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Epigenetics and the Environment: In Search of the "Toleroasome" Vital to Execution of Ischemic Preconditioning

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Abstract

Activation and repression of gene expression are key features of ischemic tolerance. Converging lines of inquiry from several groups suggests that epigenetic proteins may transduce sublethal stresses, including bio-energetic or oxidative stress into durable (2–3 days) changes in gene expression that mediate ischemic tolerance. Here we discuss the potential mechanisms by which changes in cell state (e.g., ATP, NAD+, and oxygen) can modify specific targets including polycomb complexes, jumonji domain histone demethylases, and zinc and NAD-dependent histone decetylases and thus trigger an adaptive program. A major unanswered question is whether these proteins work in parallel or convergently as part of a "tolerosome" (*tolero* is the Latin word for tolerance), a multiprotein complex recruited to promoters or enhancers of specific genes, to mediate preconditioning. Whatever the case may be, epigenetic proteins are fertile targets for the treatment of stroke.

Keywords

Epigenetics; Ischemic preconditioning; Histone demethylase; Histone deacetylase; Polycomb

Introduction

Ischemia results from a transient or permanent blockage of blood flow to the brain. Sustained or severe ischemia, known otherwise as "stroke," can damage the brain permanently and thereby affect neurological functions attributable to the damaged region. Although the mortality rate from stroke has decreased in recent years, stroke remains the leading cause of long-term disability in the USA and costs the government more than $40

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billion in annual healthcare costs, not to mention the nonquantifiable suffering and loss of quality of life [25].

Enormous progress has been made in our understanding of the pathophysiology of stroke, and yet few protective strategies have been successful. Indeed, stroke has been likened to death by a “thousand cuts,” and therapeutic interventions downstream of vascular occlusion that address a handful of these cuts have not been successful at the human bedside [39]. A potential window into a broader, more durable and robust protective response to ischemia is the phenomenon of preconditioning. Specifically, the induction of a sublethal ischemic insult can “turn on” adaptive homeostatic responses that protect the brain from a subsequent, otherwise injurious ischemia [12, 41]. This response, known generally as ischemic tolerance, has emerged as a potential approach to “fast track” understanding of the mechanisms that protect against ischemic stroke. Identification of the effector(s) of ischemic tolerance could lead ultimately to the development of novel, more robust therapeutic strategies for stroke treatment. And since ischemic tolerance is characterized initially by the transient activation of pre-existing proteins and sustained (over days) changes in gene expression, a priority has been to understand those changes and how they evolve following a sublethal, neuroprotective stimulus in order to facilitate durable neuroprotection.

Elegant studies from a number of groups, particularly those of Frank Sharp [40], Jeffrey Gidday [12], John Zhang [54, 55], Gabriel Haddad [29], Sylvain Dore [52], and Mary Stenzel-Poore and Roger Simon [44], have shown that the ischemic-tolerant brain is characterized by both repression and activation of gene expression. These studies have been complemented by an understanding of the “proteomic signature” that is associated with such induction and repression [43]. While an understanding of the proteins involved in ischemic tolerance might ultimately illuminate “master switches” and drug targets, a focus on single genes downstream of these master switches—genes that are induced or repressed during tolerance—is unlikely to be therapeutically very meaningful. In other words, the ischemic-tolerant brain most likely relies on broad cassettes of genes or programs, and it is the ability of these gene cassettes to work coordinately at a cellular, local, and systemic level that mediates effectively the brain’s adaptive response. Accordingly, an identification of the “master switch” that turns on these programs is essential to the development of clinically beneficial strategies for stroke treatment. Selective modulators of that switch might act as vehicles for the activation of the ischemic-tolerant brain, and would remove the need to engage more potentially harmful preconditioning stimuli such as Toll-like receptor (TLR)-binding proteins and ischemia itself in order to trigger the brain’s adaptive response.

One biological intersection between the environmental changes such as those created by a sublethal stress and changes in gene expression is the area of epigenetics. Epigenetics classically refers to heritable changes in gene expression that are unrelated to differences in DNA sequence; however, it has also come to refer to the panoply of cell components that sit above the genome (DNA) and how the modification of those components by changes in the cell or organismal environment can lead to changes in gene expression [33]. This review focuses on epigenetic players in neurons and the evidence to date that suggests that direct manipulation of these players can lead to ischemic tolerance.

For epigeneticists interested in stroke neuroprotection, the $64,000 question is: How are transient changes in energy, in a cell's level of ATP routinely observed during sub-lethal ischemia, transduced into the epigenetic modifications that govern the reprogramming of genes in the ischemic-tolerant brain? This review further challenges the clinical neuroscientific community to investigate this relatively obscure area of neurobiology, rather than to examine specific genes with those that are coordinately regulated in the ischemic-tolerant brain.

The Changing Landscape of Biological Discovery Informs Mechanisms of Ischemic Tolerance: Genomics

For many years, the investigation of ischemic tolerance relied on a "one gene at a time" approach. In the past decade, scientists have employed unbiased, microarray analysis or RNA sequencing in order to paint a fuller picture of the mechanisms involved in the ischemic-tolerant brain. With microarray analyses, Stenzel-Poore and colleagues showed that hundreds of genes are regulated after an insult to the ischemic preconditioned brain [44]. Later, Stevens et al. attempted to find the convergence upstream regulators of these genes. The group induced preconditioning with two different TLR ligands and with ischemia itself, and then examined the genomic profiles that resulted from the different stimuli. Interestingly, the responses to all three stimuli converged on a shared subset of 13 genes. The promoter regions of these genes had shared sequences as well—sequences that all required interferon regulatory factor (IRF)-mediated transcription [45]. IRFs or the factors that regulate the IRFs (interferon consensus sequence binding protein) may well be "master switches" of the ischemic-tolerant brain, and should no doubt be investigated further. In addition to LPS and poly dI dC, in vivo treatment of animals with tilorone, a small-molecular weight interferon-inducing agent, is known to induce tolerance [7, 34]. Simultaneous with this treatment, mRNA silencing of IRF-7 or IRF-3 might reveal whether these factors are necessary for tilorone-mediated neuroprotection. However, while IRFs most likely play a significant role in ischemic tolerance, they are not necessarily its only effectors.

The approach taken by Stevens, Stenzel-Poore, Simon and coworkers to the investigation of ischemic tolerance is a viable one: in order to identify the larger programs that facilitate neuroprotection, it is essential first to find overlap in regulatory elements or promoter architecture between the gene groups essential for ischemic tolerance. Can this approach also be used in order to identify epigenetic "master switches?" The answer is certainly yes, because transcription factors represent important mechanisms by which positive epigenetic regulators and negative epigenetic regulators are recruited to specific gene loci. Thus, an identification of the epigenetic, transcription factor, and transcriptional basal machinery proteins that are recruited by one or more "toleroasomes" (tolero is the Latin word for tolerance and "some" refers to a complex of proteins) will provide an abundance of potential therapeutic targets. In the following sections, we detail individual epigenetic players that might mediate ischemic tolerance and how some of these are already changing our optimism for stroke protection and repair.

Potential Epigenetic Targets Involved in Ischemic Tolerance

Potential epigenetic players involved in ischemic tolerance include polycomb group proteins, zinc and NAD-dependent histone-modifying proteins (histone deacetylases (HDACs) and HATs), micro-RNAs, DNA methylases, and histone demethelyases. Which of these modulators, if any, plays the largest role in ischemic tolerance? Could a member of these broad families act as a protective “master switch?”

Polycomb Group Proteins

Polycomb group proteins (PcG) may well be one of the central modulators of ischemic tolerance. These proteins compose a family of transcriptional repressors. Though PcG were thought originally to regulate the transcription of only a few genes, including the homeotic (*Hox*) genes, it has been shown in recent years that PcG target genes are involved in the regulation of many genes involved in diverse functions including in electron and glucose transport, as well as with endopeptidase, oxidoreductase, and G-protein-coupled receptor activity [20, 53]. The *Zhou lab* has shown that the induction of a non-injurious ischemic insult leads to increases in the quantity of specific components not only polycomb complexes, but other epigenetic proteins in the brain [43]. These PcGs, sex comb on midleg homolog 1 (SCMH1) and B lymphoma Mo-MLV1 insertion region 1 (BMI1), are part of a larger complex called poly-comb repressive complex 1 (PRC1), which monoubiqitinates both histone 2A and 2B [53] (see Table 1). The exact role of this monoubitination in ischemic tolerance-induced transcriptional repression has been unknown, but these changes likely contribute to repression of genes that consume ATP, including voltage-gated potassium channels [43]. The findings of increased levels of more than half a dozen proteins, many of which are involved in epigenetic regulation (histone H1 and H2 variants, BMI1, and SCMH1) provides a compelling reconciliation of the simultaneous need for de novo transcription ischemic tolerance and the prominent role that gene repression appears to play. Indeed, one could argue that the convergent regulators of transcription or activity of the polycomb complex might represent druggable targets for regulating preconditioning. It will be of interest to know whether PRC1 component induction is a common mechanism for preconditioning induced by many stimuli (e.g., LPS, hypoxia, NMDA receptor activation).

Histone Phosphorylation via AMP Kinases

Adenosine monophosphate-activated protein kinases (AMPK) have been shown to help neurons and other cell types cope with lethal ischemic injury after a sublethal ischemic insult. These kinases are activated by high AMP levels under conditions of energetic stress [6]. As cells consume ATP out of proportion to its production, AMP levels rise; cells must therefore recruit AMPK in order to activate catabolic processes and inhibit anabolic ones, all with the goal of preserving energy balance. Thus, AMPKs play a powerful role in the regulation of cellular and organismal energy homeostasis. Recent evidence suggests that low levels of activation of AMPK lead to neuroprotection while sustained or high levels lead to death [24, 31]. Indeed, the latter appears to dominate in stroke, a condition in which deprivation of oxygen and glucose leads to loss of ATP and cell death. AMP kinase has a growing list of targets, including epigenetic proteins. In an elegant study from Craig

Thompson's lab, it was shown that metabolic and genotoxic stressors activated transcription through AMPK-dependent phosphorylation of a specific H2B Serine residue, Ser^{36} (see Table 1). Substitution of Ser^{36} with an alanine decreased transcription, recruitment of RNA polymerase II, and cell viability under conditions of cellular stress [6]. These results suggest that AMPKs regulate cellular adaptation to stress not only in the cytosol, but through epigenetic modification as well, and that the direct epigenetic modulation is essential for cell viability.

After an ischemic insult, it has been shown that the AMP/ATP ratio in neurons to increases, along with neuronal recruitment of AMPK. That AMPK might facilitate energy conservation through epigenetic modifications makes it another attractive candidate as a "master switch" of ischemic tolerance. Exactly how the phosphorylation of H2B Ser^{36} promotes cell viability under conditions of stress—and whether this phosphorylation occurs at all in the brain following conditions in which ATP is depleted, such as stroke —are pressing questions that should be investigated further. Prior studies from Louise McCullough, Gabrielle Ronnett and colleagues suggest that preconditioning leads to a downregulation of AMPK and that activation of AMPK by metformin can abrogate the salutary effects of preconditioning, so it is possible that other substrates besides histone H2B dominate in mediating the effects of this adenine nucleotide sensor in the brain. Such work confers a more prodeath role for the sensor in the brain as opposed to the prosurvival bias seen in cancer cells.

Histone Demethylation via Jumonji Domain Proteins, Iron, 2-Oxoglutarate, and Oxygen-Dependent Dioxygenases

Modifications of N-terminal histone tails in histones by acetyltransferases and methyltransferases has been shown to affect chromatin structure as well as recruitment of complexes involved in transcriptional repression, activation or elongation [47]. Methylation at specific sites was once considered to be a stable modification responsible for heritable changes in gene expression not attributable to differences in the DNA code [17, 27]. However, studies over the past decade have shown that this histone modification is remarkably dynamic and underlies environment-induced changes in gene expression. One class of enzymes identified that mediate changes in histone methylation are the Jumonji domain proteins (see Table 1). These proteins are iron, 2-oxoglutarate, and oxygen-dependent dioxgyenases. The dependence of these proteins on 2-oxoglutarate (an intermediate in mitochondrial metabolism) and oxygen (which is reduced during ischemia) suggests that these proteins might be sensors for ischemia and comprise another class of potentially viable ischemic tolerance mediators [46].

Small molecule inhibitors of the jumonji domain histone demethylases such as desferoxamine (an iron chelator) and 2-oxoglutarate analogs (e.g., DMOG) have already been shown to reduce infarct size when given prior to a stroke, and only some of these effects can be attributed to their ability to modulate the stability of the adaptive transcription factor, HIF-1α [4, 11, 14, 50]. Thus, future studies should investigate whether jumonji containing demethylases are inhibited by hypoxia during ischemia, and the transcriptional consequences of modulating this function. The analysis might be complex, as HIF-1α,

which is stabilized in some regions of the brain following stroke [9], can regulate the expression of specific jumonji containing demethylases [18]. Therefore, the roles of jumonji domain containing demethylases, of which there are several, might critically depend on the time after stroke, the location of the stroke, and the predominant cell types affected [48].

Histone Modifications and Ischemic Tolerance via Zinc-and NAD-Dependent Histone Deacetylases

The HDAC family of proteins has been implicated most directly in ischemic tolerance via the studies of a number of groups, including those of Chuang [8], Langley and Ratan [19], Zukin [1], and Meisel and Andres [51]. Class I and II HDACs are Zn^{2+} dependent and share significant structural homology with one another [42]. Class III HDACs, known otherwise as Sirtuins, are NAD^{+} dependent, and these will be discussed below. Data from several groups suggests that nitric oxide or other changes in the redox balance could mediate inhibition of class I or II HDACs in ischemic preconditioning [37, 38] (see Table 1).

As mentioned previously, it has been reported that the ischemic-tolerant brain relies on a balance of gene expression, activation, and repression. And while some of the genes that are activated in the ischemic-tolerant brain may ultimately facilitate transcriptional repression themselves (please see above on polycomb group proteins), it is undisputed that durable, ischemic tolerance requires de novo transcription. Histone deacetylase inhibitors, or HDACi, may well contribute to this new transcription. Class I and II HDAC inhibitors all possess a cap, linker, and chelator moiety that allows them to inhibit selectively the zinc hydrolase domain of HDACs [5]. A host of these compounds have been developed and are in phases II or III human clinical trials for cancer. HDACi induce gene expression by favoring histone acetylation—a process which, through the addition of an acetyl moiety to a histone lysine residue, loosens the DNA wound around the histone core and subsequently leads to transcriptional activation [15].

The most common inhibitors of classes I and II HDACs, hydroxamates such as trichostatin A and short-chain fatty acids such as sodium butyrate, have been shown to ameliorate stroke in rodent models when delivered before the insult; such protection confirms their potential as a master switch of ischemic tolerance [19, 50]. The simple model is that these compounds enter multiple tissues inside and outside of the brain, enhance histone acetylation and transcriptional activity, and thereby confer resistance to ischemia, oxidative stress, DNA damage, and inflammation [2, 3, 49]. HDAC inhibitors have also been shown to prevent white matter damage and preserve ATP levels, a “holy grail” for ischemia treatment [2, 3]. A catalog of genes has been implicated in this protection, including gelsolin, HSP70, peroxiredoxin, and PGC1α, and it is likely that all contribute in one way or another to ischemic tolerance. An area of active investigation is whether a specific isoform of the zinc HDAC family dominates in ischemic preconditioning [56].

Exciting additional questions involve the role of HDAC modifications of cytoplasmic and mitochondrial proteins. It has been shown that HDAC 1 [16] and HDAC 4 [23] can shuttle between nucleus and cytoplasm under conditions of neuronal stress. Do ischemic-tolerant

neurons show a movement of HDAC1 into the axon or a movement of HDAC4 from the cytosol into the nucleus? These interesting questions have yet to be explored.

Sirtuins: NAD Sensors that Mediate Resistance to Ischemia

Class III HDACs, or sirtuins, have also been implicated in preconditioning. Studies of that implication, primarily those of the Perez-Pinzon lab, have been reviewed elegantly elsewhere. This model posits that ischemic preconditioning increases the NAD/NADH ratio and thereby leads to an increase in the activity of sirtuins, NAD dependent deacetylases. Sirtuins are named after Sir2, the first sirtuin identified in budding yeast, and include seven family members [28]. Their activity depends on NAD^+, a vital oxidizing agent of the glycolytic and Krebs Cycle, and are found in the nucleus, cytosol, and mitochondria [26]. Raval et al. showed that SIRT1 is activated in the ischemic preconditioned brain and that such activation is neuroprotective against oxygen/glucose deprivation (OGD)-induced cell death [35, 36]. Many targets activated by SIRT1 have been proposed that might lead to this protection. These include PGC1α and HIF-2α [10]. Thus, ischemic tolerance-induced changes in the Krebs Cycle or in anaerobic glycolysis (via lactate dehydrogenase) might manipulate levels of NAD^+ and might thereby facilitate deacetylation of nuclear proteins (e.g., HIF-2α, PGC1α) or mitochondrial proteins that switch metabolism away from glucose and toward fatty acids. Sirtuin 3 is also resident in the mitochondria and appears to be involved in lysine demalonylation, and lysine desuccinylation [13]. An obvious question is how inhibition of zinc dependent HDACs could be protective while activation of NAD+-dependent HDACs is protective. This important question has not been completely answered, though emerging evidence suggests that acetylation of some lysine residues promotes transcription, and that deacetylation of other distinct lysine residues also promotes transcription. Future studies will clarify how sirtuins- and zinc-dependent HDAC inhibitors mediate ischemic tolerance, and whether the dominant effect of these proteins is nuclear, cytosolic, mitochondrial, or all of these.

Do Global Changes in Post-Translational Modifications of Epigenetic and Other Proteins Mediate Epigenetic Reprogramming in the Ischemic Tolerant Brain?

As ischemic preconditioning possibly involves many proteins in the nucleus, cytoplasm, endoplasmic reticulum, and mitochondria working in concert to maintain viability, it is also possible that the master switch is not a single “toleroasome” but rather reflects adaptive activity changes in hundreds of proteins. A candidate master regulator of protein function via posttranslational modification in the ischemia resistant brain is sumoylation. There are three small ubiquitin-like modifier (SUMO) isoforms in humans: SUMO-1, SUMO-2, and SUMO-3. SUMOylation involves the binding of these proteins to other proteins and thereby regulates nuclear-cytosolic transport, transcriptional regulation, and stress response. Lee et al. investigated SUMOylation as a potential master switch of ischemic tolerance. The group observed that cultured cortical neurons preconditioned by sublethal levels of OGD had elevated levels of SUMO-1 conjugation in contrast to non-preconditioned cells [21, 22]. Moreover, cells in which the SUMO-1 and 2 genes were over-expressed showed increased

survival after OGD in vitro and in vivo, and depletion of endogenous SUMO-1 sensitized cells to OGD [21, 22]. These results implicate SUMO-1 conjugation as a component of ischemic tolerance.

It has been reported that SUMO-1 is involved in protein trafficking between the cytosol and the nucleus [57]. Does SUMO-1 mediate ischemic tolerance at the cytosolic, or the transcriptional level, or both? More broadly, the exact pathways governed by SUMOylation remain elusive. Does SUMO-1 conjugation activate a known protective mechanism or a novel one? What are the known SUMO substrates and are these proteins regulators of the ischemic tolerant genomic signature?

Conclusion

This review has focused on delineating the putative mechanisms by which changes in energy substrates and reactive nitrogen or oxygen species can be transduced via epigenetic proteins to mediate gene transcription changes associated with resistance to ischemia. Our discussion has not been exhaustive, as we have not discussed the role of long, non-coding RNAs, miRNAs [32], transglutaminase [58], or HATs as epigenetic mediators of tolerance [30]. However, we have sought to highlight the ways in which epigenetic proteins sense sublethal stress and thus act as “master switches” in mediating adaptive responses. The identities, functions, and regulatory frameworks of epigenetic proteins are becoming increasingly clear. Future studies will define whether a monolithic complex of proteins, which we term the “toleroasome,” exists, and how that complex might be manipulated therapeutically. Alternatively, many of the proteins described may work in parallel to mediate both cross-tolerance and remote preconditioning.

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Table 1

Ischemic tolerance targets, sensors, and proteins modified

Epigenetic and cytosolic protein effectors	Physiological state change sensed by the effector	Epigenetic modification
AMP Kinase	High levels of cellular AMP or altered AMP to ATP ratios	Phosphorylation of H2B-Ser^{36}
SCMHI and BMI1, part of the PRC1 polycomb complex	Unknown	Monoubiquitination of H2A and H2B
Jumonji domain histone demethylases	Lowered levels of oxygen, 2-oxoglutarate, vitamin C and cellular iron lead to alterations in histone methylation	Mono, di, and trimethylation of histone N terminal tails (histone H3, lysines 4, 9, 27 36; histone H4 lysine 20)
Zinc Dependent Histone (lysine) deacetylases (Class I and II HDACs)	Inhibited by nitric oxide and dowstream of glutathione depletion	Lysine acetylation on N-terminal tails of histones and transcription factors as well as a an array of cytosolic proteins (e.g., peroxiredoxin)
Sirtuins, NAD+ dependent histone (lysine) deacetylases located in the nucleus, cytoplasm and mitochondria (class III HDACs)	Altered NAD+/NADH ratio or consumption of NAD+	Lysine acetylation on N-terminal tails of histones and transcription factors. Lysine malonylation and succinylation in mitochondria