Genomics of Preconditioning

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Abstract—Brief episodes of ischemia can protect against subsequent damaging ischemic events; however, the molecular mechanisms responsible for protection are poorly understood. Identifying genes involved in this process could provide insight into cell survival and treatment of stroke. We developed a murine model of ischemic preconditioning and subsequent stroke and used gene expression profiling to identify genes that may be involved in neuroprotective pathways. Middle cerebral artery occlusions were performed in mice for 15 minutes, (preconditioning), 60 minutes (stroke), or 15 minutes, followed 72 hours later with 60 minutes (preconditioning plus stroke) of middle cerebral artery occlusions. RNA from a region of cortex that is protected by ischemic preconditioning was hybridized to oligonucleotide microarrays. Follow-up experiments used patch clamp to examine cell conductance in cultured neurons exposed to oxygen–glucose deprivation. Stroke, ischemic preconditioning, and ischemic preconditioning plus stroke all induced gene changes that overlapped little among conditions. Stroke induced robust upregulation of gene expression whereas preconditioning followed by stroke resulted in a marked downregulation. Genes upregulated by stroke suggested activation of stress/inflammatory pathways and increased metabolism and ion channel function. Preconditioning tended to decrease genes involved in these pathways. Follow-up experiments show that preconditioning decreased voltage-gated potassium currents in vitro and increased bleeding time. Preconditioning reprograms the response to ischemic injury via transcriptional changes that may suppress metabolic pathways and immune responses, reduce ion channel activity, and decrease blood coagulation. These changes resemble evolutionarily conserved responses to decreased blood flow and oxygen availability that occur during hibernation. (Stroke. 2004;35[suppl I]:2683-2686.)

Key Words: gene expression m middle cerebral artery occlusion

Ischemic preconditioning is a phenomenon whereby brief exposure to ischemia provides robust protection against injury from subsequent prolonged ischemia. This has been demonstrated in animal models of stroke,1 and an analogous process has been suggested in humans wherein previous transient ischemic attacks are associated with improved clinical outcome and reduced infarction size from subsequent stroke.2–4 Understanding the cellular mechanisms by which ischemic preconditioning affords neuroprotection is essential to develop therapeutic treatment strategies.

Methods

All animal procedures were performed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility; protocols met National Institutes of Health guidelines with the approval of the Oregon Health & Science University Institutional Animal Care and Use Committee.

Mouse Model of Cerebral Ischemic Tolerance

Cerebral focal ischemia was induced by middle cerebral artery occlusion (MCAO) as published previously.5 C57BL/6J mice (male, age 8 to 10 weeks, n=8 to 10 per group per time point) received 15 minutes of occlusion (preconditioning alone), 60 minutes of occlusion (stroke alone), or 15 minutes of occlusion, followed 72 hours later with 60 minutes of occlusion (preconditioning plus stroke). At time of euthanization, mice were anesthetized and perfused with heparinized saline. A 1-mm coronal slice of the brain was removed (4 mm from rostral end, corresponding to approximately bregma of Franklin & Paxinos atlas6) for infarct area analysis by 2',3'-5'-triphosphonitrozolium chloride staining. From the remaining frontal 4 mm of each hemisphere, the dissected cortex was snap-frozen in liquid nitrogen. This cortical sample contains the region of the ipsilateral hemisphere consistently spared from ischemic injury by preconditioning.

RNA Isolation

Total RNA was isolated from individual cortices using Qiagen RNeasy (Qiagen Inc). RNA from 2 to 3 cortices was pooled to generate 3 paired samples (ipsilateral and contralateral) for each of the 5 experimental groups.

Microarray Analyses

Total RNA was labeled and hybridized to MG_U74Av1 GeneChip oligonucleotide array as described previously7 and in the Affymetrix GeneChip Expression Analysis Technical Manual. Data were processed using Affymetrix Microarray Suite 5.0 (MAS 5.0) with the appropriate masking of the inaccurate probe sets present on version 1 of this chip design. These data have been analyzed and reported previously using MAS 4.0.7 Although individual genes regulated differ between analyses, the overall pattern of gene changes and our conclusions remain the same.

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Microarray Statistical Analyses
Genes were considered differentially regulated if they met 2 criteria: statistically based call values were different (P<0.0025, 1-sided Wilcoxon signed rank test) for 6 of 9 comparisons and average fold change compared with contralateral hemisphere ≥1.7.

Validation of Microarray Analyses
Gene regulation was validated on a subset of genes by real-time polymerase chain reaction using β-actin as the housekeeping gene. Genes were quantified based on a standard curve included in all measurements. In 35 determinations (7 genes × 5 experimental conditions), we found 3% false-positive rate and 40% false-negative rate in gene selection. We verified corresponding protein regulation of a gene subset by Western blot. Protein levels for OPN, TNFRp55, HSP70, and GFAP were consistent with gene expression.

Gene Function Assignment
We determined putative gene function using Gene Ontology,8 the Stanford Online Universal Resource for Clones and EST web site (http://genome-www5.stanford.edu/cgi-bin/SMD/source/) and literature review. Approximately 30% of regulated genes had unknown function.

In Vitro Tolerance Model
Cortical neuronal cultures were prepared from 1- to 3-day-old Sprague–Dawley rat pups as described.7 We used oxygen–glucose deprivation (OGD) to model hypoxia–ischemia as described.7 Experimental conditions were: (1) preconditioning: 30-minute OGD, 24-hour recovery in MEM; (2) damaging OGD: 2-hour OGD, 24-hour recovery; (3) preconditioning plus damaging OGD: 30-minute OGD, 24-hour recovery followed by 2-hour OGD, 24-hour recovery; and (4) control: cultures maintained in MEM for 48 hours.

Electrophysiology and Bleeding Time
Whole-cell recordings on cultured rat cortical neurons were performed as described.7,9 At 1, 3, or 5 days after preconditioning or after sham, the last 3 mm of the tail was incised. Tails were immersed in saline (37°C), and the time until bleeding stopped was measured. A maximum bleeding time of 10 minutes was allowed.

Results
Ischemic Preconditioning Protects Against Subsequent Damaging MCAO
We developed a murine model of ischemic preconditioning using brief exposure to focal ischemia. In the absence of preconditioning, 60 minutes of MCAO causes extensive infarction of the ipsilateral cortex and striatum (data not shown). Ischemic preconditioning 72 hours before MCAO reduced infarct size by 72% (P<0.0001) (Figure 1) and confined damage primarily to the striatum.

Ischemic Preconditioning Alters Gene Expression Before and After Stroke
We analyzed cortical RNA from mice 3 or 24 hours after each condition (Figure 2a, small arrows). Of ∼7500 genes interrogated, 437 genes showed differential regulation versus contralateral in at least 1 condition (Figure 2b, 2c). Three hours after all ischemic events, nearly all regulated genes were increased (Figure 3); however, only 19 of the 117 regulated genes were shared among all conditions (Figure 2b). This indicates that gene changes do not represent a generalized stress response but are specific to the stimulus and state of the cell. Twenty-four hours after stroke alone, upregulation of genes predominates. In contrast, after preconditioning and preconditioning plus stroke, the majority of genes are downregulated (Figure 3). This is not a generalized phenomenon because housekeeping genes are not differentially regulated. These data suggest that preconditioning alters the transcriptional response to stroke via a specific pattern of gene suppression.

Preconditioning Reprograms the Response to Ischemia
 Pronounced differences in gene function emerged among conditions. The transcriptional response to stroke alone was dominated by upregulation of genes that coordinate immune and stress responses and those involved in metabolism, with genes involved in cellular transport and synaptic transmission also upregulated strongly (Figure 3). In contrast, after preconditioning and preconditioning plus stroke, genes involved in metabolism and transport/synaptic transmission were predominantly downregulated (Figure 3). These results suggest...
that preconditioning reprograms the subsequent response to ischemia, leading to dampened cellular activity.

**Preconditioning Induces Cellular Adaptations Seen in Hibernation and Hypoxia Tolerance**

The transcriptional response to preconditioning suggests suppression of cellular energy use and attenuation of ion channel activity. Similar changes occur when oxygen availability is limited (eg, hibernation, anaerobiosis, estivation). In these situations, controlled arrest of cellular functions preserves cellular homeostasis. Specifically, metabolic suppression leads to reduced glucose oxidation, reduced protein turnover, and channel arrest. Immunosuppression and hypocoagulation also are thought to contribute to neuroprotection during hibernation and hypoxia-tolerant states.

**Ischemic Preconditioning Decreases Potassium Ion Channel Function In Vitro**

ATP-driven pumps and ion channels represent a significant cellular energy sink. Several genes involved in channel function are downregulated in preconditioning: potassium voltage-gated channel Kv1.5, ionotropic glutamate receptors, adenosine A2a receptor, ATPase Na+/K+ transporting α1, and FXYD ion transport regulator 6. We speculated that energy-conserving adaptations, such as suppressed ion channel function, which exists in neurons from hypoxia-tolerant species, occur in mammalian neurons after preconditioning.

We tested ion channel function using an in vitro neuronal culture system of OGD to model ischemia/hypoxia (see Methods section). In our model, preconditioning attenuated cell death from prolonged hypoxia by 50% (data not shown). Preconditioning alone or followed by injurious OGD decreased whole-cell potassium current density and whole-cell conductance across membrane potentials from -40 mV to 80 mV (Figure 4a, 4b).

**Hypocoagulation Accompanies Ischemic Preconditioning In Vivo**

During hibernation, blood flow is reduced up to 90%. Typically, low blood flow increases the risk of thrombus formation; however, prolonged blood clotting time during hibernation minimizes this risk. The similarities in gene regulation and cellular responses between preconditioning and hibernation led us to speculate that preconditioning might confer other neuroprotective adaptations associated with hibernation such as hypocoagulation. We measured bleeding times and found that preconditioning prolonged bleeding times 5-fold compared with control (sham-operated mice...
were not different from control at any time point) (Table). In those animals in which bleeding continued past the 10-minute time interval, blood loss was stopped by external pressure; thus, median bleeding time of pre-conditioned mice is likely underestimated.

**Discussion**

Preconditioning genetically reprograms the response to ischemia and may underlie the basis of tolerance. The genetic profile of ischemic tolerance is characterized by dampened expression of numerous genes involved in metabolism, cell cycle regulation, and ion channel abundance. Moreover, our data indicate that during preconditioning, decreased transcription of genes involved in membrane transport has functional consequences, decreasing potassium currents and whole-cell conductance. Alterations in the density of ion transport molecules may modulate electrical gradients across the cell membrane to control excitability and consequently restrict ATP turnover. Such changes would be expected to confer neuroprotection during prolonged periods of ischemia.

Our finding that in the absence of ischemic injury preconditioning prolongs bleeding times suggests that this hypocoagulation mimics that seen in hibernation and may be neuroprotective. The mechanism of the prolonged clotting time induced by preconditioning is unclear, although it is likely that alterations in platelet aggregation are involved.

Collectively, these features mimic hibernation and hypoxia tolerance and suggest that conserved endogenous adaptations to oxygen limitation enhance survival. Nonhibernating animals may have retained such adaptations to improve survival in conditions in which brief periods of oxygen deprivation are likely to occur (eg, perinatal period). Understanding the molecular mechanisms involved in these pathways may yield therapeutic strategies for treatment of stroke.

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**Ischemic Preconditioning Increases Bleeding Time**

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<th></th>
<th>Control†</th>
<th>1 Day</th>
<th>3 Days</th>
<th>5 Days</th>
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<tr>
<td>Bleeding time*</td>
<td>1.67 (0.7–4.73)‡</td>
<td>10.0 (1.25–10.0)</td>
<td>8.0 (0.95–10)</td>
<td>5.99 (1.15–10)</td>
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*Median bleeding time in minutes (range).
†Sham-operated mice not different from control at any time point.
‡N=8–16 mice/group.