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Genetic Animal Models Of Preconditioning

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Abstract

The preconditioning phenomena have been well established in heart as well as brain. In this review, we detail some of the original studies on preconditioning as well as studies from our lab using rodents and a genetic model system (fruit fly). We have used *Drosophila* in our lab to solve some of the questions related to tolerance or susceptibility to hypoxia. We believe that these pro-survival strategies and genetic pathways help us understand some of the preconditioning mechanisms that protect the brain from ischemia or ischemia.

Keywords

Preconditioning; genetic model; fruit flies; hypoxia tolerance

Introduction

Preconditioning refers to the notion when an organism becomes resistant or tolerant to a severe stress after being exposed to a milder form of that particular stress (Figure 1). The terms “preconditioning” and “tolerance” were first described by Janoff in 1964 where he referred to the phenomenon of stress-induced tolerance against traumatic or endotoxin insults.

The preconditioning phenomena have been well established in heart as well as brain. Brain's resistance to injury can be augmented by prior exposure to a preconditioning stimulus [1–4]. The preconditioning stimulus results in the initiation of a cascade of gene signals and endogenous adaptive mechanisms which result in the development of tolerance [5]. In addition, a number of stimuli, such as hypothermia, hyperthermia, pharmacological agents, low doses of endotoxin such as LPS and hypoxia, which can initiate preconditioning [6] with an ability leading to cross-tolerance since one stimulus can induce preconditioning for another. Understanding these overlapping mechanism(s) through model systems of preconditioning would help us identify the genes and proteins when designing future drug targets for prevention of cardiac or CNS injury (Figure 1).

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Conflict of Interest statement

The authors declare that they have no conflict of interest.

***In vivo* and *In vitro* models of Hypoxic/Anoxic Preconditioning**

As early as 1960's, a study by Dahl and Balfour showed that rats survived anoxia well after being exposed to a milder hypoxic stress [7, 8]. Since then, numerous *in vivo* and *in vitro* studies have demonstrated that neurons exposed to brief periods of sublethal anoxia or hypoxia develop resistance to subsequent stronger insults [9, 10, 4, 11–14]. A number of mammalian models such as mice, rat and pigs have been utilized and have shown protection from injury by preconditioning *in vivo* [15, 16]. The animal models of hypoxic preconditioning generally involved either whole-body exposure or single-organ exposure to hypoxic stress [4]. Depending on the organism, various levels of oxygen concentrations were used. For example, in murine models, generally the range is from 8% to 13% O₂ [17–19, 6]. These models measured neuroprotection as either early or late preconditioning [20]. It was commonly observed that ischemic protection was temporal. An early phase would last minutes or hours [21, 22] and is usually mediated by changes in metabolism or activation of existing proteins. The late one required hours or days to develop and provided longer protection. This phase may involve changes in transcriptional and translational metabolic pathways or changes in ion-channel activity [23]. These studies have also indicated that significant degrees of protection can occur in various parts of the brain, such as the hippocampal CA1 subfield and cortex.

We have also studied the fate of neuronal cellular activity after rats were exposed to hypoxia *in vivo*. We have found actually that exposure of rats to chronic hypoxia made neocortical neurons (NCX) *more susceptible* in subsequent acute hypoxic stress to injury as compared to naïve NCX [24]. In particular, a) the baseline membrane properties of cortical and hippocampal CA1 neurons were not significantly different from those of naïve neurons and b) long-term hypoxia renders neurons *more vulnerable* to hypoxic stress [25]. From these studies, we caution that “stress duration”, “severity” are critical parameters for preconditioning and that it is possible that an earlier stress does not lead to preconditioning.

In the fly model system, with long-term experimental selection for survival under low O₂ over many generations, we obtained a *Drosophila melanogaster* strain that can live perpetually in extremely low, normally lethal, O₂ condition (as low as 4% O₂) [26]. When we tested these adapted flies under 1% O₂ as well as anoxia, they survived much longer and recovered much faster than the wild type flies [26]. This observation suggested to us that a phenotype of preconditioning may take place also in the *Drosophila* and a deeper knowledge of the genetic interactions would facilitate our understanding of preconditioning.

***Drosophila* as a model to study hypoxia tolerance**

Our understanding of the mechanism(s) by which preconditioning protects the brain can be facilitated by studying hypoxia tolerant species. For example, we have discovered that the adult *Drosophila melanogaster* is tolerant to a low O₂ environment, withstanding ~3–4 hours of total O₂ deprivation or anoxia without showing any evidence of cell injury [27–30]. We could not distinguish, using light and electron-microscopy, between cells and cellular organelles in the central nervous system of flies that were exposed to anoxia for hours from normoxic control flies. This is clearly remarkable, especially that 3–4 hours of the life of a fly is much longer than this specific absolute time if one translates this in terms of “human” time! This model has served well not only in the discovery of the relation of such genes to diseases but also in the understanding of how such genes respond to physiological changes [31]. Indeed, in spite of many advances in monitoring oxygenation, there is still considerable morbidity and mortality arising from conditions with O₂ deprivation leading to hypoxic/ischemic damage, especially, in brain. Part of this failure is related to the complexity of the cascade of events that ensue after hypoxia. Hence, we have used *Drosophila* to solve some

of the questions related to tolerance or susceptibility to hypoxia. We believe that these pro-survival strategies and genetic pathways help us understand some of the preconditioning mechanisms that protect the brain from ischemia.

Currently, we study the molecular mechanism(s) of hypoxia tolerance in *Drosophila* under various paradigms – such as adaptation of flies to hypoxia over many generations, exposure of naïve flies to acute hypoxia (constant as well as intermittent), or exposure of naïve flies to chronic hypoxia. In one study where we exposed naïve flies to acute hypoxia, we focused on gene expression changes associated with severe (1% O₂) short term (2.5 hours) constant (CH-1% O₂) or intermittent hypoxia (IH-1%-21% O₂) in adult flies [32]. Our microarray analysis has identified multiple gene families that are up- or down-regulated in response to acute CH or IH. We observed distinct responses to IH and CH in terms of gene expression that varied in number of genes and also in the type of gene families. The microarray results showed that there were many fewer significantly altered genes following acute IH as compared to acute CH (about 1 to 10 ratio of genes). We discovered that there were several gene families that were over-represented in CH treated flies, such as those involved in the response to unfolded proteins, chitin, lipid, carboxylic acid, amino acid-metabolic processes, and immunity. Indeed, the heat shock protein family was the most up-regulated group in CH and this was exclusive to this treatment. In contrast, during IH, biological processes primarily involved in neurotransmitter transport and defense response were over-represented and these were exclusively altered in IH.

With the use of P-elements and EP lines, (A P element is a transposon that is present specifically in the fruit fly *Drosophila melanogaster* and is used widely for mutagenesis. These lines have single transposon insertions whose precise location in the affected gene is known) we further studied the role of candidate genes (that were up or down-regulated in arrays) in the phenotype of adult survival under either paradigm (1.5 O₂ % CH or IH) for longer periods (7–10 days) of exposure [32]. We observed significantly increased adult survival (as compared to controls) of P-element lines over-expressing Hsp70 and Hsp23 genes during CH (Figure 2). The survival rate was 55% for Hsp23 and ~70% for the Hsp70Aa and Hsp70Bbb P-elements as compared to control (31%) (P<0.05, Figure 2). In addition, when the Hsp70 P-elements were precisely excised, their increased survival was eliminated. Interestingly, when we used fly lines that had no copies of Hsp70 or even half of the copies for Hsp70Bb, Bbb, and Bc expressed, adult flies had markedly reduced survival and even lower survival than controls (Figure 2). This suggested that the increased transcript levels of these genes as observed in the array data after acute hypoxia play an important role under severe hypoxia. Furthermore, we proved that these genes play a role in hypoxia tolerance in specific tissues. For example, when we over-expressed Hsp70 specifically in fly brains the flies showed significantly higher survival as compared to control flies (Figure 3). This is intriguing since stress proteins are thought to be potential targets associated with cerebral preconditioning [33, 34]. For example, in gerbils and rats models a strong correlation has been found in the production of Hsp70 and degree of protection [21]. Similar to our study, in Gerbils it was observed that blocking Hsp70 leads to loss of tolerance [21, 35, 36]. It has also been shown that *Drosophila* nerve terminals treated with a prior heat shock (and higher than normal levels of Hsp70) were better able to regulate their pre-synaptic calcium concentrations at higher temperatures as well as protect synaptic transmission than those flies who expressed regular levels of Hsp70 [37]. It is important to realize that heat shock proteins have been shown to be one of the major effectors in the signaling cascades of preconditioning [20].

The genomic studies on hypoxia tolerant species as well as cerebral preconditioning also suggest striking similarities in the signaling pathways, genes as well as mechanism(s) of cerebral tolerance acquired under both scenarios [38]. The mechanism(s) involved in

preconditioning such as suppression of metabolism, cell cycle regulation, and ion channel activity, defense against oxidative stress appear to mimic adaptive neuro-protective strategies seen in hypoxia-tolerant species. In hypoxia selected flies (obtained over many generations), we also found suppression in the TCA cycle, lipid β -oxidation, and respiratory chain complex genes [26]. In other hypoxia tolerant animal models, there are similarities in the involvement and regulation of ion channels such as Delta opioid receptors and NMDA receptors as seen in preconditioning [39]. The inflammatory as well as stress response are also similar under both models [40]. Particularly, HSP70 upregulation results in multiple, beneficial actions during preconditioning including chaperonic function, maintaining mitochondrial physiology, inhibition of apoptosis, role in inflammatory response or reduction in oxidative stress [40]. We have shown in our previous study that over-expression of heat shock proteins in hemocytes in flies can lower ROS levels under hypoxia [41]. Hence, lowering ROS levels and injury may be one of the mechanism(s) of protection provided by heat shock proteins.

With respect to clinical application of preconditioning models in humans, the preconditioning studies in model systems as well as naturally adapted organisms will likely advance our knowledge of ischemic tolerance or insults. Studying the mechanism(s) of neuro-protective agents such as Heat shock proteins in model systems provide an opportunity to study the physiologic pathways involved in its protective effects. Such effector targets found in the genomics or proteomics studies can then be translated into drugs that can likely to be used for prophylactic treatments to minimize neurological injury associated with ischemic insults.

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Figure 1. Concept of Preconditioning

Understanding its gene targets and mechanism(s) and designing prophylactic drug targets.

Figure 2. Role of Heat shock Proteins in survival under 1.5 % O₂

a) The microarray results were clustered using GenMapp and viewed with Treeview. Upregulated genes are shown in red and downregulated genes are shown in green. Heat shock proteins specifically Hsp23, Hsp68 and Hsp70, are significantly over-expressed during acute constant hypoxia (CH1, CH2 and CH3) as compared to normoxia controls (NC1, NC2 and NC3) and intermittent hypoxia replicates (IH1, IH2 and IH3).

b) Functional testing of Hsp23 and Hsp70 genes during CH. Percent survival of adult flies after exposure to 1.5% O₂ CH for 7 days. CS and yw serve as controls. Hsp70 and Hsp23 P-element lines showed significantly higher survival than CS, yw controls (P<0.05). In contrast, P-element excision lines (Ex) and Hsp70- lines showed survival similar to or less than controls. Each bar represents the average of at least three tests for each line and error bars represent the standard errors. * with unpaired t-test P<0.05. (Azad P, Zhou D, Russo E, Haddad GG (2009). PLoS ONE 4(4): e5371. doi:10.1371/journal.pone.0005371).

Figure 3. Over-expression of Hsp70 in brain increases survival of flies under 1.5% O₂ constant hypoxia

The graph represents the percent survival of CS, yw (controls) and the F1 progeny obtained by crossing UAS-Hsp70Bbb with c736(Brain)-GAL4 driver. * with unpaired t-test $P < 0.05$ (Azad P, Zhou D, Russo E, Haddad GG (2009). PLoS ONE 4(4): e5371. doi:10.1371/journal.pone.0005371).