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Mediators of ischemic preconditioning identified by microarray analysis of rat spinal cord

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Abstract

Spinal ischemia is a frequent cause of paralysis. Here we explore the biological basis of ischemic preconditioning (IPC), the phenomenon in which a brief period of ischemia can confer protection against subsequent longer and normally injurious ischemia, to identify mediators of endogenous neuroprotection. Using microarrays, we examined gene expression changes induced by brief spinal ischemia using a rat balloon occlusion model. Among the nearly 5000 genes assayed, relatively few showed two-fold changes, and three groups stood out prominently. The first group codes for heat shock protein 70, which is induced selectively and robustly at 30 min after brief ischemia, with increases up to 100-fold. A second group encodes metallothioneins 1 and 2. These mRNAs are increased at 6 and 12 h after ischemia, up to 12-fold. The third group codes for a group of immediate–early genes not previously associated with spinal ischemia: B-cell translocation gene 2 (BTG2), the transcription factors early growth response 1 (egr-1) and nerve growth factor inducible B (NGFI-B), and a mitogen-activated protein kinase phosphatase, ptpn16, an important cell signaling regulator. These mRNAs peak at 30 min and return to baseline or are decreased 6 h after ischemia. Several other potentially protective genes cluster with these induced mRNAs, including small heat shock proteins, and many have not been previously associated with IPC. These results provide both putative mediators of IPC and molecular targets for testing preconditioning therapies.
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Keywords: Spinal cord; Ischemia; Preconditioning; Hsp70; Metallothioneins; Neuroprotection; Microarrays

Introduction

A common cause of spinal cord ischemia is cross-clamping of the aorta during repair of thoracoabdominal aneurisms. Clinical studies have demonstrated that the incidence of paraplegia in patients who underwent such procedures ranges between 0% and 40% depending on the series and patient risk factors (Cox et al., 1992; Verdant et al., 1995). In addition, several syndromes of vascular insufficiency have been described, including anterior spinal artery syndrome (Castro-Moure et al., 1997) and spinal cord compression (Ducker et al., 1984). Finally, spinal cord ischemia may participate in secondary cell death after traumatic injury (Mautes et al., 2000; Tator and Fehlings, 1991). Blood flow in the spinal cord falls dramatically after experimental injury, and posttraumatic ischemia correlates strongly with neurological loss (Tator and Fehlings, 1991). Taken together, these causes of spinal cord ischemia affect hundreds of thousands of people each year. The large number of people affected and the severity of their disability call for strategies to protect the spinal cord from ischemic damage.

Taira and Marsala (1996) previously described a simple, reproducible, and clinically relevant rat spinal ischemia model employing balloon occlusion of the descending aorta. Aortic occlusion causes a low blood flow state in the spinal cord. In this model, 10 min of spinal ischemia leads to development of spastic paraplegia and selective loss of interneurons in the intermediate zone of lumbosacral segments. Ischemic intervals shorter

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than 8 min have no long-term effect on motor function or histopathological appearance of the spinal cord. Here we use this model to investigate an endogenous form of neuroprotection called ischemic preconditioning (IPC). IPC describes the phenomenon that a short period of ischemia confers tolerance of a tissue to a subsequent longer period of ischemia. Rats were exposed either to brief ischemia or to sham surgery, various periods of normal perfusion (reflow), and then 10 min of ischemia. Locomotor scores were measured over the subsequent week. Three minutes of ischemia protects rats at an early time after ischemia (30 min reflow) and a late time (24 h reflow) but not an intermediate time (2 h reflow). Six minutes of ischemia provides protection only at 24 h of reflow, and this protection is greater than that afforded by 3 min IPC at the same reflow period (Cizkova et al., 2004).

The biphasic protection observed after 3 min of ischemia has been observed in other preconditioning models (Shohami et al., 1987). Although the early and late phases of ischemic tolerance share common mechanisms, they also have significant differences. One important distinction is that although early tolerance does not require production of new proteins, delayed preconditioning does (Barone et al., 1998; Matsuyama et al., 2000). In keeping with this, cardiac preconditioning was abolished by the addition of the transcription inhibitor Actinomycin D (Strohm et al., 2002). By employing microarrays to show changes associated with brief ischemia, we focused our investigation on the late phase of ischemic preconditioning, which likely depends on mRNA changes.

Others have used genomic tools to provide insight into neuronal ischemia in general (Jin et al., 2001; Majda et al., 2001; Soriano et al., 2000) and ischemic tolerance in particular (Bernaudin et al., 2002; Omata et al., 2002). Previously, we employed Affymetrix microarrays to characterize traumatic spinal cord injury (Carmel et al., 2001; Nesic et al., 2002). To better characterize the molecular mediators of spinal cord IPC, we used microarrays to survey mRNA changes after brief ischemia. In this study, we used spotted oligonucleotide-based microarrays to survey gene expression differences between the spinal cords of rats with or without ischemic preconditioning. Selected results were validated using Q-RT-PCR. Our goal was to identify possible mediators of the preconditioning effect that can be subjected to further testing to determine their role in IPC and their protective potential.

Materials and methods

All animal experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego.

Induction of spinal ischemia

Male Sprague–Dawley rats (300–365 g; Harlan Industries, Indianapolis, IN) were used. A previously described technique was used to induce spinal cord ischemia (Taira and Marsala, 1996). Animals were anesthetized with 3% halothane in an O_2/room air mixture (1:1) in an induction box and were maintained with 1–1.5% halothane delivered by an inhalation mask. A probe was inserted 6 cm into the rectum for core temperature measurements. Following anesthetic induction, a 2F Fogarty catheter was passed through the left femoral artery to the descending thoracic aorta so that the tip reached the level of the left subclavian artery. This level corresponds to a distance of 10.8–11.4 cm from the site of insertion. To measure distal blood pressure (DBP; i.e., below the level of occlusion), the tail artery was cannulated with a PE-50 catheter. The left carotid artery was cannulated with a 20-gauge polytetrafluoroethylene catheter for blood withdrawal. To induce spinal cord ischemia, the intra-aortic balloon catheter was inflated with 0.05 ml of saline. Proximal hypotension (40 mm Hg) was maintained by withdrawing blood (10.5–11 cm^3) from the carotid artery. The efficacy of occlusion was evidenced by an immediate and sustained drop in the DBP measured in the tail artery. After ischemia, the balloon was deflated, and the blood was reinfused over a period of 1 min. After blood reinfusion, 4 mg of protamine sulfate was administered subcutaneously. Stabilization of the arterial blood pressure was then monitored for additional 10 min after which arterial lines were removed and wounds closed. In control animals, the balloon catheter was placed into the descending thoracic aorta but was not inflated. At the end of the survival period, animals were terminally anesthetized with pentobarbital, cervical and lumbosacral spinal cord segments were removed by hydroextrusion, and samples were immediately frozen in −70°C pentane.

Microarrays

Design

The 4967 probes on our custom microarrays contain a collection of 4854 oligonucleotides specific for 4803 rat cDNA clusters purchased from Compugen, Inc. (Jamesburg NJ) and a set of 113 oligos designed and synthesized by MWG-Biotech AG (Ebersberg, Germany) on the basis of a set of GenBank accession numbers selected by us. The probes, 65–70 nt long, are standardized for melting temperature and minimal homology. All bioinformatics for the oligonucleotides are provided on our web site, http://www.ngelab.org.

Printing and processing

Microarrays were printed on poly-L-lysine-coated glass slides using an OmniGrid microarrayer (GeneMachines, San Carlos CA) and quill-type printing pins (Telechem, Sunny-

vale, CA). Oligonucleotides were resuspended to 40 μM in 3 × SSC and printed at 24°C with a relative humidity of approximately 50%. After printing, arrays were stored overnight and post-processed by standard procedures. Slides were stored at room temperature in a desiccator flushed with nitrogen and were used between 3 weeks and 3 months after printing.

RNA preparation

Frozen lumbar spinal cords were suspended in ice-cold Trizol (Invitrogen, Carlsbad CA) and homogenized with a tissue grinder. Chloroform was added to the Trizol homogenate and a phase extraction performed. A small volume (0.5 ml) of the resulting aqueous phase was adjusted to 35% ethanol and loaded onto an RNeasy column (Qiagen, Valencia CA). The column was washed and RNA eluted following the manufacturer's recommendations. RNA was quantified by spectroscopy, with A_{260}/A_{280} ratios at pH 8.0 between 1.9 and 2.1 for all samples. Samples of 6-min ischemia cord RNA and sham controls were subjected to capillary electrophoresis on an Agilent (Palo Alto CA) 2100 Bioanalyzer; all samples demonstrated sharp ribosomal RNA bands (not shown).

The same RNA preparations were used for both microarray and Q-RT-PCR assays, except that each control channel in the microarray assays represents a "reference" pool of the control RNAs (Yang and Speed, 2002). Initial studies used six sham control rats—half received sham surgery 30 min before sacrifice, the others 24 h before. Real-time PCR experiments showed no differences in gene expression between these two control groups for HSP70, metallothionein, GAPDH, and all other genes measured (not shown). Under the presumption that time-matching sham and control animals was unnecessary, equivalent amounts of RNA were pooled from all sham animals, and the same pooled reference RNA was used for all microarray experiments.

Hybridization target was prepared using the Genisphere 3DNA dendrimer system (Stears et al., 2000; Genisphere, Inc., Hatfield, PA). Total cellular RNA (2 μg) was reverse-transcribed from a "capture-sequence"-containing oligo-$d(T)_{18}$ primer using Superscript II (Invitrogen) and then alkaline hydrolyzed to destroy RNA. Automated hybridizations were performed using a Ventana Discovery System (Ventana Medical Systems, Tuscon AZ) following protocols designed by us. The sequence-tagged target was hybridized for 12 h at 58°C, and microarrays were washed twice in 2× SSC for 10 min at 55°C and once in 0.1 × SSC for 2 min at 42°C. Fluorescent dendrimer was then applied and incubated at 55°C for 2 h. The microarrays were washed with 2× SSC for 10 min at 55°C and then removed from the instrument and washed vigorously three times for 1 min each in Reaction Buffer (Ventana Medical Systems) and then once in 2×SSC for 1 min. Arrays were spin-dried in a centrifuge and scanned on an Axon GenePix 4000B (Axon Instruments, Union City CA).

Dye flip controls were performed in a separate experiment. A group of seven microarrays was hybridized with Ambion rat brain and liver control RNAs, with three arrays labeling liver RNA with Cy3 and four arrays labeling liver RNA with Cy5. We examined the pairwise Pearson correlation coefficients for the valid observations on each slide (approximately 4950) to determine data reproducibility. No difference was found among correlations within each dye group [0.931 ± 0.013 (3) and 0.963 ± 0.003 (4)] and correlations from one dye group to the other [0.925 ± 0.011 (12); each listed as mean ± SEM (n)]. Therefore, we concluded that the inclusion of dye flips within our experimental design was not necessary under these conditions.

Data analysis

Image files were processed using the Axon GenePix 4.0 software, resulting in text files containing median fluorescence intensities, median local backgrounds, and flags of the few spots with overlaid background. Results were imported to the public microarray database BASE (Saal et al., 2002). Normalization and data analysis were conducted in GeneSpring (Silicon Genetics, Redwood City CA) using a custom ODBC interface to the BASE database (DeLong and Hart, unpublished). We used the Lowess method of normalization (Yang et al., 2002). The ratio of signal intensities was calculated only if the spot was not flagged, and replicates were averaged. All microarray data are available on a public web site (http://base.rutgers.edu).

Statistical analysis

To better elucidate groups of changed genes, we employed k-means clustering, beginning with a filtered group of genes (see text). We employed the GeneSpring Standard Correlation as our similarity metric. The reproducible difference between mean ratios and a ratio of unity (expressed as the t test P value) was assessed using GeneSpring's Global Error Model of replicates, an implementation of the Rocke–Lorenzato model.

Quantitative real-time PCR

We confirmed selected microarray results by comparison with relative mRNA levels obtained by quantitative reverse transcription PCR (Q-RT-PCR) using gene-specific primer pairs (Table 1). RNA was reverse-transcribed with Superscript II (Invitrogen, Carlsbad CA) and random primers as suggested by the manufacturer. The PCR reactions were carried out using 20 ng of cDNA, 67 nM of each primer, and SYBR Green master mix (Applied Biosystems, Foster City CA) in 10 μl reactions. Levels of Q-RT-PCR product were measured using SYBR Green fluorescence (Ririe et al., 1997; Wittwer et al., 1997) collected during real-time PCR on an Applied Biosystems 7900HT system. A control cDNA dilution series was created for each gene to establish a standard curve. Each reaction was subjected to melting

Table 1
Oligonucleotide primers for Q-RT-PCR

Gene	Description	GenBank	Forward	Reverse
ATF3	activation transcription factor 3	NM_012912	CGCCATCCAGAACAAGCAC	GACTCCAGCGCAGAGGACAT
Cryab	alpha B crystallin	NM_012935	CGAACATGGCTTCATCTCCA	GCTGGGATCCGGTACTTCCT
Egr-1	early growth response 1	NM_012551	CAGCAGCGCTTTCAATCCTC	TGCTCGTAGGGTGM2TTCGC
Fos	fos	X06769	CTTCTCAATGACCCTGAGCCC	AATGTTCTGM2ACCGGCTCCA
GlyRA1	glycine receptor A1	NM_013133	GATGCCAGGATCAGACCCAA	GCAACTCACGTTCACAGGAGG
HemeOX	heme oxygenase	NM_012580	AGAGCGAAACAAGCAGAACCC	TCCTCAGGGAAGTAGAGCGG
Hsp27	heat shock protein 27	NM_031970	TGCCCAAAGCAGTCACACAA	CGAAAGTGACCGGAATGGTG
Hsp70.1	heat shock protein 70.1	NM_031971	TTCAATATGAAGAGCGCCGTG	GCTGATCTGM2CCCTGM2AGACC
Hsp70.2	heat shock protein 70.2	Z75029	AGAGGCTCTTTCTGGCGCTC	GGCCACCCATCTGTCTCCTAG
Nfkbia	NF kappa B inhibitor, alpha	X63594	AGGCACTTCTGAAAGCTGGC	TTCCTCGAAAGTCTCGGAGC
MPZ	myelin protein zero	NM_017027	CCCCAGTAGAACCAGCCTCA	TCCAGGCCCATCATGTTCTT
MRG-3	memory-related gene	U95149	ACATGACAGGACAGCATGGC	CATGM2GTCTGCACACCTCTTTTT
MT-1	Metallothionein 1	J00750	CTGCTCCAAATGTGCCCAG	CACTGM2TCCGAGGCACCTTT
MT-2	Metallothionein 2	M11794	TGCAAGAAAAGCTGCTGTTCC	GGAGCACTTCGCACAGCC
MT-3	Metallothionein 3	NM_053968	TGAGACCTGCCCCTGTCCTA	CATTGM2TCCGAGCAGGTGC
Oazi	ornithine decarboxylase antizyme inhibitor	NM_022585	AATTCCGCCGAAAAAGAGAAG	GATAACGGCCCAAAGAGTCG
Syn7	synaptotagmin 7	NM_021659	TCTGTCTCGGACCTCGTCAAC	GGAGAGCATGAGCATCTCGC
Ttpa	tocopherol transfer protein alpha	NM_013048	GCGTTATTCCCATGACCCG	CCAAAGACTTGM2GTTTCCCG
UVB	ultraviolet B radiation-activated gene	U12526	GGACAACTGAGTAGGACTTCGGG	TAGCGGGCCTTAGAGGTGAC

Primers were designed using PrimerExpress software (Applied Biosystems, Inc.) from the same GenBank accession records as the microarray probes except for genes that had a NCBI reference sequence for the same UniGene cluster (Cryab, Oazi, and Syt7). Gene descriptions are from NCBI.

point analysis to confirm single amplified products. Reactions were run in duplicate, and results were averaged. Each value was normalized to GAPDH to control for variations in the amount of input cDNA. Fold-change values represent a mean of four ischemic samples divided by the mean of the six sham controls described above. Change between ischemic and sham animals was determined significant by Student's *t* test using a *P* value of less than 0.05.

Northern blot

A separate group of rats was used for Northern blot experiments. Animals were subjected to 6 min of spinal cord ischemia as described above. At the end of the survival period (4, 18, or 24 h), rats were sacrificed and their spinal cords collected. Cervical segments of the spinal cord (C2–C5; non-ischemic) were pooled (three segments per extraction) as were the lumbar segments (L2–L5; ischemic). Total RNA was extracted using guanidine thiocyanate extraction buffer (Puissant and Houdebine, 1990). RNA was separated by electrophoresis in 1% agarose–formaldehyde gels, transferred onto nitrocellulose membranes, and hybridized with a 3′-end labeled hsp70 oligonucleotide probe ($2–5 \times 10^6$ cpm/ml buffer) at 42°C overnight. The blot was washed to high stringency and autoradiographed with two intensifying screens (DuPont, Wilmington DE) at −70°C for 1–14 days. Each blot was stripped and rehybridized with a control 18S oligonucleotide rRNA probe. Bands were quantified with an MCID analysis system (Imaging Research Inc., St. Catharines, ON, Canada) and results were expressed in arbitrary optical density units. Values were corrected for loading and expressed as fold change over sham-operated controls.

Results

To survey mRNA changes induced by brief ischemia, we applied ischemic and sham control samples to oligonucleotide microarrays. Animals (n = 4 at each time point) were exposed to either 3 or 6 min of spinal cord ischemia and were allowed to survive for various times: 30 min, 6 h, 12 h, or 24 h RNA from individual ischemic cords was compared to reference-pooled RNA from sham controls. Results were expressed as a fold change ratio of net hybridization signal for ischemic samples to sham controls.

The results of the 3- and 6-min ischemia microarray experiments are graphed in Figs. 1A and B, respectively. The relative ischemia/sham ratio is plotted on the y-axis against the time of reflow after brief ischemia on the x-axis. Each plotted line represents the result from a single oligonucleotide probe on the microarray. Of the probes assayed, few showed large changes, and two groups stood out prominently. In the 3-min ischemia experiment, three mRNAs were found to be strongly increased over sham controls at 30 min of reflow (Fig. 1A). All three of the probes showing strong differences at this time point are specific for the same protein, HSP70. Two probes (hsp70 and hsp70.1) detect the same mRNA; another probe (hsp70.2) hybridizes with an HSP70-encoding mRNA having a different 3′ untranslated region (Table 2). For animals that received 3 min of ischemia, hsp70 mRNA was increased maximally at 30 min of reflow, had smaller increases at 6 and 12 h, and returned to sham baseline levels by 24 h. The three hsp70 probes also showed the largest ratios following 6 min of ischemia (Fig. 1B). Six-minute preconditioned spinal cords showed even greater ratios of hsp70 at 30 min than the 3-min ischemia–30-min

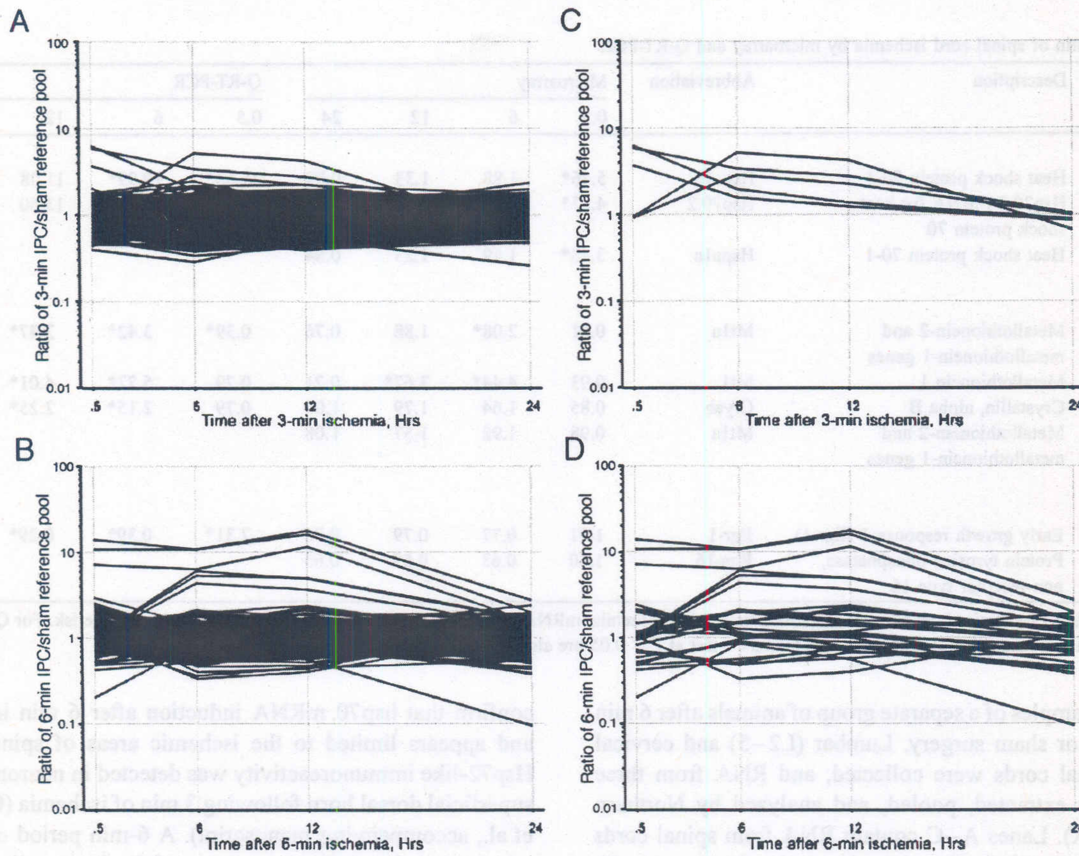


Fig. 1. Graph of microarray results comparing brief ischemic and sham controls. Animals received either brief ischemia (3 or 6 min) or sham operation. The animals were allowed to survive for various times and were sacrificed. RNA was prepared from lumbar spinal cords, reverse transcribed, hybridized to oligonucleotide microarrays, and detected using fluorescent probes. Arrays were scanned, and replicate results ($n = 4$) averaged. Lowess normalized ratios (Normalized Intensity; y-axis) of ischemia-treated fluorescence hybridization signal to sham control signal are depicted at various times after ischemia (Time; x-axis). Each line represents the ratio of ischemic–sham hybridization for a single probe over time. All microarray results have been posted to a public web site (http://base.rutgers.edu). (A) 3-min ischemia results. (B) 6-min ischemia results. Results were filtered using three criteria: fold-change greater than two, signal intensity approximately two-fold above background, and t test with a P value less than 0.05. (C) Filtered genes from 3-min ischemia experiment ($n = 5$). (D) Filtered genes from 6-min ischemia experiment ($n = 31$).

reflow group, and levels continued to rise at 6 and 12 h before declining at 24 h.

We measured the same RNA preparations by Q-RT-PCR to confirm microarray results (Tables 3 and 4 and Figs. 2B and 3B). Hsp70 mRNA changes were far greater by Q-RT-PCR than by microarray, consistent with the superior dynamic range of Q-RT-PCR. In 3-min ischemia animals, hsp70.1 and hsp70.2 mRNA levels rose to approximately 25 times the levels of sham-operated controls at 30 min after preconditioning (Fig. 2B and Table 3). These levels fell over the course of 1 day and were not significantly different from sham by 24 h. After 6 min of ischemia, spinal cords showed robust induction of hsp70 at 30 min with further rises at 6 and 12 h to a peak of approximately 100-fold change from sham animals (Fig. 3B and Table 4). As with the microarray results, 6 min of ischemia produced more intense and prolonged increases in hsp70 mRNA than did 3 min of ischemia.

To test whether increased hsp70 mRNA was a local response to ischemia or whether it might be elevated elsewhere in the spinal cord, a Northern blot was performed on

Table 2
Relationship of hsp70 microarray probes, Q-RT-PCR primers, and sequence identity

Probe	GenBank accession	Unigene cluster	Q-RT-PCR forward primer	Q-RT-PCR reverse primer
Heat shock protein 70	L16764	Rn.1950	5′-TTCAATATGAAGAGCGCCGTG-3′	5′-GCTGATCTGM2CCCTGM2AGACC-3′
Heat shock 70 kD protein 1A	NM_031971			
Hsp70.2 mRNA for heat shock protein 70	Z75029	Rn.81083	5′-AGAGGCTCTTTCTGGCGCTC-3′	5′-GGCCACCCATCTGTCTCCTAG-3′

Microarrays contain three probes for the same protein, HSP70. Q-RT-PCR primers were designed for one member of each Unigene cluster.

Table 3
Results after 3 min of spinal cord ischemia by microarray and Q-RT-PCR

GenBank	Description	Abbreviation	Microarray				Q-RT-PCR			
			0.5	6	12	24	0.5	6	12	24
Cluster 1										
L16764	Heat shock protein 70-1	Hspa1a	5.46*	1.88	1.33	0.89	26.68*	10.99*	11.08	0.84
Z75029	Hsp70.2 mRNA for heat shock protein 70	Hsp70.2	4.87*	3.05*	2.50*	1.01	26.34*	11.75*	13.60	0.91
NM_031971	Heat shock protein 70-1	Hspa1a	3.43*	1.49	1.23	0.84				
Cluster 2										
M11794	Metallothionein-2 and metallothionein-1 genes	Mt1a	0.81	2.08*	1.88	0.76	0.39*	3.42*	2.47*	1.11
J00750	Metallothionein 1	Mt1	0.93	4.44*	3.67*	0.71	0.79	5.72*	4.01*	1.20
M55534	Crystallin, alpha B	Cryab	0.85	1.64	1.79	1.04	0.79	2.15*	2.25*	1.12
M11794	Metallothionein-2 and metallothionein-1 genes	Mt1a	0.98	1.92	1.57	1.08				
Cluster 3										
NM_012551	Early growth response 1 (Egr-1)	Egr-1	1.91	0.77	0.79	0.76	2.31*	0.39*	0.29*	0.32*
U02553	Protein tyrosine phosphatase, non-receptor type 16	Ptpn16	1.60	0.63	0.59	0.65				

Listed is the ratio of ischemic/sham animals at various times after ischemia. mRNAs altered by 2-fold or greater are marked with an asterisk. For Q-RT-PCR results, genes that are significantly different by Student's t test at $P < 0.05$ are also marked with an asterisk.

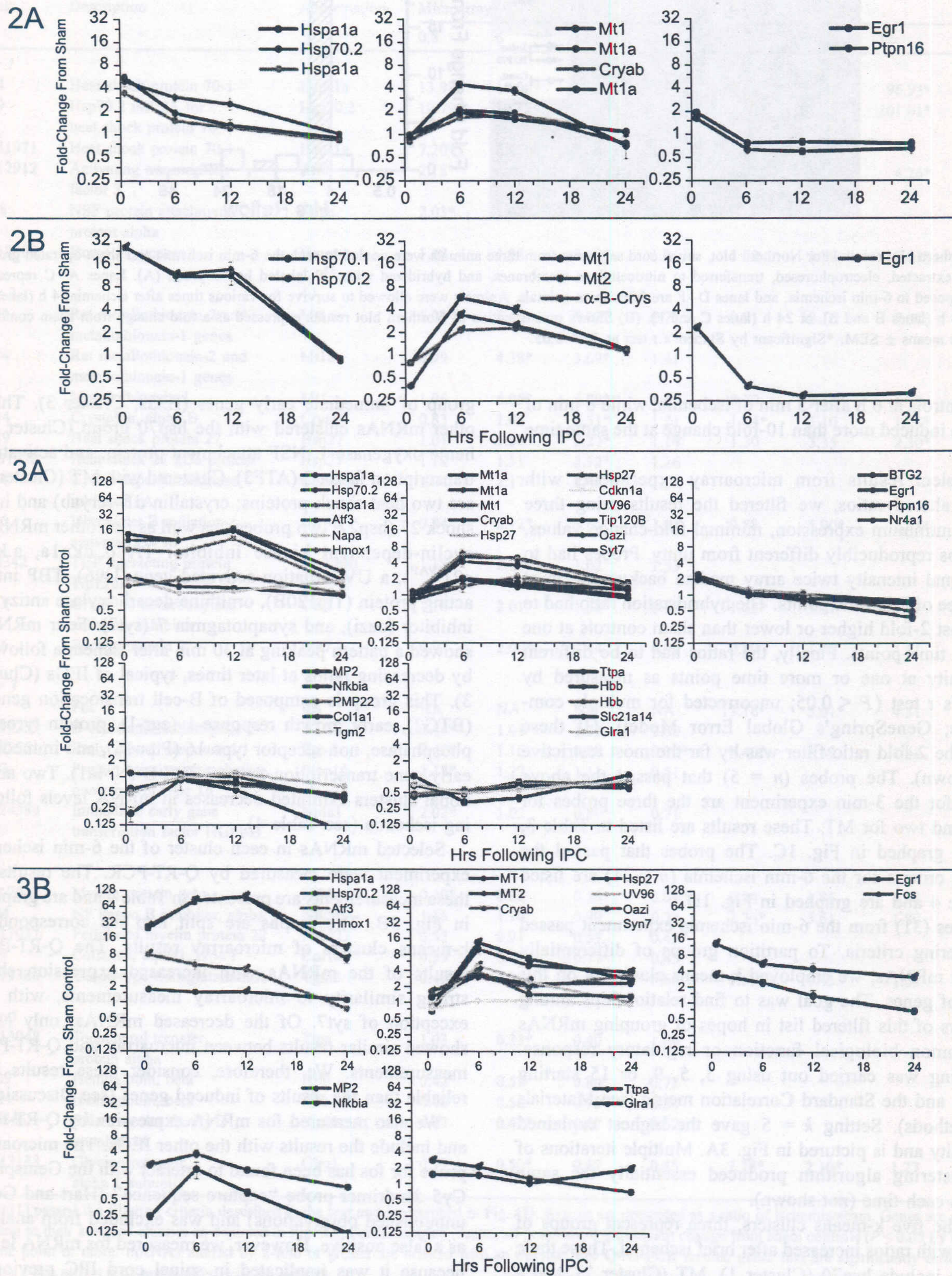
spinal cord samples of a separate group of animals after 6 min of ischemia or sham surgery. Lumbar (L2–5) and cervical (C2–5) spinal cords were collected, and RNA from three animals was extracted, pooled, and analyzed by Northern blot (Fig. 4A). Lanes A–C contain RNA from spinal cords that received 6 min of ischemia and lanes D–F were run with RNA from sham-operated rats. Animals were sacrificed at 4 h (lanes A and D), 18 h (lanes B and E), and 24 h (lanes C and F) after ischemia. Northern blot results are shown in Fig. 4B and quantifications are expressed as fold-change from sham controls. A single band of hybridization was observed under all conditions, suggesting that the Hsp70 mRNA was not alternatively spliced or otherwise changed in sequence throughout the experiment (although Hsp70 mRNA is normally unspliced). Robust accumulation (approximately 25-fold) of hsp70 mRNA was seen 4 h after preconditioning in the lumbar spinal cord, but no change was seen at 18 or 24 h. Cervical cord segments showed no induction of hsp70 mRNA at any of the reflow time points. These results confirm that hsp70 mRNA induction after 6 min is robust and appears limited to the ischemic areas of spinal cord. Hsp72-like immunoreactivity was detected in neurons of the superficial dorsal horn following 3 min of ischemia (Cizkova et al., accompanying manuscript). A 6-min period of ischemia extended staining to neurons of the intermediate zone, consistent with a protective function in these cells.

Another group of microarray probes, increased at 6 and 12 h, stand out prominently in Figs. 1A and B. In the 3-min ischemia experiment, only one of these stood apart (Fig. 1A), whereas three showed robust increases in the 6-min ischemia group (Fig. 1B). The three probes detect metallothioneins (MT-) 1 and 2 mRNAs. In both 3- and 6-min ischemia groups, MT-1 and MT-2 mRNA levels were not increased above sham at 30 min of reflow. Levels rose sharply by 6 h of reflow and remained elevated at 12 h before falling towards baseline by 24 h. As with hsp70, MT-1 and MT-2 mRNA levels were more highly increased following 6 min of ischemia than with 3 min.

Fig. 2. Clustering and validation of 3-min ischemia results. (A) Anchor gene clustering. To find genes with similar expression patterns to three genes of interest—hsp70, MT, and egr-1—an "anchor gene" clustering (Carmel et al., 2001) was performed. The 3-min results were filtered at 1.5-fold change to create a subset of 159 genes. The expression patterns for hsp70, MT, and egr-1 were used to find genes with similar patterns using the standard correlation metric with values of 0.90 or greater. Each hsp70 probe identified the two other hsp70 probes as close correlates. Likewise, each MT probe was closely correlated to the other two as well as to the small heat shock protein cryab. The expression pattern for egr-1 was correlated with ptpn16, another IEG. (B) Validation of anchor genes by Q-RT-PCR. Samples of the same RNA preparation used for microarrays were assayed by Q-RT-PCR. Results (mean ± SEM; $n = 4$) were calculated as fold-change from mean sham to match microarray results.

Fig. 3. Clustering and validation of 6-min ischemia results. (A) k-means clustering. To partition the 31 genes that passed our filters (see text), we used k-means clustering with $k = 5$. Similarity was measured with the standard correlation metric, and clustering was performed iteratively until stable clusters were found. The identity of the genes in each cluster is listed in Table 4. Cluster 1 contains the three probes for hsp70 as well as for heme oxygenase and activating transcription factor 3 (ATF3). Grouped with MT-1 and MT-2 in cluster 3 are the small heat shock proteins cryab and hsp27 among others. Cluster 3 contains BTG2, egr-1, ptpn16, and NGFI-B, all IEGs. Clusters 4 and 5 contain genes that are decreased with ischemia and show inverse patterns. (B) Q-RT-PCR validation of selected genes. Results are graphed in groups according to k-means clusters. Most induced mRNAs show validation of microarray findings with a greater dynamic range by Q-RT-PCR than microarrays. With the exception of MPZ, none of the decreased genes was validated. Results are mean ± SEM ($n = 4$).

Like hsp70, MT mRNAs also showed greater fold-changes by Q-RT-PCR than by microarrays (Tables 3 and 4). MT-1 mRNA levels rose more than 5-fold at 6 h after 3 min of ischemia; after 6 min of ischemia, MT-1 rose more than 13-fold over sham controls. MT-2 showed a similar time course, increasing more than three times the levels of



(Yang et al., 2000). Fos mRNA elevation (13-fold) was greater and more sustained than other IEGs measured in this study (Table 4 and Fig. 3B).

We asked whether the three clusters with increased ratios in the 6-min ischemia experiment might have homologous patterns in the 3-min ischemia experiment. The hsp70 group (three probes) and a MT group (two probes) are contained in the filtered set of probes from the 3-min results (Fig. 2B). However, IEGs and other genes identified in the 6-min clustering fell below our arbitrary 2-fold ratio restriction. To test whether similar genes clustered in the 3-min experiment, we first created a set of less stringently filtered genes by setting the fold change cutoff at 1.5 ($n = 159$). k-means clustering with this group identified an hsp70 cluster, a MT cluster, and a cluster with several IEGs (data not shown). However, the explained variability of this clustering was low compared to that of the stringently filtered 6-min k-means, and the patterns were less easily distinguishable on visual inspection. The magnitude of effects following 6 min of ischemia was more pronounced and the patterns of response were more easily partitioned by the k-means algorithm into meaningful groups than the results of the 3-min ischemia.

To better isolate the hsp70, MT, and IEG groups in the 3-min data, we employed an "anchor gene" approach (Carmel et al., 2001). This clustering technique involves handpicking a gene of interest and then using the time series of that gene to find close correlates in hopes that retrieved genes might also be biologically meaningful or coordinately regulated. The Standard Correlation metric was used with a correlation coefficient of 0.90. Representative anchor genes from Clusters 1–3 (hsp70, MT, and egr-1, respectively) of the 6-min clustering were used to find close correlates in the 3-min data. We anticipated that of the 159 genes in the filtered subset, each anchor would more likely be paired with genes that clustered together in the 6-min results. This held true without exception. The microarray results of these correlations are graphed in Fig. 2A, and the Q-RT-PCR results in Fig. 2B; both sets of results are included in Table 3. The temporal pattern of each hsp70 probe was highly correlated with the other two, as was the case for the three MT probes. In addition, the pattern for cryab was also associated with each MT probe; these genes were clustered together in the same 6-min k-means. For egr-1, the gene whose expression correlated significantly was ptpn16. This pairing was also found with the 6-min k-means clustering. Thus, similar clusters were found in both 3- and 6-min ischemia experiments.

Discussion

Investigation of ischemic preconditioning in the rat spinal cord may reveal molecular mechanisms that can be exploited to protect people from paralysis after aortic aneurism repair or spinal contusion injury. Although spinal cord IPC has been demonstrated in experimental models, the basis for the tolerant state has not been well elucidated. Here we employ microarrays to survey gene expression associated with IPC and to identify target genes for future study. Our studies of gene expression after short ischemia identify several genes and gene families that are changed significantly. These include hsp70, MT, small heat shock proteins, IEGs, and other possible neuroprotective agents, including several not previously reported.

Spotted oligonucleotide microarrays have been demonstrated to be a reliable technique for assessing a large number of mRNAs (Hughes et al., 2001; Wang et al., 2003). Most microarray results were validated by Q-RT-PCR measurements, and expression profiles generally have similar patterns by the two measures. Microarrays tended to underestimate changes, an observation that has also been made by others (Rajeevan et al., 2001). All microarray results showing 3-fold or greater changes were validated. All increased genes, except synaptotagmin 7, were validated by Q-RT-PCR. Several probes showed divergent results, and these were mostly the decreased genes, including Ttpa, IκB, GlyRA1, and MRG-3. Of the decreased genes, only MPZ showed similar (although not statistically significant) changes by Q-RT-PCR. The reasons for this disparity are puzzling—results such as these would normally be indicative of microarray dye-specific false signals. However, our dye-flip experiments showed high correlation coefficients among dye-flipped pairs (see Materials and methods).

Heat shock protein 70

The greatest increases were seen in hsp70 mRNA, up to 100-fold higher after 6-min ischemia than sham controls. The inducible heat shock protein 70 (HSP70) is a molecular chaperone that aids in the folding of nascent proteins and binds proteins during times of cell stress, including hyperthermia and ischemia (Mestril and Dillmann, 1995). HSP70 was first identified in Drosophila exposed to hyperthermic preconditioning that protected against a lethally hot environment (Arrigo and Welch, 1987; Tomasovic et al., 1985). Increases in HSP70 have been associated with models of IPC, including spinal cord IPC (Matsumoto et al., 2001; Matsuyama et al., 1997). Other interventions that protect the spinal cord, including whole body hyperthermia (Zhang et al., 2000), local cooling (Motoyoshi et al., 2001), and pharmacological stress (Perdrizet et al., 2002), all increase HSP70 levels. Finally, in models of cerebral ischemia, overexpression of HSP70 protects neurons from ischemic insult (e.g., Hoehn et al., 2001; Kelly et al., 2001), while loss of HSP70 abrogates the conditioning effect (Nakata et al., 1993). In a companion acticle, Marsala et al. report an analysis of HSP70 expression and localization after brief spinal ischemia (Cizkova et al., 2004).

Metallothioneins

Two other mRNAs that show robust increases following brief ischemia are MT-1 and MT-2. Metallothioneins are a

family of cysteine-rich, low molecular weight proteins that bind transition metals, such as zinc and copper (Hamer, 1986). MT-1 and MT-2 are induced by heavy metals, oxygen-free radicals, glucocorticoids, cytokines, and immediate early genes (Palmiter, 1998). MTs have been proposed as detoxifying agents of reactive metals and free radicals (Liu et al., 1991), both of which contribute to ischemia-induced cell damage (Chan, 1996). MT-1 and MT-2 are induced robustly by ischemia, including cerebral ischemia (Ebadi et al., 1995; Gerlai et al., 2000; Sharp et al., 1993), and MT has been identified as an ischemia-induced gene by other genomic screens, including serial analysis of gene expression (Trendelenburg et al., 2002) and microarrays (Onody et al., 2003). MT-1 and MT-2 have also been implicated in the delayed phase of ischemic preconditioning of the heart (Chen et al., 1997) and brain (Emerson et al., 2000).

Several groups have examined the effects of MT gain- and loss-of-function to elucidate its role in cellular protection. Induction of MT by application of transitional metals protects the heart against oxidative damage (Satoh et al., 1988) and human primary CNS cultures against irradiation damage (Cai et al., 2000). Cardiac myocytes overexpressing MT-1 were protected from ischemia by inhibition of the cytochrome *c*-mediated apoptotic pathway (Wang et al., 2001). van Lookeren Campagne et al. (1999) compared the effects of transient middle cerebral artery occlusion (MCAO) in transgenic mice that overexpress MT-1 (Iszard et al., 1995) and wild-type mice. Lesion volume and sensorimotor deficits were significantly decreased in MT-1 overexpressing mice compared with controls. In loss-of-function studies, repression of MT exacerbates cell damage. In the mouse model of familial amyotrophic lateral sclerosis that lacks superoxide dismutase, further deletion of MT-1 and MT-2 by crossbreeding resulted in earlier onset of clinical signs and death. Using the MT-1 and MT-2 double knockout (KO) mice, Trendelenburg et al. (2002) found KO mice had approximately three-fold larger cerebral infarcts and significantly worse neurological outcome than wild-type controls in response to transient ischemia. These studies substantiate a cell protective role for MT.

Although hsp70 and MT induction constituted the most prominent mRNA changes that we observed in these experiments, brief ischemia affected other genes as well. We found the use of k-means clustering instructive in trying to group these genes. Several clusters included genes known to have similar function, suggesting that the groupings may follow biological likeness.

Genes clustering with MT

Grouped in the same cluster as MT were the small heat shock proteins hsp27 and αB crystallin (cryab). These two genes encode proteins that assemble into chaperone complexes, which bind and protect proteins at times of cell stress (Dillmann, 1999). Cryab, first characterized as a major component of the vertebrate lens, is involved in many cellular processes, including oxidative stress responses in the heart and lung, cellular differentiation in the eye, and a variety of neurodegenerative disorders (Dillmann, 1999). Striated muscles, including the heart, express high levels of cryab (Iwaki et al., 1990). Overexpression of cryab protects myocytes from ischemia and also provides cardioprotection (Martin et al., 1997). Mice expressing a mutated form of the protein show increased damage to myocytes made ischemic (Martin et al., 2002).

Brief ischemia also induced the small HSP, hsp27, in the spinal cord. Hsp27 is induced by multiple cell stresses, including heat shock, ischemia, and seizures (Dillmann, 1999). As with αB crystallin, hsp27 expression in the stressed brain is primarily localized in astrocytes and rises more slowly and for longer periods of time than hsp70 (Akbar et al., 2001). Gain- and loss-of-function studies point to a protective role of hsp27. In a neuronal cell line and primary neuronal cultures, overexpression of hsp27 protected against exposure to heat shock and withdrawal of nerve growth factor (NGF) (Wagstaff et al., 1999). Overexpression of hsp27 also protects cardiac myocytes against simulated ischemia (Martin et al., 1999). The same adenoviral vector encoding an hsp27 antisense oligonucleotide significantly decreased HSP27 levels and increased injury in cultured myocytes (Martin et al., 1999). Hsp27 also appears to play a key role in sensory neuron survival after axotomy or neurotrophin withdrawal (Lewis et al., 1999). Finally, Akbar et al., 2003 recently showed that mice overexpressing human hsp27 were protected from damage to hippocampal neurons due to kainate-induced seizures. Identification of hsp27 mRNA increases following IPC is suggestive of a protective role in the spinal cord as well.

The immediate–early gene cluster

Another group of genes revealed by k-means clustering, Cluster 3, includes four immediate–early genes: B-cell translocation gene 2 (BTG2), the transcription factors early growth response 1 (egr-1) and nerve growth factor inducible B (NGFI-B), and a mitogen-activated protein kinase phosphatase, ptpn16, an important cell signaling regulator. Egr-1 and ptpn16 also cluster together in the 3-min ischemia cluster. This cluster of genes shows an expression pattern of early induction followed by either return to baseline or repression at 6 h and later time points. The expression profile of these genes and their known function identifies them as possible regulatory genes that may affect downstream changes.

BTG2 was originally identified as a transcript induced by p53 in response to genotoxic stress (Rouault et al., 1996). Several studies also point to a role for BTG2 in neuronal differentiation. Similar to egr1 and NGFI-B, BTG2 levels are increased in PC12 cells after induction of differentiation by NGF (Bradbury et al., 1991). BTG2 is expressed during the production of postmitotic neurons and is considered a marker of neuronal birth (Iacopetti et al., 1999). Importantly,

expression of BTG2 appears to protect differentiated neural cells from apoptosis, as antisense oligonucleotides to BTG2 trigger programmed cell death (el-Ghissassi et al., 2002). The increased expression of several NGF-induced genes in this assay points to common mechanisms, and the protective role of BTG2 makes it a promising target for future study.

Egr-1 (also known as NGFI-A, krox-24, or zif268) is a transcription factor and an immediate early gene (for review see Beckmann and Wilce, 1997). Egr-1 is induced in the rat brain by a large number of stresses, including glutamate–NMDA stimulation (Beckmann et al., 1997), long-term potentiation (Worley et al., 1991), focal traumatic brain injury (Honkaniemi et al., 1995), and a variety of cerebral ischemia models (Abe et al., 1991; Collaco-Moraes et al., 1994). Egr-1 was also shown to be elevated in the spinal cord in response to noxious peripheral stimulus (Lanteri-Minet et al., 1993; Wisden et al., 1990) and sciatic nerve lesion (Herdegen et al., 1993). Recently, Rybnikova et al. (2002) showed that mild hypoxic preconditioning increased the level and duration of egr-1 expression in the rat brain following severe cerebral hypoxia. Other authors suggest that prolonged egr-1 expression after ischemic insult is associated with delayed neuronal degeneration (Honkaniemi and Sharp, 1996; Honkaniemi et al., 1997). The egr-1 gene codes for a zinc finger nuclear phosphoprotein that binds to GC-rich sequences in the promoter region to affect transcription of a diverse set of genes (Beckmann and Wilce, 1997). These include genes with potential protective roles, such as platelet-derived growth factor (Khachigian et al., 1996), transforming growth factor β (Khachigian et al., 1996), and NGFI-B (Williams and Lau, 1993). Some genes modulated by egr-1 may have deleterious effects on neuronal survival. These include NMDAR1 (Bai and Kusiak, 1995), tumor necrosis factor (Kramer et al., 1994), and two prothrombotic genes: tissue factor (Cui et al., 1996) and thrombospondin 1 (Shingu and Bornstein, 1994). Thus, whether egr-1 plays a protective or deleterious role may depend on the timing of its expression and its relationship to other transcriptional events, particularly those regulated by other IEGs.

NGFI-B was found in the same genomic screen as egr-1 (NGFI-A) of PC12 cells exposed to NGF (Milbrandt, 1988), and k-means clustering paired the two genes in this study as well. NGFI-B has a zinc finger domain and belongs to the thyroid–steroid receptor family. It is an immediate early gene that is stimulated by growth factors (Hazel et al., 1988), depolarization (Yoon and Lau, 1993), and seizures (Watson and Milbrandt, 1989). The pattern of induction by hypoxia (Gubits et al., 1993), global ischemia (Neumann-Haefelin et al., 1994), and focal ischemia (Lin et al., 1996) parallels those of other immediate early genes (Johansson et al., 2000). NGFI-B is known to regulate expression of corticotropin-releasing factor (CRF), vasopressin, oxytocin, and steroid 21-hydroxylase in vitro (Chan et al., 1993; Wilson et al., 1991, 1993), all of which may be increased by stress. Although this gene is increased at times of stress and likely induces other stress-related genes, how NGFI-B might participate in neuroprotection remains to be elucidated.

In addition to egr-1, ptpn16 (aka mitogen-activated protein kinase phosphatase-1) also shows induction with both 3 and 6 min of ischemia. This gene encodes a dual-specificity protein phosphatase that acts on tyrosine and threonine residues of mitogen-activated protein (MAP) kinases, inactivating the kinases (Alessi et al., 1993; Sun et al., 1993). Phosphorylation of MAP kinases is a key regulatory step in cell signaling, particularly during times of stress (for review, see Irving and Bamford, 2002). Multiple stressors induce ptpn16, including oxidative stress, heat shock, seizures, and brain ischemia (Boschert et al., 1998; Keyse and Emslie, 1992; Wiessner et al., 1995). Two genomic screens identified this gene as induced by focal cerebral ischemia (Soriano et al., 2000) and hypoxic preconditioning (Bernaudin et al., 2002). Of the MAP kinase families, ptpn16 has stronger affinity for p38 and JNK than ERK (Camps et al., 2000). JNK and p38 signaling has several effects on cell survival, but most reports suggest deleterious effects of these pathways on neuronal survival (Chihab et al., 1998; Maroney et al., 1999). Thus, by inhibiting these pathways, increased ptpn16 expression would likely enhance neuronal survival.

Although the fos probe was excluded from microarray results because of cross reactivity with the labeling molecule (see Results), fos mRNA levels were assayed by Q-RT-PCR and found to be the most highly induced of the IEGs. In brain ischemia paradigms, fos protein appears to be induced in neurons more resistant to ischemia (e.g., hippocampal CA3 neurons) than in the more susceptible CA1 neurons (Johansson et al., 2000; Nowak et al., 1990). Fos mRNA is also induced by short ischemic periods that are protective in brain IPC models (Truettner et al., 2002). Marsala et al. have shown that Fos protein was elevated by 6 min of spinal cord ischemia (Yang et al., 2000). The protein expression peaked at 2 h, and the expression at 4 h was limited to the susceptible interneurons in laminae V–VII. The spatial correlation of protein expression with protection and the robust, early, and sustained mRNA expression make fos an IEG worthy of further study.

Another transcription factor, activating transcription factor 3 (ATF3), is robustly induced (8-fold by Q-RT-PCR) at 30 min after 6 min of ischemia and remains elevated until returning to baseline by 24 h (Figs. 3A and B, Table 4). ATF3 is a member of the CREB family of transcription factors that recognizes the cyclic AMP response element (CRE) site and forms dimers by binding at the leucine zipper region. ATF3 represses transcription as a homodimer (Chen et al., 1994) and activates transcription as a heterodimer with jun (Chu et al., 1994; Hai and Curran, 1991). ATF3 is induced in a variety of stressed tissues (reviewed in Hai et al., 1999), including the ischemic heart (Yin et al., 1997) and post-seizure brain (Chen et al., 1996). ATF3 is strongly induced by sciatic nerve lesion in the dorsal root ganglia and spinal motor neurons (Tsujino et al., 2000).

Overexpression in the heart (Okamoto et al., 2001), pancreas (Allen-Jennings et al., 2001), and liver (Allen-Jennings et al., 2001) shows detrimental effects in these organs. Whether ATF3 may be acting as a functional activator or repressor of transcription in IPC remains to be investigated, but its marked induction and association with cell stress make it an enticing target for further study.

Comparison of 3 vs. 6 min of IPC

While 3 and 6 min IPC treatments have somewhat differing protective outcomes (Cizkova et al., 2004), most mRNA effects were similar in the two models, with differences in magnitudes. The clustering of MT and αB crystallin was conserved between the 3- and 6-min IPC experiments, and pairing of the IEGs egr-1 and ptpn16 was similarly conserved. In addition, the similarity of the clusters resulting from the 3-min ischemia and the 6-min experiments implies that increasing the length of conditioning ischemia may modulate the amplitude, rather than the mechanisms, of the neuroprotective effect. Six minutes of spinal ischemia induces similar changes in gene expression as 3 min, but the changes are more robust.

Summary

Most of the mRNA changes caused by brief ischemia of the spinal cord appear to have potentially beneficial effects. The genes that are activated by brief ischemia may affect all stages of IPC, including signal transduction (e.g., ptpn16), transcription (e.g., ATF3, fos, egr-1), and effectors of neuroprotection (e.g., HSPs and MT). This study increases the number of candidate neuroprotective genes. It also places changes in suspected mediators (e.g., hsp70) in the context of overall mRNA changes. It is likely that some of these changes will participate directly in the neuroprotective state, while others will not. Among the criteria important for selecting potential protective genes for study are the magnitude of change, the known biological function of the gene, and the novelty of the association. Hsp70 mRNA shows enormous induction by preconditioning (up to 100-fold), and the product has proven beneficial effects in CNS ischemia. So hsp70 is an attractive target, although it is the most widely studied gene in spinal ischemia and preconditioning. MT-1 and MT-2, on the other hand, have not been studied in spinal cord IPC, although their protective effects in brain ischemia are well documented. The magnitude of mRNA induction of these genes and their dual roles in heavy metal binding and free radical scavenging make them very appealing targets for study in this system. Among the more novel genes whose expression is induced by brief ischemia are the IEG transcription factors, including fos, egr-1, and ATF3. Although induced more moderately than hsp70 and MT, these genes may serve as gatekeepers to transcriptional events that confer neuroprotection. Identification of these responses to brief ischemia provides good justification for detailed, mechanistic studies with this limited set of genes.

The molecular mediators found by the study of IPC may appear to have the greatest clinical applicability in the prevention of paralysis that occurs due to cross-clamping during aorta surgery. Our study provides a practical, functional genomics endpoint against which one may assay putative phamacologic preconditioning agents. Patients undergoing such surgery could receive pharmacological preconditioning to modulate spinal cord ischemic tolerance before the blood supply is cut off, a practical impossibility for people who suffer spinal cord injury. However, evidence from one HSP70 study (Hoehn et al., 2001) suggests that this mediator of preconditioning may also be effective as treatment after the onset of ischemia. This raises the hope that mediators of ischemic tolerance may also have wider clinical importance for people who suffer spinal ischemia.

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