4). All genes regulated after stroke in the setting of OPN administration were also regulated after stroke in the absence of OPN administration. This indicates that OPN acts as a neuroprotector by acute suppression of the damaging response in stroke rather than induction of a novel protective set of gene transcripts (reprogramming) as observed in preconditioning.

Conclusions

Genomic studies provide an unbiased view of the molecular basis of complex biologic systems and thereby reveal surprising insights into hidden mechanisms that might otherwise remain elusive. Recent studies in the setting of ischemia have revealed novel genes that appear to be effectors of injury in the brain. Genomic studies of preconditioning paradigms in the brain are relatively few, but have given us important clues into neuroprotective mechanisms (eg, heat shock proteins, hypoxia-inducible factor dependent genes, glutamate receptors) that have a role in tolerance to ischemic injury. Here we have used such an approach to elucidate endogenous neuroprotective pathways of ischemic and LPS preconditioning. Our results support several novel features of preconditioning that may not have been identified otherwise. First, our results suggest that preconditioning reprograms the transcriptional response to ischemic injury. Evidence for reprogramming comes first from ischemic preconditioning and is confirmed more recently with genomic studies of LPS preconditioning and hypoxic preconditioning in the brain. Further support for reprogramming lies in the fact that both ischemic

Figure 3. LPS preconditioning alters the inflammatory response to stroke injury by suppressing pro-inflammatory and increasing anti-inflammatory mediators. LPS preconditioning before stroke modulates the expression of a unique subset of genes associated with defense responses. Gene regulation is displayed from samples taken 24 hours after stroke in the presence or absence of LPS preconditioning. Gene expression: red indicates increased expression; green indicates decreased expression; gray indicates no change in expression. The shaded red area represents genes associated with type I interferons. Genes were assigned to Biological Function categories based on the Gene Consortium database and literature review. IL6 indicates interleukin 6; Mip1a, macrophage inflammatory protein 1α; Il1r, interleukin 1 receptor; Traf6, Tnf receptor-associated factor 6.

Figure 4. Osteopontin does not induce unique gene regulation in response to stroke. Venn diagram showing unique gene regulation (percentage) 24 hours after stroke in mice treated with osteopontin vs mice given a stroke without osteopontin treatment. Osteopontin plus stroke and overlap shown in the green circle and stroke alone represented by the blue circle. Osteopontin treatment reduced the number of genes with changes in expression by 4-fold.
and LPS preconditioning induce a new genomic signature comprised of novel sets of endogenous molecules that serve a neuroprotective function in response to ischemia.

Second, the emergence of unique endogenous neuroprotective phenotypes appears to be directed by the nature of the preconditioning stimulus. Brief periods of ischemia induce pathways that are neuroprotective in the setting of reduced blood flow and oxygen availability, conditions that parallel those seen in hibernating animals. However, preconditioning with an inflammatory stimulus such as LPS allows the induction of pathways that confer protection by countermanding deleterious inflammatory responses induced in ischemic injury and permitting neuroprotective cytokine responses to dominate. Protection against uncontrolled inflammatory responses may be an important advantage that enabled to limit excessive inflammatory mediators that may lead to severe organ damage and death.

Finally, there is new evidence indicating the genomic response to preconditioning that leads to neuroprotection is distinct from the genomic response induced by acute neuroprotective therapy. Neuroprotection induced by preconditioning involves a novel genomic response to ischemia that occurs by reprogramming the response to injury, whereas acute neuroprotection induced by OPN occurs by dampening the response to ischemia without induction of novel pathways of neuroprotection.

The cellular and molecular mediators activated by preconditioning set into motion a complex cascade of signaling events leading to new synthesis of proteins that ultimately reprograms the cellular response to subsequent ischemic injury. That preconditioning may lead to a fundamental reprogramming event, which, in turn, can confer a neuroprotective phenotype is a novel concept in the field of ischemic tolerance. Moreover, the concept that preconditioning stimuli confer neuroprotection via distinct effectors that differ according to the nature of the preconditioning stimulus offers the hope that multiple, nonoverlapping neuroprotective pathways may be identified as potential therapeutic approaches for neuroprotection. This idea is supported further by genomic preconditioning studies in the heart wherein gene expression profiles were found to differ substantially between distinct forms of preconditioning (ischemic versus anesthetic). Over-all, this idea is broadly relevant to injury states in general. Thus, identification of the central mediators involved in preconditioning may define essential pathways responsible for this important cellular injury response program and thereby provide new and effective therapeutic strategies for stroke as well as other central nervous system disorders.

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