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Tolerance to Ischemia – an increasingly complex biology

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Abstract

In this review we identify and discuss some of the genomics studies of preconditioning and the ischemic tolerance phenomenon. Such studies have been attempted in multiple species, using different array technologies and with different preconditioning and tolerance models. In addition, studies are starting to reveal epigenetic mechanisms and modifiers of tolerance and preconditioning. Together these studies are starting to reveal some of the immense complexity of the ischemic tolerance phenomenon, yet further studies await to be performed.

INTRODUCTION

There have been many advances in our understanding of the pathology associated with ischemia in brain and other organs. However, we still do not have effective therapies to reduce such cell damage. Pharmacological approaches to reduce brain damage following ischemia and other neuropathological insults have been disappointing in clinical trials (O'Collins et al. 2006). Great promise was offered by glutamate receptor blockers, but in clinical trial these have been limited by their narrow therapeutic window, H⁺ gating of the receptor and other physiological consequences (Biegon et al. 2004).

It is therefore understandable that great emphasis has been placed on the phenomenon of preconditioning and ischemic tolerance, whereby a brief non-harmful stimulus results in the activation of endogenous protective mechanisms resulting in robust protection against a normally harmful exposure to ischemic stress. This conserved biological phenomenon of tolerance is observed across species and can occur at the level of an organism, organ or single cell. The protection observed following preconditioning is as potent as any neuroprotective agent tested to date. Genomic studies of preconditioning and tolerance have been reported by a number of groups. The first genomic study of tolerance was reported in 2003, and yet since this initial lead, ischemic tolerance genomics research has failed to provide a conclusive breakthrough. Most disappointingly, genomics studies have failed to further advance our understanding of tolerance as a biological concept. The field has not

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moved much beyond the three identified stages of the tolerance process as proposed in 2003 by Dirnagl, Hallenbeck, and Simon (Dirnagl et al. 2003).

Inducer

Cellular and molecular mechanisms which activate transducers of tolerance (e.g. receptor signaling cascades, protein kinase activation).

Transducer

Regulates changes in gene expression and protein synthesis (e.g. transcription factors, microRNA's).

Effector

The net effect of the preconditioning: a broad -based cellular change resulting in tolerance to harmful stresses (eg regulation of cell cycle, repression of inflammation).

This set of processes has focused research on the identification the key effectors or the key transducers of tolerance. Our scientific biases have resulted in this being a search for the key single effector protein molecule or gene. What has not been as useful from this model is a more global understanding of what the biological phenotype of tolerance would be. It was speculated in 2003 that ischemic tolerance involved the reprogramming of the brains response to ischemia, and the induction of a hibernation-like state (Stenzel-Poore et al. 2003). Although the reprogrammed response to stress in tolerant brain is supported by other studies, the hibernation biological phenomenon appears limited to ischemic tolerance, as tolerance to other stresses results in a different phenotype. For example, seizure tolerance is denoted by changes in patterns of genes associated with excitotoxicity and calcium handling (Jimenez-Mateos et al. 2008). While these programmatic/pathway phenotypes appear stimulus specific, a key conserved feature between these studies, show gene downregulation as a common tolerance phenotype. Common processes involved in the acquisition of tolerance, i.e. that process activated by preconditioning are yet to be identified.

Since we are starting to enter a clinical phase of preconditioning studies (Koch et al. 2011), we feel it is timely to address some of the advances in our understanding of the biology of tolerance as revealed by “omics” research. Upon review of previous studies it becomes apparent that this is only the beginning of our understanding of a biological process that is highly complex. As newer techniques and more powerful computational approaches become available, we can push preconditioning “omics” research to a leading role for translational discovery.

ISCHEMIC TOLERANCE DEFINITIONS

In order to be clear when discussing ischemic tolerance and preconditioning, we wish to define what we mean by each.

Preconditioning is a non-injurious stress applied to a cell/tissue/organism that activates a protective program that results in the cell/tissue/organisms being subsequently protected against a normally injurious dose of a stressor. Therefore, studies considering the molecular mechanisms of preconditioning should focus on genomic changes up to the point prior to the presentation of the injurious challenge. Understanding the key pathways of preconditioning will identify molecular targets for drug therapy to induce protections, which will have utility when ischemic events can be predicted or there is a higher risk of such an event, such as cardiac or vascular surgery (Barber et al. 2008).

Tolerance is the response of a preconditioned tissue to a harmful stress. Molecular studies of tolerance will identify how the normal response to a harmful stress is “reprogrammed” or changed by the preconditioning event, such that the cell/tissue/organism has less damage. Understanding the key pathways active in tolerance will identify molecular targets for therapy to reduce injury following a harmful ischemic event.

When considering the genomics of ischemic tolerance studies one must be careful to identify whether one is investigating the response to preconditioning, or the tolerance response following harmful ischemia in tissues that were subjected to preconditioning. As such the use of carefully chosen controls are critical, to determine whether an effect is due to preconditioning, or exposure of a preconditioned tissue to ischemia.

Some of the temporal definitions of brain ischemic tolerance can become confusing because of the comparison to studies of ischemic tolerance in heart. Typically, it has been reported that preconditioning evokes two time windows of protection. Most studies of ischemic tolerance have looked at delayed ischemic tolerance, which occurs 24–72 h following the preconditioning event. The first time window (30–60 min post preconditioning) is usually called rapid ischemic tolerance. Some studies have used the term classic or early preconditioning, however this is confusing because classic tolerance in heart is actually rapid tolerance in brain. In addition, the term rapid preconditioning would imply a shorter preconditioning event, when in fact the same stimulus for rapid and delayed tolerance is commonly used. For example, in our studies we have used a 30 min oxygen and glucose deprivation preconditioning event to induce ischemic tolerance both rapid, at 1 h following the preconditioning event, and delayed, 24 h following the preconditioning event (Meller et al. 2005; Meller et al. 2006a). One key difference between rapid ischemic tolerance and delayed ischemic tolerance is the requirement for new protein synthesis. Rapid ischemic tolerance is not blocked by the protein synthesis inhibitor cycloheximide, where as delayed ischemic tolerance is blocked by cycloheximide. Hence genomic studies have focused on gene expression changes that may mediate delayed ischemic tolerance. However molecular events initiated within the rapid ischemic tolerance time window may play a role in delayed ischemic tolerance.

Previous genomics studies of ischemic preconditioning-induced neuroprotection and ischemic tolerance

Multiple studies have been published that investigate gene expression in various models of preconditioning, including studies where ischemia, hypoxia and seizures were used as the preconditioning stimulus. One of the first studies was published in 2003 in the Lancet (Stenzel-Poore et al. 2003). The 2003 study used Affymetrix Mg_U74AV1 mouse genome chips. Gene expression levels in the ipsilateral cortex genes were compared to the contralateral (non-ischemic) cortex. Genes were considered differentially regulated if they had a 2.2 fold or greater change and a significant of $P < 0.05$ or lower (ANOVA with repeated measures post-hoc test). Interestingly, no common overlap of genes regulated by harmful ischemia and preconditioning ischemia, or genes commonly regulated by harmful ischemia and tolerant brain were observed (Stenzel-Poore et al. 2003). In ischemic tolerance the expression of high energy-utilizing cellular processes, such as potassium ion channels and transporters, were inhibited, leading the authors to hypothesize that ischemic tolerance evoked a hibernation like phenotype in brain to conserve energy. This study proposed that following preconditioning, a reprogramming event is central to ischemic tolerance such that the normal response to ischemia change (Stenzel-Poore et al. 2007). This study suggests that the re-programming event must be mediated by the genomic response to the preconditioning event, such that in response to normally harmful ischemia, a differential “pattern” of gene expression is activated, although the molecular mechanism of the reprogramming event or “switch” was not described.

Feng et al (2007) reported the genomic response to in ischemic tolerance in rats using a global ischemia model. As a control they also treated some animals with the NMDA antagonist MK801 prior to the preconditioning event to remove epi-phenomenon effects of gene expression (Bond et al. 1999; Chen et al. 2008), however, it should be noted that other studies suggest ischemic tolerance does not require NMDA receptor activation (Wrang and Diemer 2004). Hippocampi were isolated 1, 4 and 24h following the injurious ischemia event. RNA samples were pooled and hybridized to Affymetrix Rat Genome 230 2.0 microarrays. In this study RNA from 5 animals was pooled and hybridized to a single array. Statistical analysis of microarray data proceeded in three steps: (1) low-level analysis of raw data, (2) ranking of genes according to the four-way analysis of gene expression levels (between preconditioned and non-preconditioned animals), and (3) statistical analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene ontology (GO) terms represented by the sorted list of genes with a 1.25-fold change. Their analysis revealed regulation of MAP kinase signaling pathway, Toll receptor pathway, TGF-beta signaling pathways, and pathways associated with ribosome function and oxidative phosphorylation. Interestingly they reported a decrease in inflammation and enhanced expression of energy metabolism genes, which is opposite of the observation of Stenzel-Poore et al (Stenzel-Poore et al. 2003).

Preconditioning has been studied following hypoxic preconditioning in developing rat brain (Bernaudin et al. 2002; Gustavsson et al. 2007) and adult mice (Tang et al. 2006). The genomic response to hypoxia was first shown in a rat neonatal rat model using 8% O₂ for 3 hours, and recovered for 0, 6, 18 and 24 hours following the preconditioning event (Bernaudin et al. 2002). The group analyzed the RNA from 2 animals per group and RNA was used to create cDNA for hybridization to the Affymetrix rat U34A array, (an earlier and smaller chip than the 230A chip). Genes were deemed regulated if they showed a 1.5-fold change. In addition, only genes with present calls in both hypoxic and control samples with similar direction of regulation were considered. Gene regulation was analyzed using Statistical Analysis of Microarray (SAM) software and gene hierarchical clustering was performed using Genespring. This study reported few regulated genes. Compared to control, more genes show upregulation at 6 and 18 h post preconditioning, compared to control, and more downregulation at 6 h than that 24h. The study was focused on hypoxia and Hif1a-associated target genes, supporting a role of the hypoxia regulated transcription factor Hif1a in hypoxic tolerance, although see (Baranova et al. 2007).

In a second study (Gustavsson et al. 2007) the group sizes were expanded to include 5 animals at multiple time points (0, 2, 8, and 24 h), for a total of 40 arrays. The study also utilized the Affymetrix 230 2.0 array which contain more genes than the U34A chip. Data management, normalization, statistical analysis and gene ontology (GO) analysis were performed using the Web-based GeneSifter software (<http://www.genesifter.net>). Gene regulation was determined for genes that show a 1.2 fold change compared to control. Statistical analysis was performed using analysis of variance (ANOVA) (with 0 h set as control) with the Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons set at 0.05. There were 77 significantly regulated genes that GO ontology identified as being regulated in apoptosis, specifically related to the Bcl-2 family, JNK pathway, trophic factor pathways, inositol triphosphate (PI3) kinase/Akt pathway, extrinsic or intrinsic pathway, or the p53 pathway.

Gene expression in global ischemic tolerance was also investigated in mice (Tang et al. 2006). The preconditioning transcriptome from 2–3 animals per experimental condition were identified using Affymetrix U74A mouse arrays (MG_U74Av2). Genes were determined to be differentially regulated if they have a 1.5 fold changes compared to control. A strong HIF-1 α dependent pattern of gene expression was observed. The gene

expression profiles were investigated 6 hours following the hypoxia, rather than at the time point when tolerance is observed (24–72 hours post preconditioning). As such this study may actually reveal transduction pathways involved in the response to hypoxic preconditioning. Further gene ontology analysis suggests a role for cell communication, signal transduction, transcription and transport-related genes following hypoxic preconditioning in mice (Tang et al. 2006). Interestingly, most of the gene changes appear to occur immediately following hypoxia, rather than at the 24–72 hour time point when tolerance is observed.

Hirata et al (2007) reported the genomic response to hyperbaric oxygen-induced ischemic tolerance. One difficulty of comparing this study to other forms of preconditioning is that hyperbaric oxygen is administered for 1 h a day for 5 days, rather than being an acute event that promotes protection 1–3 days later. The genomic analysis was performed 6–72 h following the ischemic event (8 min forebrain global ischemia) and as such should be considered a tolerance study. Genomic analysis was performed on mRNA extracted from CA1 hippocampal cells using a Rat Oligo DNA microarray kit (Agilent). Two arrays were used per time point. Genes were called upregulated if they showed a significance of $P < 0.05$ using GeneSprints 6.2 (Welch's ANOVA with a P value cutoff of 0.05). The cut off for fold change was not mentioned. Statistical analysis of microarray data showed significant upregulation in 60 probe sets, and 7 specific genes (p75NTR, CD74, C/EBPdelta, Nrp1, Edg2, Trip10 and Igf2). The time course of these gene expression changes corresponded to hyperbaric oxygen-induced neuroprotection. Some of these gene changes were further validated by measuring protein levels by western blotting.

Gene expression changes in ischemic preconditioning have also been studied using an *in vitro* model of ischemia (oxygen and glucose deprivation: OGD)(Prasad et al. 2011; Prasad et al. 2012). Nowakowska's group used the Apoptosis and PI3K-AKT pathway finders PCR array (SA Biosciences)(Prasad et al. 2011) and then the whole rat genome G413 Agilent 60mer 4×44K microarray chip (Agilent)(Prasad et al. 2012). Data were transformed using Lowess method and statistical analysis performed using MANCOVA and FDR (Benjamini & Hochberg post-hoc test) was performed to correct for repeated measures. In addition to investigating the effect of preconditioning the group also investigated post conditioning, the post conditioning data will not be considered further. The cut off for fold changes was 1.5 and FDR was set at 0.05. Following preconditioning ischemia, the levels of 2620 genes were increased and 3008 genes were decreased 3h following re-oxygenation (reperfusion). Unlike *in vivo* studies, there was a high degree of overlap between harmful OGD and preconditioning OGD groups. The authors list the top 50 upregulated and downregulated genes. Further pathway analysis of regulated genes using ingenuity pathway analysis software, suggested that genes that map to cell cycle-regulation were upregulated and genes mapping to cAMP signaling were downregulated.

Benardete investigated the genomic response to oxygen glucose deprivation of rat hippocampal slices from adult animals (Benardete and Bergold 2009). RNA was isolated 3, 6 and 12 h following the preconditioning event. 2 µg of total RNA was obtained in triplicate or quadruplicate and between 6 and 9 slice cultures were needed to obtain 2 µg of total RNA (approx. 300 ng per slice culture). The microarray procedures were performed by Asuragen, Inc. (Austin, TX). Genes were identified on the Affymetrix rat 230 2.0 DNA microarray and analyzed using GeneSifter and Genespring software. Student's t-test was used to test for significant differences between gene expression levels under two different conditions. When gene expression levels were compared for three or more conditions, ANOVA was used. Since the number of samples at each time point was relatively small (3 or 4), the false discovery rate (FDR) correction was not used. In contrast when all the data sets were analyzed together, ANOVA was performed to determine differentially expressed genes and

a Bonferroni's post-hoc test was used. The authors used a 1.3 fold change as cut off for up- and downregulated gene expression. Differentially expressed genes were analyzed based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and gene ontology (GO) terms. Genes involved in signal transduction, transcription, and oxidative phosphorylation were reported as being differentially expressed at each time point. Their analysis suggests a role for TGF-beta, Wnt, MAPK, ErbB, Toll-like receptor, JAK-STAT, VEGF signaling pathways as playing a role in ischemic preconditioning.

Studies of lipopolysaccharide (LPS)-preconditioning induced ischemic tolerance, were recently published (Marsh et al. 2009a). This study investigated the genomic response to the preconditioning agent endotoxin (aka lipo-poly saccharide:LPS) at 3, 24 and 72h following LPS administration. Gene expression in the cortex was determined using Affymetrix MOE430 2.0 arrays. Genes were deemed regulated if a significant 2.0-fold regulation of genes was detected (2 way ANOVA between control and preconditioning followed by Benjamini and Hochberg correction to obtain false discovery rate (FDR)). These studies show a muted inflammatory signaling response via the toll receptor mediated activation of IRF3/TRIF. Following administration of LPS, 50% of regulated genes were involved with inflammation signaling, followed by metabolic processes and signal transduction. At the 72 hour time point following administration of LPS, when the harmful ischemia was performed, only 5 genes were differentially regulated. In subsequent analyses, there was no apparent overlap following comparison between the genomic response to ischemic preconditioning and endotoxin preconditioning (Stenzel-Poore et al. 2007). This study also investigated the genomic response to LPS treated mice following harmful focal ischemia (60 min MCAO). This reveals a change in Interferon 1 signaling in brain following stroke, suggesting a muted or reprogrammed inflammatory response in LPS-treated brain.

In addition to analyzing the genomic response to LPS preconditioning, Stenzel-Poore's group has also reported the genomic response to the Toll receptor agonist CpG (Marsh et al. 2009b). Mice were treated with CpG (20–40 µg;) and brains isolated 3 and 24 hours following 60 min MCAO in mice. Genes were hybridized to the MOE430 2.0 array and processed with Affymetrix GeneChip Operating Software. Probability values were adjusted for multiple comparisons using the Hochberg and Benjamini method. Significance was determined by $P < 0.05$ and a 1.5 fold change. This study focused on promoter regulation of genes following CpG administration. Promoter analysis of regulated genes using the TRANSFAC data base revealed a signature of type 1 INF response/(IRF, IRF8, ISRE, 3-hydroxy-3-methylglutaryl-1Y).

As an alternative approach to microarray studies, Valina Dawson's group used a functional cloning strategy to identify genes regulated by preconditioning ischemia, and tested their protective effects (Dai et al. 2010). A cDNA library was created from rat neuronal cultured subjected to 15 min preconditioning ischemia (OGD). The library with an insert size of 2.5 kB was inserted into a plasmid vector, and then expression was induced in fibroblasts. Cells were then tested for their resilience to menadione treatment (induced PARP dependent cell death). Cells were re-challenged with a second dose of menadione, and those clones exerting resistance were identified. Cells transfected with 95 of the isolated clones survived the two menadione challenges, and 31 independent genes were recovered for further analysis. Thirteen initially unidentified genes were designated as "neuroprotective gene" (NP1-13), the identity of which was only revealed later in their study. Twenty eight of the 31 cloned and identified genes inferred neuroprotection to cultured neurons. This study shows that precondition induced genes can confer neuroprotection to neurons, but the study acknowledged the bias of only cloning genes up to 2.5 kB insert size. Also it is unclear whether addition of multiple members of the genes identified would induce larger amounts of neuroprotection. It is however, interesting that many genes currently have an unknown

function. As a recent science editorial would suggest, there is still plenty of work to be done to characterize the function of these types of genes (Alberts 2012). This lack of knowledge certainly impairs our knowledge of what a global transcriptome response may be.

Two notable *in vivo* genomic studies of seizure-induced preconditioning have been reported in mice (Hatazaki et al. 2007) and rats (Borges et al. 2007). Animals received an intraperitoneal injection of a low dose of the seizure-inducing agent kainic acid resulting in benign preconditioning seizures. In the mouse study (Hatazaki et al. 2007), gene expression in the isolated CA3 sector was determined using Affymetrix 230 v 2.0 murine chips, a significant 1.8-fold regulation of genes was reported (t-test between control and preconditioning followed by Benjamini and Hochberg correction to obtain false discovery rate (FDR)).

In the Borges study micro dissected hippocampus was utilized (dentate gyrus, CA1 and CA3 sectors) (Borges et al. 2007). The RNA was amplified following its conversion to cDNA using a MegaScript T7 High Yield Transcription kit (Ambion). The subsequent libraries were hybridized to Affymetrix rat RAE230A chips at the NINDS NIMH Microarray Consortium at the Translational Genomics Institute in Phoenix, AZ. Multiple control and preconditioned samples were utilized (8 of each) and gene regulation differences of 25% were analyzed using eGOn software. The authors noted that in all three cell layers transcript levels from seizure animals were more often increased than decreased.

Both studies investigated gene changes in the selectively vulnerable CA3 region of the hippocampus (Borges et al. 2007; Hatazaki et al. 2007). Most of the differentially regulated genes affected tissue structure and cell signaling in rats (Borges et al. 2007), where as in mice transport and localization, ubiquitin metabolism, apoptosis and cell cycle control related genes were regulated (Hatazaki et al. 2007). Interestingly, in rats the CA1 (non-vulnerable region) was also investigated. This region showed an increase in energy metabolism related genes which may be expected during seizures (Borges et al. 2003). In an interesting comparison, the Borege study looked at genes upregulated following ischemic preconditioning (Stenzel-Poore et al. 2003), but only found 4 common genes (GFAP, calyculin, groucho-related gene 1 and DNA-directed RNA polymerase II).

The findings of an *in vitro* model of seizure-induced tolerance whereby kynurenic acid withdrawal-induced seizures are made non harmful by preconditioning seizures with the addition of MK801 (see (Meller et al. 2006b)), have also been briefly published. Gene expression was determined by genechip analysis using Affymetrix 230 v2.0 murine chips and data analyzed using GeneSifter Microarray data analysis software. As seen in other seizure preconditioning experiments, genes regulated by *in vitro* preconditioning seizures include metabolism, cell cycle and signal transduction-related genes (Simon et al. 2007).

Limitations of analysis of previous genomic studies to identify common mediators of preconditioning

There are considerable challenges with respect to meta-analysis of previous genomic studies on preconditioning. These studies were not designed to cross compare common mechanisms and as such use different animal strains (Sprague Dawley vs. spontaneous hypertensive rats) and species (mice vs. rats). Further, different microarray chips were used that have a different representation of genes (Affymetrix U34, RAE230A, rat230 2.0, mouse U74A, Mouse Genome 430 2.0 Genechip array, Agilent rat oligo DNA microarray Kit). The data were analyzed using different programs (MAS 4.0, MAS 5.0 RMAExpress 0.5, and Genesifter) and cut-off scores (1.4–2.2 fold changes). In addition, different controls were used between studies. Some studies use contralateral brain as a control (Stenzel-Poore et al. 2003), where as other studies use handled, unhandled or sham controls. Hence, determining

commonality between different preconditioning methods using such different methodologies and analysis methods is challenging.

Each preconditioning paradigm results in the regulation of genes unique to the biology of the preconditioning agent, as well as those representing a conserved response to preconditioning. In order to refine what is a critical or common mechanism of preconditioning, one must reduce from multiple observations the genomic profile of preconditioning. A study on its own attempting to identify a “common” mechanism is analogous to an $n=1$ study to identify a biological response. Each additional study identifying another genomic signature in a different system is another $n=1$ event. Therefore a common genomic signature of preconditioning is more likely to be revealed by simultaneous analysis of multiple models of preconditioning, rather than meta-analysis. This has yet to be done.

Are n numbers big enough?

One area of concern is whether genomic studies have sufficient power. Clearly the size of a study is usually confined by the level of funding available to the lab for such a study, i.e. how many samples can realistically be performed and analyzed for a given budget. Additional constraints may also come from IACUC (or non-US animal welfare committee equivalent) input on group sizes. Many reviewers want power analyses to be performed (requiring prelim data) to obtain a group size to show that sufficient power is available for a conclusion from the data.

First, for many genomic studies between 20–30K genes can be analyzed on a single microarray chip, where as sequencing technologies enables readout of the entire genome. It is typical in science to use a cut-off of $p<0.05$ as a level of significance, however this would mean accepting 1:20 of the genes represented on the array or in the genome (1–2K). A number of post-hoc analysis methods are available such a Benjamini Hochberg tests or False Detection Rate tests following an initial ANOVA to determine significantly regulated genes, however these tests require high initial significance levels for the corrected p value to be significant. Higher significance levels are best reached using higher n numbers. How many n depends on the significance that is to be accepted, and the fold change, or the size effect. It should be noted that genetic studies frequently enroll hundreds of patients for GWAS analyses.

Previous genomic studies of preconditioning and tolerance typically use a fold change of 1.5–2.2 for a gene to be determined as changed. The values utilized are not usually mathematically justified, and seem more depended to the authors having a manageable data set and sufficient a size effect for the given population size (n number) to obtain significance. However, as analysis tools become more adept at handling larger dataset, access to the original data files is necessary to relax such stringent cut off points and enable a more detailed analysis of the global biological response to preconditioning or tolerance. Scientists, tend to have a bias towards finding the gene with the largest fold change, rather than considering smaller changes to genes common to a given pathway which may have a more profound biological effect on a cell. As bioinformatics tools become capable of accepting and interrogating larger data sets, re-analysis of such data sets may reveal novel and previously overlooked biological processes being regulated in tolerance.

microRNA regulation following preconditioning ischemia

In addition to global mRNA expression levels, a number of studies have investigated microRNAs (miRNA) expression levels following preconditioning ischemia. MicroRNAs are short RNA sequences which are sequenced as 71 mers and then processed by Dicer into

21mer sequences. They associate with the argonaute family of proteins to regulate the silencing or translational arrest of genes. A number of studies were published around 2010 which investigated the role of miRNA following ischemic preconditioning. Lusardi et al investigated miRNA expression 24 h following preconditioning, harmful ischemia and tolerance (Lusardi et al. 2010). RNA was extracted using the mirVana protocol, and then hybridized to mirVana microarray chips (probeset V2: Ambion). They used their own analysis methodology which compared expression levels on the ipsilateral vs. contralateral cortex subjected to ischemia. They showed approximately 300 miRNAs regulated by preconditioning ischemia (200 decreased and 100 increase their levels). In contrast 100 were decreased following harmful ischemia and fewer were regulated in the ischemic tolerance group. Interestingly, the most unique robust response was to preconditioning ischemia. Target analysis using Miranda identified downregulated miRNAs as having a common protein/mRNA target of MeCP2, a transcriptional regulator that binds to methylated DNA (see later). They show that MeCP2 protein levels increase following ischemic preconditioning, and loss of MeCP2 using knockouts blocks ischemic tolerance.

Dharap and Vermaguti published a data set of preconditioning regulated miRNAs in rat (Dharap et al. 2009). The study follows the temporal profile of microRNAs at 6, 24 and 72 hours following the preconditioning event. They isolated miRNA using the mirVana isolation protocol, but analyzed expression levels using an LC Sciences rat miRNA chip, which contained probes to 256 miRNA sequences. Of these 59% were detected on their chip. In this study more miRNAs were upregulated vs downregulated. Further pathway analysis using KEGG ontology's of the targets to the miRNA (using the Miranda algorithm) suggest regulation of MAPK signaling and mTOR pathways, but these were not confirmed.

Lee et al reported the response of miRNAs to preconditioning and harmful ischemia 3 and 24h post ischemia in mice following MCAO (Lee et al. 2010b). Their data suggest a rapid (3h) increase in miRNA expression following preconditioning, followed by a decrease in expression at 24 h following preconditioning. In this study, miRNA was extracted using TRIZOL and hybridized to a mouse miRNA microarray 8 × 15K (Agilent). They focused their analysis on the 3h groups and found the largest increase in miRNA following ischemic preconditioning. They cloned the upregulated miRNA into Neuro2A cells and tested their neuroprotective potential. These microRNAs target PHD2 and the HIF1a pathway.

Met analysis of these studies is difficult because each study utilized different microarrays (with different species and numbers of miRNA probes). Additionally different analysis methodologies were utilized. Two studies suggested microRNAs are predominantly increased by preconditioning ischemia, and one showed an even spread of the expression data. There was some overlap of commonly regulated miRNAs, but some miRNAs show different directions of regulation in the different models.

DNA Methylation in tolerance

There is considerable interest in epigenetic regulation of gene expression following preconditioning events and in ischemic tolerance. Epigenetics refers to the modification in the expression of specific genes, but without a change in the sequence of the genomic DNA. Modifications in chromatin structuring regulate the accessibility of active transcription factors to their promoter regions. One such mechanism of epigenetic re-programming, DNA methylation is a powerful silencer of gene expression. Methylation of cytosine usually occurs in a CpG dinucleotide motif, although non CpG methylation has also been reported. Methylation of DNA is mediated by DNA methyltransferases (DNMT1 and DNMT3A/3B) and the methyl group is donated by S-adenosyl-methionine (SAM).

Data from experimental stroke models show global DNA methylation increases in the brain following harmful focal ischemia, as measured by [³H]-methyl incorporation into DNA (Endres et al. 2000). There was no change in the expression of DNMT1 or 3 following harmful ischemia. The chromatin sequences that are methylated by ischemia were not identified in this study (Endres et al. 2000). Heterozygous knockouts of DNA methyltransferase1 had reduced ischemia-induced infarction, an effect mimicked by blocking DNA methyltransferase with 5-aza-2'-deoxycytidine (AZA). Therefore reducing DNA methylation may be a protective event prior to harmful ischemia. However, in a subsequent study in 2001 Endres showed that only heterozygous DNMT1 knockouts were protected; an inducible homozygous DNMT1 knockout did not protect against ischemic injury (Endres et al. 2001). This would suggest that a reduction rather than a complete blockade of DNMT function is protective. The role of other DNMTs to compensate for DNMT1 loss of function is also not yet clear.

An increase in DNA methylation following harmful ischemia would be consistent with a recent study which showed that MECP2, a methyl CpG binding transcription repressor, was increased following brief preconditioning ischemia (see above). In addition, the DNA methylating agent methylazoxymethanol (MAM) blocks ischemic tolerance induced neuroprotection (Maysami et al. 2008). The effect of MAM in blocking ischemic tolerance was attributed to a blockade in progenitor cell proliferation, however, the ability of MAM to hypermethylate DNA such that preconditioning-induced changes in DNA methylation are blocked, cannot be ruled out. Hence, ischemia can affect DNA methyltransferase activity in brain, however it is unclear which genes are methylated in response to ischemia.

Following on from the Endres studies, a number of genes have been shown to be regulated by DNA methylation following ischemic or preconditioning events. NKCC1, a Na/K+ co-transporter expression has been shown to increase following ischemia, and this correlates to decreased methylation of the promoter (Lee et al. 2010a). Wilson and Westberry have described how the estrogen receptor is methylated following development of brain, but that this methylation is decreased following ischemia (Wilson and Westberry 2009). The decrease in methylation correlates with increased ER expression, and may account for the observation that estradiol is protective in ischemia models (Westberry et al. 2008). Interestingly, the decrease in methylation of the ER promoter only was shown in females and not males following ischemia (Westberry et al. 2008). Methylation of the promoter is followed by the binding of the transcriptional regulator MeCP2. Finally the trombospondin1 gene promoter has been shown to methylated following non-injurious ischemia, concurrent with a decrease in gene expression (mRNA) (Hu et al. 2006).

Even though DNA methylation has been described since 2000 in ischemia, the only high throughput study on DNA methylation following preconditioning/tolerance to date is a study by Miler-Delaney in a model of seizure tolerance (Miller-Delaney et al. 2012). Following preconditioning seizures, brain injury from an intra-amygdaloidal injection of kainic acid is reduced, specifically in the CA3 subfield of the hippocampus (Hatazaki et al. 2007; Jimenez-Mateos et al. 2008; Jimenez-Mateos et al. 2010). Similar to ischemic tolerance, a reduction in gene expression has been reported in seizure tolerant brain subsequently subjected to injurious seizures, suggesting gene repression mediates both seizure and ischemic tolerance (Jimenez-Mateos et al. 2008). In this study the authors investigated the methylation of a number of genes in control, harmful seizure and tolerance using CpG Island promoter plus arrays from Roche NimbleGen. These arrays represent 34 thousand promoters and CpG islands on a single chip, and utilize 2 color sample preparation to reduce variability. Interestingly 3400 positive hits in the control sample were recorded, which is in line with the genome being approximately 10% methylated.

DNA methylation patterns reveal a dynamic pattern whereby both an increase and decrease in methylation is observed, however the majority of genes show a decreased pattern of methylation (approx 50%) compared to control in both injury and tolerant brains. Of these methylation events over 60% correlated with genes, suggesting that the rest were CpG islands. The genes that were hypo-methylated showed a high degree of overlap, suggesting that both seizure treatments induce a similar degree of de-methylation on the same genes. Some genes were unique to each condition, how these selective genes are targeted for demethylation is not yet clear. Indeed it is not yet clear how genes are targeted for selective repression although the polycomb repressive complex 2 is involved in gene methylation ischemic tolerant brain (Stapels et al. 2010). It has recently been shown that some transcription factors can play a role in both repression and activation of gene expression depending on which cofactors they bind (Evans et al. 2007). Whether this is determined by the promoter region, interactions with co-factors or other signaling events is not yet clear.

The limited temporal profile of this study highlights the difficulty of mechanistically determining the role of an epigenetic event at one time point (8h following seizures) with the subsequent genomic (or proteomic) event at different time points (the original study profiled genes at 24h post seizure). The effect of preconditioning ischemia on DNA methylation and the role of DNA methylation changes in regulating ischemic tolerance are currently unknown and a more detailed profile of methylation events correlated with RNA expression data is needed.

A plan of action

Upon a review of the current literature, it becomes apparent that knowledge of the whole transcriptomic/miRNA/epigenetic response to a preconditioning event is in its infancy. While much has been learned we are still a long way off fully understanding the biology of this response. The following is a list of areas for potential further research.

New data sets from multiple sites using new technologies to obtain full data sets

One limitation of meta analysis of past preconditioning studies is the lack of methodological similarity between the studies. This is due to different species, strains etc. different methodologies and microarray chips, statistical analysis methods and cutoffs employed. Differences between different microarrays may affect which genes are detected. In addition, microarrays have the limitation of narrower dynamic windows for detection of RNA than newer sequencing or SAGE based approaches. In addition, sequencing technologies enable the identification of SNPs, isoform splicing and non-coding RNA. In a recent study it was shown that a single cell can contain multiple isoforms of the same gene (Tang et al. 2009). The higher sensitivity of the newer sequencing approaches may reveal the role of low abundance RNAs whose expression may account for nearly 50% of the transcriptome, as well as non-coding RNAs. It is interesting to note that for many of the published microarray studies, typically 4–10,000 genes were called positive, however sequencing reveals a larger number of genes. We recently sequenced neuronal cultures and found 14,000 genes identified when only using 1/16 of a SOLiD 5500 flowcell (unpublished data), sequencing at a greater depth of coverage will undoubtedly increase the number of identified genes.

A common criticism of *in vivo* stroke modeling of neuroprotectants is the variation between groups, hence multi-center examinations of preconditioning and tolerance transcriptomes may resolve this issue, whereby multiple groups collaborate together using one gene identification/quantification method. Such centers could also be utilized to compare different precondition paradigms. There can be two logical mechanisms by which multiple preconditioning stimuli work, either a common biological signature or disparate molecular programs. The consequence of each has implications for how we would translate

preconditioning into clinical use. If a common mechanism mediates all preconditioning stimuli, then tolerance could be induced clinically with the most benign preconditioning stimuli. If all preconditioning models activate different molecular mechanisms, then the addition of two preconditioning stimuli may result in additive and enhanced neuroprotection. An understanding of which molecular mechanisms are involved is essential and conserved mechanisms will direct scientists to identify novel translational targets to reduce brain injury following harmful ischemia. Therefore, understanding whether common mechanisms mediate preconditioning has significant consequences for therapeutic.

Temporal profiles

Fewer studies have focused on the temporal profile of genomic changes following preconditioning. One could speculate regarding the reasons for this (additional cost, complexity of analysis etc). Indeed our own experiences shows that investigating the time course of genomic events render a biological phenomenon complex (Stenzel-Poore et al. 2003). However, without a understanding of the temporal profiles of specific genomic events, it is hard to determine how we will obtain the full picture of preconditioning. Previous studies show temporal profiles of biochemical events following harmful ischemia that last minutes, hours or days (see (Dirnagl et al. 1999)). Preconditioning its self has two distinct temporal profiles of protection, rapid and delayed. Whilst considered as two distinct processes, it is clear that events occurring in the rapid phase of tolerance are likely to have consequences for delayed tolerance. For example multiple studies have identified immediate early genes as being activated following preconditioning ischemia (Sommer et al. 1995; Truettner et al. 2002; Rybnikova et al. 2009), and yet we expect their biological effects to be over within hours. Classic, protein synthesis dependent tolerance takes at least 24–48 hours to develop. Identification of downstream events may identify better targets for drug therapy to induce protection that may be missed by focusing on the time point when tolerance occurs.

Application of bioinformatics tools for understanding complex molecular signaling events

Following preconditioning ischemia, and in ischemic tolerance, it is becoming apparent that a single gene is not the key effector, but rather a complex interplay between multiple signaling cascades, cellular processes and epigenetic modulators. These are highly complex as for example, CREB binding protein and polycomb proteins, are both involved in tolerance and have 6000 and 1000 known chromatin binding sites respectively (Impey et al. 2004; Bracken et al. 2006). Promoter and pathway bioinformatics analysis are needed to understand the global mechanisms involved in the role of these pathways in regulating the genomic response to preconditioning. Non-biased approaches that do not favor one hypothesis, or single transcription factor/effector protein over another are essential. However, there is still hesitancy to appreciate these tools. This approach is becoming more common when dealing with large data sets, but still the relevance of KEGG ontology or “pathway enrichment” is not understood. Ultimately one limitation of these approaches is that they are not yet robust enough to stand alone, and all require further wet lab validation. However, as an example of the growing power of these bio-informatic tools, this approach identified the role of chromatin remodeling by polycomb proteins in ischemic tolerance (Stapels et al. 2010).

Clearly we are only at the beginning of our journey to understand the integration of genes, miRNAs and proteins and to understand how the protective effects of preconditioning result in tolerance. As new technologies reveal yet further layers of complexity we will require a renewed effort to unravel these processes. For example, we know that polycomb repressive complex 2 (PRC2) plays a role in marking chromatin, but how are these marks selectively targeted to specific genes? And what is the role of transcription factors in integrating

biochemical signaling cascades into gene expression and repression. A dual role of transcription factors has recently been revealed, in that KLF4 can both target genes for expression and repression via its interaction with different transcriptional machinery (Evans et al. 2007). How these biochemical cascades regulate and integrate the different genomic expression programs of tolerance is not yet clear, and whether each expression profile resulting in tolerance is temporally regulated. With regard to the temporal profile, we consider rapid ischemic tolerance and delayed ischemic tolerance as two entities. However as we understand more of these processes, it becomes apparent that events important or regulated within the one hour window of rapid ischemic tolerance may well have consequences for delayed tolerance.

The complexity of preconditioning is slowly being revealed. Given the expansion of clinical trials of preconditioning (Koch et al. 2011) it seems pertinent that more research be focused on obtaining a better understanding of how the molecular events regulating preconditioning-induced neuroprotection exert their effects. While considerable progress has been made in terms of understanding the genomic response to preconditioning events, we are still missing crucial information regarding temporal and cellular effects of preconditioning. As a therapeutic this may have implications for its translation, as only when we really understand the biology of preconditioning can we expect to be able to control it, and mimic it and harness it as a safe clinical modality.

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