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Long-term window of ischemic tolerance: An evolutionarily conserved form of metabolic plasticity regulated by epigenetic modifications?

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ABSTRACT

In the absence of effective neuroprotective agents in the clinic, ischemic and pharmacological preconditioning are gaining increased interest in the field of cerebral ischemia. Our lab recently reported that resveratrol preconditioning affords tolerance against a focal cerebral ischemic insult in mice that can last for at least 14 days in vivo making it the longest window of ischemic tolerance discovered to date by a single administration of a pharmacological agent. The mechanism behind this novel extended window of ischemic tolerance remains elusive. In the below commentary we discuss potential mechanisms that could explain this novel extended window of ischemic tolerance in the context of previously identified windows and the known mechanisms behind them. We also draw parallels from the fields of hibernation and hypoxia-tolerance, which are chronic adaptations to severe conditions of hypoxia and ischemia known to be mediated by a form of metabolic depression. We also briefly discuss the importance of epigenetic modifications in maintaining this depressed state of metabolism.

Preconditioning as an approach to reduce brain injury

Cerebral ischemia occurs in various forms such as stroke, cardiac arrest, subarachnoid hemorrhage and several neuro- and cardiac-surgeries[1]. Despite decades of pre-clinical and clinical research, cerebral ischemic injuries remain one of the leading causes of death and adult disability[1]. Numerous neuroprotective agents have been tested in the clinic yet nearly all have failed to promote significant recovery[2]. To date, a single FDA-approved drug for stroke exists, the thrombolytic agent recombinant tissue plasminogen activator (rtPA), yet it is only administered to a small percentage of stroke patients[3,4]. The failure of translation in cerebral ischemia research has been attributed at least in part to the rapid and complex cascade of events that ensue in the ischemic brain[3,5]. To combat this lack of translation, recent efforts have been redirected toward prophylactic approaches as an alternative means to prepare the body against an ischemic injury prior to its onset[6].

The brain responds naturally to lethal ischemia by rallying a host of molecular and cellular defenses that are deeply rooted in an organism's genetic makeup [7]. Distinct from these mechanisms, the brain also responds to non-damaging ischemia, in anticipation of a lethal ischemic event to follow, by inducing evolutionarily conserved pathways that conjure some degree of protection[7] (Figure 1). Many have drawn parallels to the likes of Nietzsche who stated "that which does not kill us, makes us stronger"[8]. While this was simply observation, the first scientific documentation of this phenomenon with regards to ischemia came in 1986 when Murry et al. discovered that in the heart, brief cycles of ischemia prior to a prolonged ischemic event actually lessens myocardial infarction[9]. In the same year, Schurr et al. reported that when rat hippocampal slices were pre-exposed to a short anoxic episode, they recovered from a subsequent longer ischemic insult while control slices did not[10]. Kitagawa et al. also showed in 1991 that brief interruption of carotid blood flow 2 days prior to global ischemia prevented CA1 pyramidal cell death in the gerbil hippocampus. Here, they coined the term ischemic tolerance[11]. The application of brief, non-damaging ischemia as a therapeutic intervention is now known as ischemic preconditioning (IPC) and has been demonstrated across species and tissues[12,13]. IPC has been extensively studied in rodent animal models as well as in *in vitro* cell models[14,15]. Interestingly, it was shown that numerous stimuli other than non-injurious ischemia can precondition the brain into a state of ischemic tolerance. These stimuli include hypoxia, hyperoxia, hypothermia, hyperthermia, inflammation, neurotoxins and many pharmacological agents[6]. Additionally, preconditioning with one stimuli can promote tolerance against an injurious dose of another stimuli, a phenomenon known as crosstolerance[6]. A promising pharmacological preconditioning agent that has been extensively studied by our group and others is resveratrol[16-18], a natural polyphenol found on the skin of grapes, berries among other plants as well as in red wine[19]. Interestingly, resveratrol has not only shown promising results in models of cerebral ischemia, but it is currently in clinical trials for Alzheimer's disease and have shown very promising preclinical results in other neurodegenerative as well as cardiovascular disorders[19-21], which further underscores the importance of understanding its mechanism of action.

Windows of ischemic and pharmacological preconditioning

The ischemic tolerance mediated by preconditioning is observed within two transient windows[22] (Figure 2). The first window, which is known as the rapid or short-term, appears minutes after preconditioning and lasts for a few hours. This window is thought to be

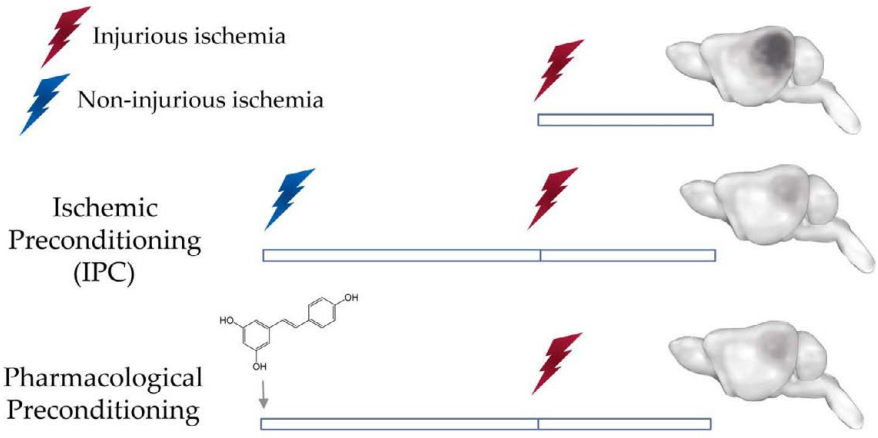

Figure 1: Preconditioning induces Ischemic Tolerance in the brain.
A non-injurious ischemic insult can protect the brain against a subsequent injurious ischemic insult. This phenomenon can be mimicked by the administration of some pharmacological agents such as resveratrol through a phenomenon known as pharmacological preconditioning.

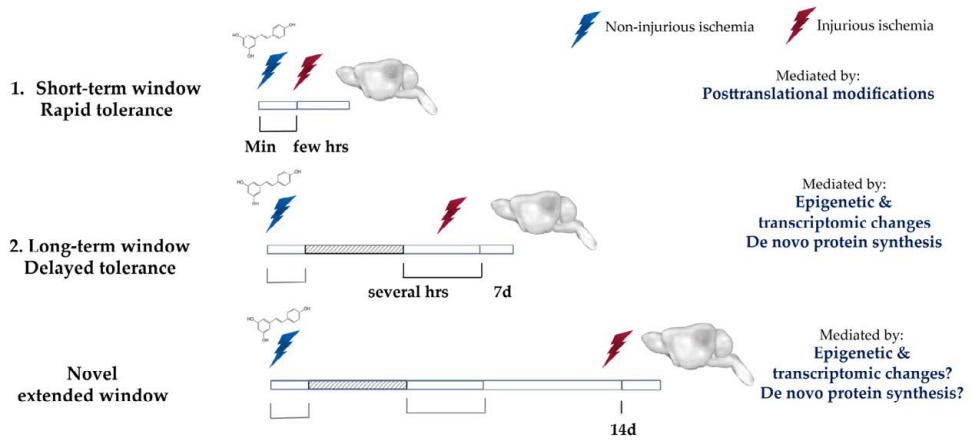

Figure 2: Ischemic Tolerance is observed within two transient time-windows.
The ischemic tolerance mediated by preconditioning is observed within two transient time-windows. The first window, known as the rapid or short-term, develops within minutes after the exposure and lasts for only a few hours after. This rapid protection is attributed to the transient post-translational modifications. The second window, known as the delayed or long-term, develops within days after the exposure but diminishes over the course of a week. The tolerance observed in the long-term window is attributed to epigenetic and transcriptomic modifications and de novo protein synthesis. Our laboratory has recently identified a novel extended window of ischemic tolerance that can last for at least 14 days in vivo after a single administration of resveratrol. Highlighted region represents a gap between the two windows where no protection has been observed. Min: minutes, hrs: hours, 7d: 7 days, 14d: 14 days.

mediated by posttranslational modifications to cellular components[6,9,23,24]. The second window, which is known as the delayed or long-term window, appears within a day after preconditioning and was thought to last for a maximum of 7 days after[6,11]. This window is known to be mediated by transcriptomic and epigenetic modifications as well as de novo protein synthesis[22,25]. Efforts have been made previously to extend the preconditioning window beyond 7 days. An interesting study revealed that repetitive hypoxic stimuli can actually extend the therapeutic window against cerebral ischemic for a remarkable period of 8 weeks in mice[26]. While repetitive hypoxia may lack translational value, IPC by way of remote limb preconditioning is a promising alternative for inducing ischemic tolerance[27]. Remote preconditioning is currently being clinically evaluated in the cardiac field[28-30]. Our lab has also made an effort to extend the preconditioning window. We previously showed that preconditioning with resveratrol (10 mg/kg), induces neuroprotection against middle cerebral artery occlusion (MCAo) in mice as well as against asphyxial cardiac arrest (ACA) in rats when administered two days prior to the insult[14,16]. Interestingly, we recently discovered that a single injection of resveratrol was sufficient to induce protection against an MCAO that lasts for at least 14 days *in vivo*, as shown by a reduction in infarct volume as well as an enhancement in the neurological score. This novel extended window of ischemic tolerance is currently the longest window of ischemic tolerance discovered to date by a single administration of pharmacological preconditioning[31]. Ongoing studies in our lab are aimed to determine the maximum time frame that a single resveratrol preconditioning (RPC) treatment can afford neuroprotection.

The fact that other studies in the field have not reported longer windows of ischemic tolerance via preconditioning could be explained by the nature of the preconditioning stimulus, its frequency of administration, and most notably its dose or intensity[32]. Higher doses of preconditioning agents do not necessarily increase the level of protection or its duration[16,33]. Studies in the field have reported an inverted U or J shape in response to preconditioning agents which highlights the importance of dose optimization in order to maximize adaptive responses and study mechanisms for potential clinical applications[32].

Genomic reprogramming in ischemic preconditioning

Knowing that resveratrol is an activator of the epigenetic enzyme SIRT1 (Sirtuin1), an NAD^+-dependent histone deacetylase[34], and taking into account the long-term therapeutic time-window observed, along with the extensive research supporting the importance of epigenetic modifications and transcriptomic changes in mediating the delayed window of ischemic tolerance[35-39], we surmise that this extended long-term window afforded by RPC is mediated by genomic reprogramming events. Multiple epigenetic marks have been studied in the brain after ischemic preconditioning including DNA methylation[40], histone acetylation[41], histone methylation[42] and histone phosphorylation[43], among others[35]. So far the most promising and consistent results came from targeting histone deacetylases (HDACs) [5,44,45]. Paradoxically, inhibting class I, IIa, IIb, and IV HDACs, which are zinc-dependent, showed promising results by decreasing infarct volume and enhancing behavioral outcome, while activating class III HDACs (sirtuins), which are NAD^+-dependent, showed the same result[35]. Additional support to these observations came from the study which showed that zinc-dependent HDACs inhibitors and sirtuin activators can synergistically

reduce the infarct volume as well as the neurological deficits[41]. Results from our lab also support a potential role for SIRT1 in mediating the observed tolerance within the long-term window of preconditioning. We have detected a significant upregulation in the protein levels of SIRT1 within the long-term window as well as increased binding to the promoter region of known mediators of preconditioning BDNF (brain-derived neurotrophic factors) and UCP2 (uncoupling protein 2) as revealed by chromatin immunoprecipitation[31]. Increased binding of SIRT1 to these promoters correlated with altered protein levels for BDNF and UCP2. Furthermore, the addition of sirtinol, a SIRT1 inhibitor, abolished the protection induced by RPC in organotypic hippocampal slices and in rodent animal models[16,18]. Ongoing studies in our lab will reveal more conclusive results about the importance of epigenetic modifications induced by SIRT1 in mediating this window of ischemic tolerance.

The first study to address the transcriptomic changes in the ischemic preconditioned brain was performed by Stenzel-Poore et al. in 2003. They reported a global downregulation in gene expression in the ischemic preconditioned brain mainly in genes involved in ion channel activity, metabolic processes, inflammation, as well as other energy-consuming pathways[37,46]. The study concluded that preconditioning reprograms the brain into a cellular state which resembles that seen during hibernation[37,46]. This cellular state of reduced metabolic activity is known to render the tissues and organs of hibernating animals resistant to hypoxic and hypoglycemic states experienced during the torpor phases of hibernation[47,48]. Subsequently, several other studies have aimed at identifying the transcriptomic changes in the ischemic preconditioned brain and have identified several common pathways which are misregulated after preconditioning including genes involved in excitotoxicity, apoptosis, inflammation, cytoskeleton remodeling, ribosome formation and ion channel activity, among others. Yet, an interesting observation common to many of these studies is a global downregulation of gene expression observed in the ischemic preconditioned and tolerant brain, reminiscent of the global downregulation in gene expression observed in hibernating and hypoxia-tolerant organisms[7,22,49].

Metabolic depression an adaptation against hypoxic and ischemic injuries

Metabolic depression, which can reach 1-20% of resting metabolic rate, is a conserved survival mechanism observed among many organisms across the animal kingdom. It is a common element observed among hibernation, torpor, estivation, anoxia, freeze tolerance, and diapause[47,48]. Animals enter this state when exposed to stressful environmental conditions such as extreme temperatures, oxygen limitation, food scarcity, or dehydration in order survive these unfavorable conditions over extended periods of time, ranging from weeks to months[47,48]. During the torpor phase of hibernation, the levels of oxygen consumption can be reduced to as low as 2 or 3% of basal rates, and heart rate reduced to an astonishing 3 to 10 beats per minutes[50]. Additionally, the rate of cerebral glucose consumption can reach 1-2% of active animals[51], and cerebral blood flow is reduced as low as 10% of euthermic state[52]. Still, these animals tolerate the severe states of hypoxia and hypoglycemia for prolonged periods of time and recover from them with no noticeable damage to their organs. Such physiological conditions when experienced by humans, such as in the case of stroke, result in detrimental consequences and irreversible damages[53]. Researchers have been studying the mechanisms behind metabolic depression for decades in the hope of identifying endogenous adaptive mechanisms that could be induced in humans for possible therapeutic applications[54]. It is believed that inducing a less extreme "hibernation-like" state will protect stroke patients against the ischemia-mediated tissue damage[54].

Hibernation falls on the extreme end of broad spectrum of phenotypes that involves metabolic depression as a form of energy conservation, yet other less extreme forms of metabolic plasticity are known to occur in other mammals. For example, slow-wave sleep which constitutes a milder form of metabolic depression is common to all mammals[55]. Thus metabolic plasticity exists among all mammals yet have different levels of flexibility[55]. The molecular basis of metabolic depression involves a controlled and coordinated suppression of ATP-consuming metabolic processes (such as transcription, translation, ATP-dependent ion pumps among others) as well as ATP generating processes so that a new homeostatic milieu is achieved that has a lower net rate of ATP turnover[56]. Additionally, the cells reprioritize the reduced ATP-availability to sustain vital cellular process such as increased antioxidant and chaperones to protect and maintain cellular components[57].

The last decade or so has experienced an increased appreciation of the role of epigenetic modifications in maintaining cellular homeostasis in response to a changing environment[58]. The epigenetic machinery allows the cells to couple various intra- and extra- cellular signals to changes in gene expression in order to maintain a stable intracellular milieu[58]. Since metabolic depression is marked by a global suppression in the transcriptional and translational rates of cells over prolonged periods of time[47,48], it is highly likely that these changes are maintained by reversible epigenetic modifications including DNA methylation, histone posttranslational modifications, and non-coding RNA such as microRNA, which are well known mechanisms that regulate transcriptional and translational

rates within a cell[58]. Support to this hypothesis came from recent studies performed on the skeletal muscle and brown adipose tissue of the hibernating thirteen-lined squirrels[59,60]. These studies showed an increase in the expression and activity of histone deacetylases along with a reduction in the histone acetyl marks, which are known marks of active transcription, during the torpor phase of hibernation compared to the euthermic state thus supporting the reduced transcriptional rates observed during hibernation[59,60]. Similar observations were reported after prolonged anoxia in the freshwater turtle *Trachemys scripta elegans* which is also known to use metabolic depression as a survival mechanism to endure hypoxic conditions[61,62].

Does preconditioning induce a depressed state of metabolism?

As mentioned previously the first study to assess the transcriptomic profile of preconditioned mouse brains reported a global suppression of gene expression after IPC specifically in genes involved in glucose metabolism, protein turnover, and ion channel abundance among others[37,46]. Consistent with their transcriptomic results, the authors also showed that preconditioning of cortical neuronal cultures reduces their whole-cell conductance as well as potassium-channel activity thus further supporting a depressed state of metabolic activtiy[37,46]. A subsequent study by Stapels et al. in 2010 showed that the transcriptional repressors known as the polycomb group proteins (PcG) are upregulated in the ischemic tolerant brain after IPC and are required to mediate the observed ischemic tolerance [49]. The authors showed that these PcG proteins associate with the promoter region of two potassium channels whose expression is reduced in the ischemic tolerant brain, and the knockdown or overexpression of these PcG protein in neuronal cultures increased or decreased the activity of voltage-gated potassium channels respectively[49]. The authors further show that the knockdown of these channels was sufficient to protect cells from ischemic injuries[49]. These studies support the role of metabolic depression regulated by epigenetic modifications as a mechanism behind ischemic tolerance seen after preconditioning. Additional support to the potential role of preconditioning in inducing a depressed state of metabolism comes from a study by Bracko et al. in 2014 who showed that ischemic tolerance seen in rats 3 days after preconditioning with 3-nitropropionic acid (NPA) correlates with a reduced state of metabolism in the brain as revealed by a significant reduction in the cerebral blood flow concomitant with a decrease in the brain's energy charge potential as revealed by the abundance of the adenosine tri-, di-and mono-phosphates[63]. Another study has also reported a similar finding after preconditioning with cortical spreading depression and have attributed the observed ischemic tolerance to a reduction in the rate of the metabolism[64]. Furthermore, a recent study by Cui et al. in 2015 showed that repetitive exposure to hypoxia alters the proteomic profile of mouse brains causing a downregulating in the expression of genes involved in ATP (adenosine triphosphate) synthesis and citric acid cycle while upregulating those linked to glycolysis, which is similar to the adaptation used by hypoxia-tolerant organisms during their states of metabolic depression[65]. Moreover, several studies have showed that preconditioning reduces the brain's energy expenditure by reducing its electrical activity as revealed by an alternation in the neurotransmitter signaling[61]. Preconditioned brains reduce their excitatory glutamate signaling while increasing the release of inhibitory neurotransmitters GABA and adenosine[61]. All these studies support a role for preconditioning in inducing a state of depressed metabolism in the brain which could explain the observed ischemic tolerance, whether resveratrol preconditioning also induces some form of metabolic plasticity in the brain within the long-term window of ischemic tolerance will be revealed by future studies in our lab.

Conclusion

The existence of a novel 14 day window of ischemic tolerance induced by resveratrol preconditoinng is reminescent of the long-term adaptations seen in nature in hiberanting and hypoxia-tolerant organisms against severe hypoxic and ischemic states. These adaptations might be evolutionary conserved at different levels across the animal kingdom and rely on a form of metabolic plasticity. Taking into account the importance of epigenetic machinery in mediating these adaptations, and in allowing a balanced homeostatic milieu in response to a continuously changing environment, we see the importance of understanding similar epigenetic changes in the context of ischemic tolerance and especially in the context of preconditoining. Understanding the molecular mechanisms behind this novel long-term window of ischemic tolerance might provide new insights into previously unexplored pathways and adaptations of ischemic tolerance.

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