

Mechanisms of disease

Effect of ischaemic preconditioning on genomic response to cerebral ischaemia: similarity to neuroprotective strategies in hibernation and hypoxia-tolerant states

Mary P Stenzel-Poore, Susan L Stevens, Zhigang Xiong, Nikola S Lessov, Christina A Harrington, Motomi Mori, Robert Meller, Holly L Rosenzweig, Eric Tobar, Tatyana E Shaw, Xiangping Chu, Roger P Simon

Summary

Background Molecular mechanisms of neuroprotection that lead to ischaemic tolerance are incompletely understood. Identification of genes involved in the process would provide insight into cell survival and therapeutic approaches for stroke. We developed a mouse model of neuroprotection in stroke and did gene expression profiling to identify potential neuroprotective genes and their associated pathways.

Methods Eight mice per condition were subjected to occlusion of the middle cerebral artery for 15 min (preconditioning), 60 min (injurious ischaemia), or preconditioning followed 72 h later by injurious ischaemia. RNA was extracted from the cortical regions of the ischaemic and non-ischaemic hemispheres. Three pools per condition were generated, and RNA was hybridised to oligonucleotide microarrays for comparison of ischaemic and non-ischaemic hemispheres. Real-time PCR and western blots were used to validate results. Follow-up experiments were done to address the biological relevance of findings.

Findings Microarray analysis revealed changes in gene expression with little overlap among the conditions of injurious ischaemia, ischaemic preconditioning, or both. Injurious ischaemia induced upregulation of gene expression; 49 (86%) of 57 genes regulated showed increased expression in the ischaemic hemisphere. By contrast, preconditioning followed by injurious ischaemia resulted in pronounced downregulation; 47 (77%) of 61 regulated genes showed lower expression. Preconditioning resulted in transcriptional changes involved in suppression of metabolic pathways and immune responses, reduction of ion-channel activity, and decreased blood coagulation.

Interpretation Preconditioning reprogrammes the response to ischaemic injury. Similar changes reported by others support an evolutionarily conserved endogenous response to decreased blood flow and oxygen limitation such as seen during hibernation.

Lancet 2003; **362**: 1028–37

See Commentary page 1007

Department of Molecular Microbiology and Immunology

(M P Stenzel-Poore PhD, S L Stevens BSc, H L Rosenzweig BA, E Tobar MSc, T E Shaw BSc), **Oregon Stroke Center** (N S Lessov MD), **Vaccine and Gene Therapy Institute** (C A Harrington PhD), and **Biostatistics and Bioinformatics Shared Resource Cancer Institute** (M Mori PhD), **Oregon Health and Science University, Portland, OR, USA; and Robert S Dow Neurobiology Laboratories, Legacy Research, Portland** (Z Xiong MD, R Meller PhD, X Chu MD, R P Simon MD)

Correspondence to: Dr Mary P Stenzel-Poore, Department of Molecular Microbiology and Immunology, L220, Oregon Health and Science University, 3181 Sam Jackson Park Road, Portland, OR 97239, USA (e-mail: poorem@OHSU.edu)

Introduction

Ischaemic preconditioning is the process by which brief exposure to ischaemia provides robust protection, or tolerance, against the injurious effects of a long period of ischaemia. Preconditioning occurs after other distinct stimuli (eg, endotoxin exposure, anaesthesia)^{1,2} and in various organs,³ which has led to the view that the process activates a fundamental response to cellular stress.⁴ Cerebral ischaemic preconditioning in animal models of stroke provides striking neuroprotection against subsequent ischaemic injury.⁵ An analogous process is believed to exist in human beings; previous transient ischaemic attacks are associated with better clinical outcome after subsequent stroke.⁶ Thus, the cellular mechanisms of neuroprotection induced by ischaemic preconditioning offer attractive targets for the development of therapeutic approaches. However, these molecular processes are poorly understood.

Many organisms use adaptations that allow survival during periods of severe oxygen deprivation—processes that lead to a state of tolerance to injury. Hypoxia-tolerant and hibernating species tolerate periods of very low oxygen and glucose delivery, yet maintain cellular homeostasis,⁷ partly through controlled metabolic suppression and altered ion-channel activity, both of which inhibit cellular function.⁸ Such neuroprotective adaptations may also underlie preconditioning strategies and lead to reversible “cellular arrest”. We hypothesise that preconditioning elicits gene expression changes leading to an analogous state that is refractory to ischaemic injury. Identification of these changes would provide insight into endogenous mechanisms of neuroprotection. Accordingly, we used DNA OLIGONUCLEOTIDE microarrays to examine preconditioned and not preconditioned ischaemic brain to elucidate the genomic changes that occur in this neuroprotective state.

Methods

All animal procedures were done in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The animal protocols met National Institutes of Health guidelines with the approval of the Oregon Health and Science University Institutional Animal Care and Use Committee.

Mouse model of cerebral ischaemic tolerance

Cerebral focal ischaemia was induced by occlusion of the middle cerebral artery with an intraluminal suture.⁹ C57BL/6J mice (male, aged 8–10 weeks; Jackson Laboratories, Bar Harbor, ME, USA) were allocated to five groups with seven or eight animals per group. Groups 1 and 2 underwent 15 min occlusion, groups 3 and 4 60 min occlusion, and group 5 15 min occlusion followed 72 h later by 60 min occlusion. Mice of groups 1, 3, and 5 were killed 24 h after the start of the final occlusion. Groups 2 and 4 were killed 72 h after the start of occlusion. Just before death, the mice were anaesthetised,

Gene	Forward primer	Reverse primer	TaqMan probe (5' FAM, 3' TAMRA)
β actin	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT	CACTGCCGCATCCTCTTCTCC
OPN	TGGCAGTGATTGCTTTTTGC	TGGGTGCAGGCTGTAAGCT	TGTTTGGCATTGCCTCCTCC
GFAP	ACAGGAGAGCAGGACTTCCGT	CCATCCGCTCAGGTCATCTT	CACTGGGCAGGGTACAGATGTGTCTCAG
Calpactin	GGAGAACGCCTTCCTGAACC	GGCAAGGGGACTCGAGACA	TCCAGTGCATCCAGAACAGGCC
FXD7	CCTGTAAGTCGGAACTGCC	GCCAGATGCGAGGAACCAGA	CTCAGCCCCCTGGAGGTGGCG
Alcohol dehydrogenase	CTTTGGAGGGTGGAAAGAGCA	TGATAACCCACACCTTGCCTT	ACTGGTGACTGACTGAGTTCCTGGAAAAGAAGTTTGA
NAGA	TGATGTCCCACTGGTGAGGA	GGACATGGCAGGGCTCTGT	CCAAGAATGACAGGCTACGGCAGCC
TNFRp55	CATAGCTTTTCTCAGCCGCC	GCTGAGGGTGCCAAAGACAG	ACTTGTGAGTGGCAGGGAGATGTACCATCA

Table 1: Primer and probe sequences for real-time PCR

and heparinised saline was infused. The brains were removed, and the olfactory bulb and cerebellum were dissected away. The brain was then placed in a preformed mouse-brain mould calibrated for removal of 1 mm slices with regular intervals. A 1 mm coronal slice was removed (4 mm from rostral end, corresponding roughly to bregma of Franklin and Paxinos atlas¹⁰) for analysis of the infarct area by the 2,3,5-triphenyltetrazolium chloride staining method. The infarct area of a coronal section taken at this position is closely correlated ($r^2=0.96$) with the volume of infarct in this model.⁹ The remaining frontal 4 mm was then removed from the mould for isolation of cortical tissue. The cortex from each hemisphere was separately dissected away from the striatum, then snap-frozen in liquid nitrogen. This cortical sample contains the region of the ipsilateral hemisphere consistently spared from ischaemic injury by preconditioning. The striatum and other internal structures were discarded.

Total RNA was isolated from individual cortices with the Qiagen RNeasy kit (Qiagen Inc, Valencia, CA, USA). For microarray analysis 30 samples were examined. RNA was pooled to generate three paired samples (ischaemic and non-ischaemic) for each of the five experimental groups by combining cortices from two or three mice.

Isolated total RNA was labelled and hybridised to a GeneChip oligonucleotide array as described in the manufacturer's technical manual (Affymetrix, Santa Clara, CA, USA). Labelled RNA was hybridised to a test array containing control probe sets. Samples that did not meet empirically defined cut-off values within the project were remade. Quality-tested samples were hybridised to the MG_U74Av1 array. Results were processed with Affymetrix Microarray Suite 4.0 (MAS 4.0) with appropriate masking of the inaccurate probe sets present on version 1 of this chip design. Genes showing an absence call on all 30 arrays were removed from the analysis. In addition, we did MAS 4.0 comparative analysis for each paired sample (ischaemic and non-ischaemic hemispheres) and obtained the estimate of the fold change for each pair.

The cube-root transformation was applied to normalise the data and to retain the genes with negative signal values (average difference). For each gene, we did repeated-measures ANOVA with the following effects in the model: within-animal effect (hemisphere), between-animals effect (group), and the interaction between hemispheres and group. A contrast statement was used to obtain the p value for the degree of expression between ischaemic and non-ischaemic hemispheres in each group. For a gene to be considered differentially regulated, two criteria had to be

met: first, a 2.2-fold difference of expression between the ischaemic and non-ischaemic hemispheres in at least two of the three pools of RNA based on the fold change obtained by MAS 4.0 comparative analyses; and second, a p value of less than 0.05 from the repeated-measures ANOVA comparing differences between hemispheres. The criteria combined statistical significance and biologically meaningful differences, leading to a better set of potentially regulated genes than any one criterion alone.

Validation of gene regulation was undertaken on a subset of genes by real-time PCR. Primers and probes (Integrated DNA Technologies, Coralville, IA, USA) were designed based on the target sequences supplied by Affymetrix (table 1). β actin was used as the housekeeping gene, and genes were quantified from a standard curve included in all measurements. We also verified corresponding protein regulation of a subset of genes by western blot or immunohistochemistry.

In-vitro tolerance model

Cortical neuronal cultures were prepared from 1–3-day-old Sprague-Dawley rats. Cortices were dissected, dissociated with papain (Worthington Biochemicals, Lakewood, NJ, USA), and plated out at 400 000 cells per coverslip in Neurobasal-A/B27 media. Cells were used after 7–10 days in culture, when cultures consist of 60–80% neurons.

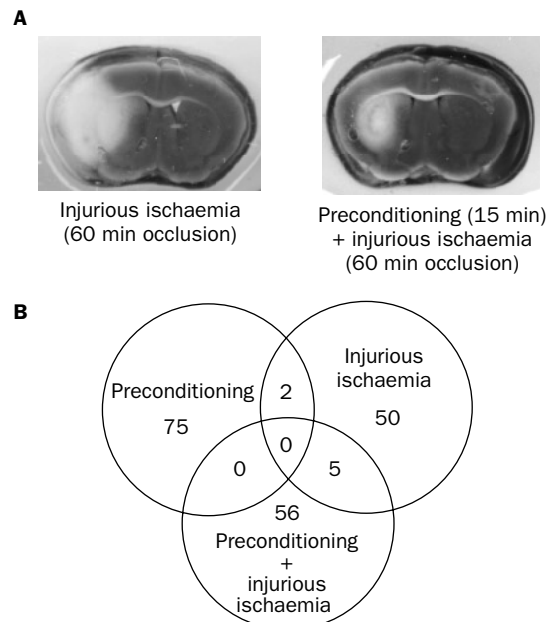


Figure 1: Effect of preconditioning on gene expression

A: Coronal brain sections stained with 2,3,5-triphenyltetrazolium chloride for visualisation of the infarct. Mean % infarct in eight animals per group 47% (SE 4) for injurious ischaemia, 14% (3) with preconditioning. B: Venn diagram showing the numbers of genes meeting the regulation criteria. To show more clearly the differences among the conditions, only the 24 h point is shown (72 h after preconditioning and injurious ischaemia was not collected).

GLOSSARY

AESTIVATION

A state of dormancy.

CHANNEL ARREST

Inhibition of channel function.

OLIGONUCLEOTIDE

A short polymeric chain of nucleotides.

Modelled hypoxia-ischaemia by use of oxygen and glucose deprivation was done to test in individual cells functional changes predicted by the in-vivo genomic data obtained by ischaemic preconditioning. Oxygen and glucose deprivation was induced by replacing the medium with phosphate-buffered saline supplemented with 0.5 mmol/L calcium chloride and 5 mmol/L magnesium chloride, pH 7.4, and placing the culture dishes in an anaerobic chamber (Forma Scientific, Marjetta, OH, USA) with an atmosphere of 85% nitrogen, 5% hydrogen, and 10% carbon dioxide at

35°C. Oxygen and glucose deprivation was terminated by replacing the medium with minimum essential medium (MEM) and moving the cultures into a normoxic incubator. Experimental conditions were: preconditioning (30 min oxygen and glucose deprivation and 48 h in MEM); damaging oxygen and glucose deprivation (2 h deprivation followed by 24 h recovery); preconditioning plus damaging oxygen and glucose deprivation (30 min deprivation, 24 h recovery in MEM, and 2 h oxygen and glucose deprivation, followed by 24 h recovery); and control (cultures were

24 h	72 h	Genbank number	Description	p	24 h	72 h	Genbank number	Description	p
Defence/stress response					Cell cycle regulation/apoptosis				
↑	..	M13660	Interferon- α (IFN- α -1-9)	0.03	↑	..	U28423	Protein kinase inhibitor p58	<0.01
↑	..	U09874	SKD3 mRNA, suppressor of K ⁺ transport defect 3	0.01	↑	..	AW228036	Traf and Tnf receptor associated protein	0.02
↑	..	M12571	Heat-shock protein, 70 kDa 3	0.01	↑	..	AA940430	vz48h05-r1	0.03
..	↑	AW048883	Small heat-shock protein HSPB2	0.02	↑	..	AB021961	Mutant p53	0.02
..	↑	Y12879	β -chemokine receptor D6	0.03	↑	..	X62154	Mini chromosome maintenance deficient	0.04
..	↑	X98113	Lymphocyte-activation gene 3	0.04	↑	..	AW048763	NMDA receptor-regulated gene 1	0.01
..	↑	AF110520	Major histocompatibility complex region	0.04	↑	..	AB009245	Stem-cell growth factor	<0.01
↓	..	M96827	C57bl/6J ob/ob haptoglobin	0.03	↑	..	A1836641	UI-M-AP0-abh-c-11-0-UI-s1	<0.01
↓	..	L76193	Paraoxonase 3	0.04	↑	..	D30743	Wee1 kinase	0.03
..	↓	U23921	Osmotic stress protein 94	0.04	..	↑	X66449	Calcyclin	0.02
Metabolism					Signal transduction/synaptic transmission				
↑	..	AB020202	Adenylate kinase isozyme 2	0.02	↓	..	D17583	Proprotein convertase subtilisin/kexin	<0.01
↑	..	AF024570	DNA polymerase δ 1, catalytic domain	<0.01	..	↓	AA866668	Sox 3 SRY-box containing gene 3	0.04
↑	..	AW047953	Flightless I homolog (FLI1)	0.03	..	↓	AF060490	TLS-associated protein TASR-2	0.02
↑	..	U61362	Groucho-related gene 1 protein (Grg1)	0.01	Structural proteins and skeletal development				
↑	..	D16580	Peptidyl arginine deiminase (PDI2)	0.02	↑	..	A119347	Calmodulin 4	0.04
↑	..	M34141	Cyclo-oxygenase 1	0.03	↑	..	AF058799	14-3-3 protein γ	0.02
↑	..	AL009226	Ring finger protein 3	0.01	↓	..	D13266	Glutamate receptor, ionotropic, δ 2	0.02
↑	..	D49473	SRY-box containing gene 17	0.01	..	↓	L41495	Protein-serine/threonine kinase	<0.01
↑	..	X81464	Translin	0.04	Not determined				
↑	..	U64445	Ubiquitin fusion degradation 1 - like	<0.01	↑	..	AW060819	Twisted gastrulation protein pending	0.02
↑	..	AB006036	Serine/arginine-rich protein specific kinase 2	0.02	↑	..	A1607813	ub58e11-x1	0.01
↑	..	A1836977	UI-M-AJ0-aaz-d-10-0-UI-s1	0.01	↑	..	A1877157	uc54h09-r1 end	0.03
↑	..	U49046	Zinc finger protein 64	0.02	↑	..	A1852340	UI-M-BH0-ajo-b-02-0-UI-s1	<0.01
↑	..	U31510	ADP-ribosyltransferase 1	<0.01	↑	..	A1854774	UI-M-BH0-aka-d-01-0-UI-s1	<0.01
↑	..	U87868	Phospholipase D1	0.03	↑	..	AW045743	UI-M-BH1-akq-h-05-0-UI-s1	0.04
..	↑	A1451564	Highly similar to Arnt	0.01	↑	..	AW121624	UI-M-BH2-2-aog-g-11-0-UI-s1	0.01
..	↑	D50834	Cytochrome P450, subfamily IV B, polypeptide 1	0.01	↑	..	A1785422	uj42a07-x1	0.04
↓	..	A1840733	Prostaglandin D2 synthase (PTGDS)	0.02	↑	..	AW215585	up06c04-x1	0.04
↓	..	U89924	Protein phosphatase 1 binding protein PTG	0.02	↑	..	AA727410	vu99g08-r1	0.02
↓	..	A1845735	Similar to DNA-directed RNA polymerase II	0.02	↑	..	AA982124	SWI/SNF related -SMARCA5	0.03
↓	..	AF061555	Ubiquitin-protein ligase e3	<0.01	..	↑	A1842705	UI-M-A01-aeo-d-09-0-UI-s1	0.01
↓	..	U31966	Carbonyl reductase	0.03	..	↑	A1852022	UI-M-BH0-aiq-g-11-0-UI-s1	0.04
↓	..	AF017175	Carnitine palmitoyltransferase 1	<0.01	..	↑	AA612146	vo02h09-r1	<0.01
↓	..	U19880	Dopamine receptor 4	0.02	↓	..	A1182917	uc68h10-r1	<0.01
↓	..	L07063	FKBP65 binding protein	0.03	↓	..	A1840824	UI-M-AH0-adb-d-03-0-UI-s1	<0.01
↓	..	C79210	Geranylgeranyl diphosphate synthase 1	<0.01	↓	..	A1839743	UI-M-AP0-abe-e-06-0-UI-s2	0.04
↓	..	M11988	Homeo box A6	0.03	↓	..	A1854607	UI-M-BH0-ake-c-10-0-UI-s1	0.04
..	↓	Y14334	Arachidonate 12-lipoxygenase, 12R type	0.01	↓	..	AW049125	UI-M-BH1-amq-a-03-0-UI-s1	0.01
..	↓	AF004108	Arylalkylamine N-acetyltransferase	<0.01	↓	..	AW214136	uo40c11-x1	0.04
..	↓	M74516	GA repeat binding protein, β 1	0.01	↓	..	AW208818	uo64d02-x1	0.04
..	↓	M25487	Histone H2b	0.04	↓	..	AA790307	vw17a10-r1	0.04
Transport					↓	..	AF068246	SA protein mRNA	0.01
↑	..	AW049193	UI-M-BH1-amq-g-11-0-UI-s1	0.04	↓	..	AJ005560	Small proline-rich protein 2B	0.02
↑	..	AW122731	UI-M-BH2-2-aot-e-08-0-UI-s1	0.04	↓	..	AA959291	ua14g10-r1	0.01
↑	..	AF003999	Golgi SNARE GS15	<0.01	↓	..	AW047736	UI-M-BH1-alk-a-11-0-UI-s1	0.03
↑	..	J03398	P glycoprotein 2 (PGY2)	0.01	↓	..	AF037312	untitled	<0.01
↑	..	A1042802	uc84d07-x1	0.01	↓	..	AA756275	w40d02-r1	0.01
↑	..	AA833514	uc91f12-r1	<0.01	↓	..	AI646411	w51d12-x1	0.01
↑	↓	M75135	Glut-3, solute carrier family 2	0.01	↓	..	AA797843	vy04c12-r1	0.04
..	↑	L24191	Gastric intrinsic factor	0.01	↓	..	AA409520	EST01388	0.03
..	↑	U08439	Cytochrome c oxidase subunit VIaH	0.01	↓	..	A1849500	UI-M-AH1-agt-d-10-0-UI-s1	0.02
↓	..	AW060509	Torsin family 1, member B	<0.01	↓	..	A1842362	UI-M-AM1-agc-a-03-0-UI-s1	<0.01
↓	..	J00413	Haemoglobin, β adult major chain	<0.01					
..	↓	AB015800	OCTN2	<0.01					
..	↓	D12713	Secretory protein SEC23 related gene (<i>Saccharomyces cerevisiae</i>)	0.04					
..	↓	D37793	Synaptotagmin 2	<0.01					
..	↓	AA414964	vc49a08-r1	0.03					
..	↓	U51126	G-protein coupled inwardly rectifying K ⁺ channel	<0.01					

↑=increased expression, ↓=decreased expression. p values based on contrast statements from repeated-measures ANOVA model.

Table 2: Genes differentially regulated after preconditioning

24 h 72 h	Genbank number	Description	p	24 h 72 h	Genbank number	Description	p		
Defence/stress response				Cell cycle regulation/apoptosis					
↑	U09659	Heat-shock protein, 10, chaperonin 10	<0.01	↑	D13545	DNA primase, p58 subunit	0.04		
↑	L40406	Heat-shock protein, 105 kDa	<0.01	↑	AW048937	Cyclin-dependent kinase inhibitor 1A	0.01		
↑	M12571	Heat-shock protein, 70 kDa 3	<0.01	..	↑	X81580	Insulin-like growth factor binding protein 2	<0.01	
↑	M88242	Cyclo-oxygenase 2 (Cox-2)	0.01	..	↑	X98471	Epithelial membrane protein 1	0.02	
↑	U50712	Small inducible cytokine A12	0.02	↑	↑	X16834	Mac-2 antigen	<0.01	
↑	D82935	Pituitary adenylate cyclase-activating receptor polypeptide	0.03	↑	↑	X66449	Calcyclin	<0.01	
..	↑	X58861	Complement component 1, q subcomponent, α	<0.01	Cell adhesion/motility				
..	↑	X66295	Complement component 1, q subcomponent, c	<0.01	..	↑	D50086	Neuropilin	0.01
..	↑	M12660	Complement component factor h	0.01	..	↑	X58251	Procollagen, type I, α 2	<0.01
..	↑	X15592	Cytotoxic T lymphocyte associated protein 2 β	0.03	..	↑	AF077527	Syntenin	0.03
..	↑	M65027	Glycoprotein 49 A	<0.01	↑	↑	U04827	Brain fatty-acid-binding protein	<0.01
..	↑	U41341	Endothelial monocyte-activating polypeptide I	<0.01	↓	..	AB000636	Procollagen, type XIX, α 1	0.01
..	↑	M69069	Histocompatibility 2, D region locus 1	<0.01	Signal transduction/synaptic transmission				
..	↑	X51547	Lzp-s mRNA for lysozyme P	<0.01	↑	..	M63503	Fibroblast growth factor receptor 2	0.04
..	↑	X57796	TNFRp55	0.04	↑	..	U24160	Dishevelled 2, dsh homolog (Drosophila)	0.01
..	↑	A1504305	v104g11-x1	<0.01	..	↑	X99921	S100 calcium-binding protein A13	<0.01
..	↑	D86382	Allograft inflammatory factor 1	<0.01	..	↑	AF024637	Tyrosine kinase binding protein	<0.01
..	↑	AF068182	B cell linker protein BLNK	<0.01	↓	..	X65635	Melanocortin 1 receptor	0.04
..	↑	M14343	Protein tyrosine phosphatase receptor type C (CD45R)	<0.01	Structural proteins and skeletal development				
..	↑	X01838	β2 microglobulin	<0.01	..	↑	A1835858	Similar to A44131 tropomyosin β 2	<0.01
..	↑	AJ242663	Cathepsin Z precursor	<0.01	↑	↑	X53929	Decorin	<0.01
..	↑	L08115	CD9 antigen	<0.01	↑	↑	X02801	Glial fibrillary acidic protein	<0.01
↑	↑	A1850558	UI-M-BG1-ajj-e-07-0-UI-s1	<0.01	↑	↑	M14044	Calpactin I heavy chain (p36)	<0.01
↑	↑	AB015978	Oncostatin receptor	<0.01	Blood coagulation				
↑	↑	U18869	Mitogen-responsive 96 kDa phosphoprotein p96	0.01	↑	..	AJ002390	Annexin 8	0.03
↑	↑	X03505	Serum amyloid A 3	0.01	↑	..	AJ001633	Annexin III	0.03
↑	↑	M19681	Small inducible cytokine A2	<0.01	Not determined				
↑	↑	U49513	Small inducible cytokine A9	0.01	↑	..	AW050056	UI-M-BH1-amm-d-12-0-UI-s1	0.02
↑	↑	M22531	Complement C1q B chain	<0.01	↑	..	U73478	Acidic nuclear phosphoprotein 32	<0.01
↑	↑	M27960	Interleukin-4 receptor (secreted form)	<0.01	↑	..	A1323533	mp08c06-x1	<0.01
↑	↑	X13986	Osteopontin	<0.01	↑	..	AA684508	U22 small nucleolar (RNU22)	<0.01
Metabolism				↑	..	A1837104	UI-M-AK0-adc-d-07-0-UI-s1	0.02	
↑	..	V00835	Metallothionein 1	0.01	↑	..	AW045664	UI-M-BH1-ajg-h-10-0-UI-s1	0.03
↑	..	K02236	Metallothionein 2	<0.01	↑	..	A1747163	ul12e09-x1	0.04
↑	..	U19118	Fanconi anaemia, complementation group A (FANCA)	<0.01	..	↑	AW124337	UI-M-BH2-1-apq-f-08-0-UI-s1	0.01
↑	..	AW125516	Glucocorticoid receptor interacting protein 1	0.04	..	↑	AW122271	UI-M-BH2-2-aov-h-12-0-UI-s1	0.02
↑	..	U39060	Guanylin mRNA	0.04	..	↑	AA710564	vt43b11-r1	<0.01
↑	..	M95175	Interferon γ inducible protein 30	0.04	..	↑	A1465845	vw50c09-y1	<0.01
↑	..	U51000	Distal-less homeobox 1	0.04	..	↑	AW047476	Guanylate nucleotide binding protein3	0.01
↑	..	A1132207	Eukaryotic translation initiation factor 1A (EIF1A)	0.01	..	↑	M93275	Adipose differentiation related protein	<0.01
..	↑	AJ223208	Transcription factor LRG-21	<0.01	..	↑	M31775	Cytochrome β 558	<0.01
..	↑	L39879	Cathepsin S, partial	<0.01	..	↑	X15986	β-galactoside specific lectin (14 kDa)	<0.01
..	↑	A1844520	Ferritin light chain 1	<0.01	..	↑	X67809	Peptidylprolyl isomerase C-associated protein	0.01
..	↑	AA606587	Adenylosuccinate lyase	<0.01	..	↑	A1842277	UI-M-AM1-afz-e-02-0-UI-s1	0.01
..	↑	M19279	β-glucuronidase structural	<0.01	..	↑	A1845580	UI-M-AQ1-adx-c-01-0-UI-s1	<0.01
..	↑	U06119	Cathepsin H	0.01	↑	↑	AF020313	Amyloid β precursor protein-binding	<0.01
↓	..	J02980	Alkaline phosphatase 2	<0.01	↑	↑	AW046124	Cytochrome b-245, α polypeptide	<0.01
Transport				↑	↑	A1841894	UI-M-A00-acd-d-09-0-UI-s1	0.01	
↑	..	Y12577	ADP-ribosylation-like 4	0.03	↓	..	AB006960	mRECK	0.04
↑	..	X78445	Cyp1-b-1 mRNA for cytochrome P450	<0.01	↓	..	AW048272	SH3-binding domain glutamic acid-rich protein	<0.01
..	↑	AJ006306	Skeletal muscle calcium channel, γ-subunit	0.04	↓	..	A1846672	UI-M-AN1-afi-d-02-0-UI-s1	0.01
..	↑	L10244	Spermidine/spermine N1-acetyl transferase	<0.01	↓	..	A1845438	UI-M-AO1-aej-d-10-0-UI-s1	0.03
..	↑	A1844507	Zinc finger binding protein	0.01	↓	..	A1854265	UI-M-BH0-ajs-c-02-0-UI-s1	0.04
..	↑	X60367	Retinal binding protein1, cellular	<0.01					
↓	..	M24417	P glycoprotein 3	0.01					

↑=increased expression, ↓=decreased expression. p values based on contrast statements from repeated-measures ANOVA model.

Table 3: Genes differentially regulated after injurious ischaemia

maintained in MEM for 48 h). The proportion of cell death was assessed by staining with propidium iodide.

Whole-cell recordings on cultured rat cortical neurons were made as described previously.¹¹ Extracellular solution contained 140 mmol/L sodium chloride, 5.4 mmol/L potassium chloride, 1.3 mmol/L calcium chloride, 1.0 mmol/L magnesium chloride, 25 mmol/L Hepes, 33 mmol/L glucose, pH 7.4 (320–335 mmol/L osmolarity). Patch electrodes contained 140 mmol/L potassium fluoride, 2.0 mmol/L magnesium chloride, 1.0 mmol/L calcium chloride, 10 mmol/L Hepes, 11 mmol/L EGTA, 4 mmol/L magnesium ATP, pH 7.3

(300 mmol/L osmolarity). Potassium currents were recorded in voltage-clamp configuration with a holding potential of –60 mV. Currents were activated by 200 ms step depolarisation with increments of 10 mV. Tetrodotoxin (1 μmol/L) and nimodipine (5 μmol/L) were added to extracellular solutions to block voltage-gated sodium-ion and L-type calcium-ion channels. To limit current variation due to size differences among neurons, current density was calculated by normalising the current amplitude to cell capacitance. Access resistance and leak current were also monitored online with pClamp software. In general, only the cells with leak

24 h Genbank number	Description	p	24 h Genbank number	Description	p
Defence/stress response					
↑ D86382	Allograft inflammatory factor 1; Iba1	0-01	↓ A1852849	Integrin β 1 binding protein 1	<0-01
↑ AB015978	Oncostatin receptor	0-02	↓ L06039	Platelet/endothelial cell adhesion molecule	0-02
↓ A1841295	Glutathione S-transferase	0-03	↓ D28818	NBL4, complete cds	<0-01
↓ X64224	CD23-Fc receptor, IgE, low affinity II, α polypeptide	<0-01	Signal transduction/synaptic transmission		
↓ X53526	CD48/Bcm-1 mRNA for BCM1 antigen	<0-01	↓ AJ250491	Receptor activity modifying protein 3	0-02
Metabolism					
↑ A1153791	Zinc finger binding protein 30	<0-01	↓ M94450	Growth factor receptor bound protein 7	0-01
↑ D86726	Mini chromosome maintenance deficient 6	0-04	↓ X58289	Protein tyrosine phosphatase, receptor type, B	0-01
↓ AA409481	Proteasome 26S subunit, ATPase 3 (PSMC3)	0-02	Structural proteins and skeletal development		
↓ AF061270	Homeobox protein PKNOX1	<0-01	↑ X02801	Glial fibrillary acidic protein	<0-01
↓ AF093669	Peroxisomal biogenesis factor (Pex11a)	0-04	↑ D00613	Matrix γ-carboxyglutamate (gla) protein	0-01
↓ A1844374	Ubiquitin carboxyl terminal hydrolase 7	0-04	↓ AJ131711	Troponin T	<0-01
↓ AJ223966	N-acetyl galactosaminidase, α	0-02	Not determined		
↓ AW122036	MAF2- transcription factor	0-02	↑ AA711704	vu58c09-r1	0-04
↓ D00851	Tcp-1	0-01	↑ AA816121	vp44a01-r1	0-01
↓ M13352	Thymidylate synthase	<0-01	↑ AF083497	untitled	0-04
↓ U20257	Alcohol dehydrogenase 3 complex	<0-01	↑ A1788018	ul16c04-y1	<0-01
↓ U37386	Carboxyl ester lipase	0-03	↑ Y08361	RIL protein, reversion induced LIM gene	0-01
↓ X02578	Pancreatic α-amylose (pCEPa12)	0-049	↑ AW047476	Guanylate nucleotide binding protein 3	0-02
↓ U68535	Aldo-keto reductase	0-03	↓ A A426714	ve24c08-r1	0-03
Transport					
↓ AB030912	RING finger protein HAC1	0-03	↓ AA863768	Era (G-protein)-like 1	0-03
↓ A1844246	FXD domain-containing ion transport regulator 7	<0-01	↓ A1006319	ua70b05-r1	0-03
↓ A1845855	Zinc finger protein 289	0-01	↓ A1325802	mn24a09-y1	0-02
↓ D85037	Doc2β	0-01	↓ A1839004	UI-M-ANO-acm-c-04-0-UI-s1	0-01
↓ L22218	Potassium voltage-gated channel, Kv1-5	0-02	↓ AW048854	UI-M-BH1-amf-b-08-0-UI-s1	0-02
↓ X66117	Glutamate receptor, ionotropic, kainate 2 (β 2)	0-01	↓ AW048916	UI-M-BH1-amo-b-04-0-UI-s1	<0-01
↓ A1844507	Zinc finger binding protein	<0-01	↓ AW049139	UI-M-BH1-amq-b-08-0-UI-s1	0-02
Cell-cycle regulation/apoptosis					
↑ X16834	Mac-2 antigen	<0-01	↓ AW120628	UI-M-BH2-3-anz-a-04-0-UI-s1	0-03
↑ X84797	Haemopoietic specific protein 1	<0-01	↓ AW120971	UI-M-BH2-3-aod-d-08-0-UI-s1	0-02
↓ AF030131	Plenty of SH3s (POSH)	<0-01	↓ AW125120	Mitochondrial ribosomal protein S25	0-02
↓ AW045323	UI-M-BH1-akn-g-12-0-UI-s1	0-01	↓ AW125318	UI-M-BH2-1-apy-e-04-0-UI-s1	0-03
↓ X66449	Calcyclin	0-01	↓ C80836	est	0-04
↓ U19597	Cdk4 and Cdk6 inhibitor p19 protein mRNA	<0-01	↓ U68058	Secreted frizzled-related sequence protein 3	<0-01
↓ X76858	E4F transcription factor 1	<0-01	↓ AW050056	UI-M-BH1-amm-d-12-0-UI-s1	0-02

↑=increased expression, ↓=decreased expression. p values based on contrast statements from repeated-measures ANOVA model.

Table 4: Genes differentially regulated after preconditioning followed by injurious ischaemia

current of less than 0.1 nA at -60 mV and series resistance less than 12 MΩ were included in data analysis. Whole-cell capacitance and series resistance were electronically compensated after the measurement.

Blood tests

Bleeding times were measured 72 h after 15 min sham (n=4) or actual (n=6) occlusion of the middle cerebral artery. Unhandled control mice (n=5) were also included. The first 3 mm from the tip 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 min was allowed (50% of mice subjected to 15 min occlusion of the middle cerebral artery reached the maximum time).

Blood was collected 72 h after preconditioning from five mice and from nine unhandled control mice into

microtainer tubes coated with edetic acid for counting of platelets with a Cell Dyne 3500R counter (Abbott Laboratories, Chicago, IL, USA).

Role of the funding sources

The funding sources had no role in any feature of study design, collection, analysis, or interpretation of data, writing of the report, or the decision to submit it for publication.

Results

In the absence of preconditioning, severe ischaemia caused extensive infarction of the ipsilateral cortex and striatum (figure 1). With preconditioning, the infarct was much smaller (14% [SE 3] vs 47% [4]; proportional difference 70%, p<0.0001). The neuroprotective effect of ischaemic preconditioning was largely confined to the cortex, and the area of damage was localised to the

Gene	Preconditioning				Injurious ischaemia				Preconditioning and injurious ischaemia at 24h	
	24 h		72 h		24 h		72 h		Array	PCR
	Array	PCR	Array	PCR	Array	PCR	Array	PCR		
OPN	NC	↑	NC	↑	↑	↑	↑	↑	NC	↑
GFAP	NC	NC	↑	↑	↑	↑	↑	↑	↑	↑
Calpactin	NC	↑	↑	↑	↑	NC	↑	↑	NC	NC
FXD7	NC	NC	NC	NC	NC	NC	NC	↓	↓	↓
Alcohol dehydrogenase	NC	↓	NC	↑	NC	↑	NC	↓	↓	NC
NAGA	NC	NC	NC	↑	NC	↑	NC	↓	↓	NC
TNFRp55	NC	NC	NC	↑	NC	↑	↑	↑	NC	NC

NC=no change; ↑=increased. ↓=decreased.

Table 5: Validation of gene regulation by real-time PCR

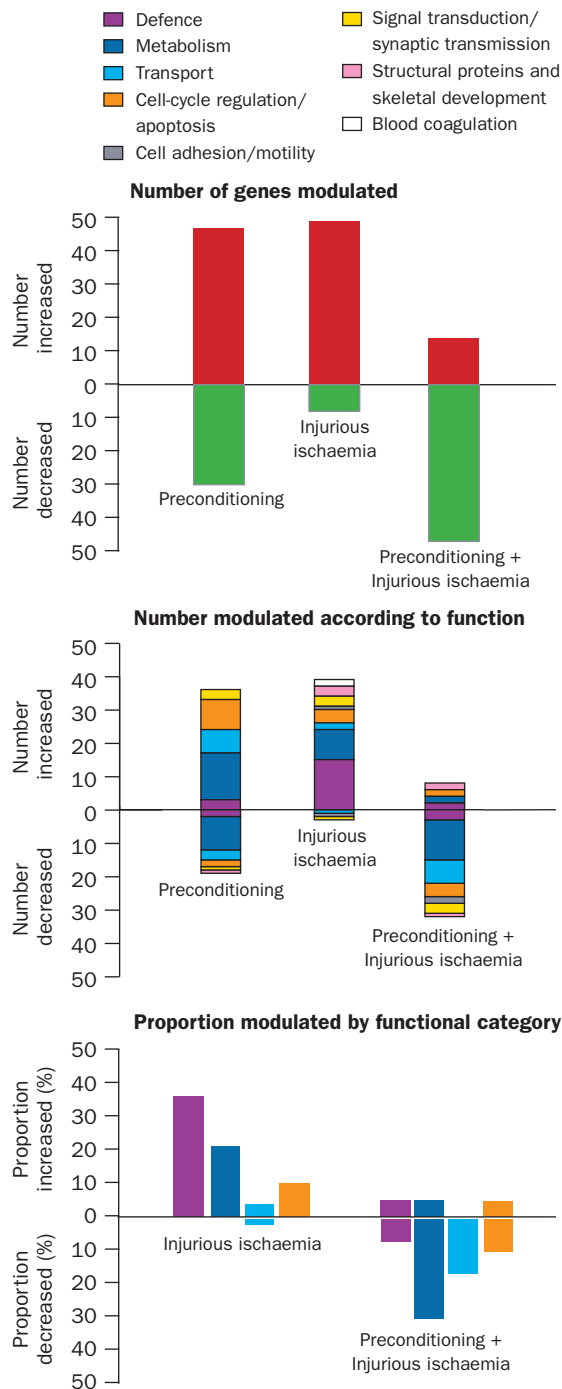


Figure 2: **Effect of preconditioning on genetic response to ischaemia**

Data shown do not include genes for which no functional information was available.

striatum. The contralateral (non-ischaemic) hemisphere showed no sign of damage after ischaemia.

To define the transcriptomes of ischaemic injury and ischaemic tolerance, we isolated RNA from the region of the cortex that in the absence of preconditioning is damaged by injurious ischaemia. High-density DNA microarray analyses showed that of the roughly 7500 genes interrogated, 267 showed differential regulation after preconditioning alone, injurious ischaemia, or preconditioning followed by injurious ischaemia compared with the non-ischaemic hemisphere (tables 2–4;

<http://www.ohsu.edu/gmsr/bbc/miame/stenzelpoore.html>). The inherent limitations of microarray gene expression data include non-normal distribution, heterogeneity of variances, limited number of replications, and issues of multiple comparisons. Accordingly, we relied on real-time PCR analyses to validate the microarray results. With the aforementioned criteria for gene selection there was very little overlap among genes regulated in the three conditions (figure 1). Validation of gene regulation was done on a subset of genes. With 35 determinations (seven genes and five experimental conditions) we found a false-positive rate of 8% and a false-negative rate of 40% in our gene selection (table 5). Thus, the criteria used for gene selection in this study limit the proportion of false-positive results while accepting substantial numbers of false-negative results.

Ischaemic preconditioning alone and injurious ischaemia alone each caused pronounced upregulation of gene expression. By contrast, preconditioning preceding injurious ischaemia resulted in striking downregulation of gene expression. Modulation of gene expression after injurious ischaemia showed that of the genes regulated, 49 (86%) were increased in expression compared with the non-ischaemic hemisphere (figure 2). By contrast, preconditioning preceding injurious ischaemia resulted in decreased expression in 47 (77%) of the regulated genes. This effect was not general, since housekeeping genes such as *GAPDH* and actin were not differentially regulated (data not shown) and 23% of the genes regulated in this condition were increased. This finding suggests that a previous stimulus of preconditioning alters the transcriptional response to injurious ischaemia via a specific pattern of gene suppression.

We then analysed each expression pattern according to gene function. We attributed the putative function of the modulated genes from the Jackson Laboratories Mouse Genome Database (<http://www.informatics.jax.org/>), the Stanford-Online Universal Resource for Clones and ESTs Website (<http://genome-www5.stanford.edu/cgi-bin/SMD/source/>), and literature review. Genes of unknown function represented about 30% of the response in each experimental condition. Among those genes for which functions have been assigned, the composition of the transcriptional response varied substantially with the ischaemic stimulus. The transcriptional response to injury by damaging ischaemia was dominated by upregulation of genes that coordinate immune responses and host defence (36% of genes with identified function; defence in figure 2). This finding contrasted with the response to preconditioning alone, when the global gene expression pattern was predominated by increased expression of genes involved in metabolic functions that affect cellular homeostasis (25%; metabolism in figure 2) and regulation of the cell cycle (16%).

The most striking difference among the gene profiles was in the response to ischaemic injury after preconditioning. In this setting, the overall transcriptional response to injury was downregulated, and most of the genes suppressed involved pathways that regulate metabolism, molecular transport, or cell-cycle control (30%, 17%, and 10%, respectively; figure 2). Thus, the response to injury in the context of preconditioning is disposed towards dampened cellular activity. Moreover, our results suggest that a preconditioning stimulus reprogrammes the subsequent response to ischaemia. A comparison of a subset of genes regulated in injurious ischaemia showed that the nature of the response is changed substantially by previous preconditioning. The response to ischaemic injury in the absence of preconditioning included heightened expression of genes

Prec 24 h	72 h	Prec + inj isch	Genbank number	Description	Prec 24 h	72 h	Prec + inj isch	Genbank number	Description
ATP use reduction-energy metabolism					ATP use reduction-protein processing				
↓	AF17175	Carnitine palmitoyltransferase 1	↓	AF061555	Ubiquitin protein ligase 3
↓	U89924	Protein phosphatase 1 binding protein	↓	AW060509	Torsin family member B
..	↓	..	Y14334	Arachidonate 12-lipoxygenase 12R	↓	L07063	FKBP65 binding protein
..	↓	..	X02578	Pancreatic α-amylase pCEPa12	..	↓	..	D12713	Secretory protein sec 23
..	..	↓	U37386	Carboxyl ester lipase	↓	AA409481	Proteasome 26s subunit ATPase 3
..	..	↓	Y14334	Peroxisomal biogenesis factor	↓	A1845855	ZFP289
..	..	↓	AF004108	Aldo-keto reductase	↓	A1844507	Zinc finger binding protein
..	..	↓	A1841295	Glutathione S transferase	Hypocoagulation				
..	..	↓	AJ223966	N-acetyl galactosaminidase	↑	M34141	Cyclo-oxygenase 1
..	..	↓	U20257	Alcohol dehydrogenase 3	..	↑	..	D50384	cytochrome P450
..	..	↓	AF004108	Arylalkylamine N-acetyltransferase	↓	L06039	PECAM
ATP use reduction-channel arrest					Immunosuppression				
↓	D13266	Glutamate receptor, ionotropic, δ 2	↓	A1840733	Prostaglandin D2 synthase
↓	U19880	Dopamine receptor 4	↓	L06039	PECAM
↑	↓	..	M75135	Glut-3	↓	X64224	CD23
..	↓	..	AA414964	Vc49a08.r1-cation transport ATPase family member	↓	X53526	CD48
..	↓	..	U51126	Girk2c	↓	D28818	NBL4
..	↓	..	D37793	Synaptotagmin 2	↓	A1852849	Integrin β 1 binding protein
..	..	↓	D85037	Doc2β					
..	..	↓	L22218	Potassium voltage-gated channel Kv1-5					
..	..	↓	A1844246	Ion transport regulator 7 (FXD7)					
..	..	↓	X66117	Glutamate receptor, ionotropic, kainate 2					

↑=increased expression; ↓=decreased expression. Prec=preconditioning. Inj isch=injurious ischaemia.

Table 6: Genes regulated suggestive of neuroprotective aspects of hibernation and hypoxia-tolerant states

required for defence and repair (eg, cyclo-oxygenase 2; osteopontin [OPN]; complement components; heat-shock proteins 10, 70, and 105), whereas preconditioning reprogrammed the response to ischaemia such that only a minor component involved defence and repair, and the net effect was reduced metabolic activity and transport (eg, N-acetyl galactosaminidase, carboxyl ester lipase, alcohol dehydrogenase, potassium voltage-gated channel Kv1-5, kainate receptor, Doc2β).

The transcriptional response to preconditioning alone or followed by injurious ischaemia had a profile that suggested

suppression of cellular energy use and attenuation of ion-channel activity. Similar changes are also observed when oxygen availability is limited (eg, hibernation, anaerobiosis, torpor, and AESTIVATION).¹² In such cases, the preservation of cellular homeostasis during oxygen deprivation is achieved through the controlled arrest of cellular functions,⁸ which may occur via a common molecular mechanism of metabolic depression.^{8,12} Several features associated with hibernation or tolerance of hypoxia could contribute to neuroprotection during limited oxygen availability, such as metabolic suppression, inhibition of protein synthesis,

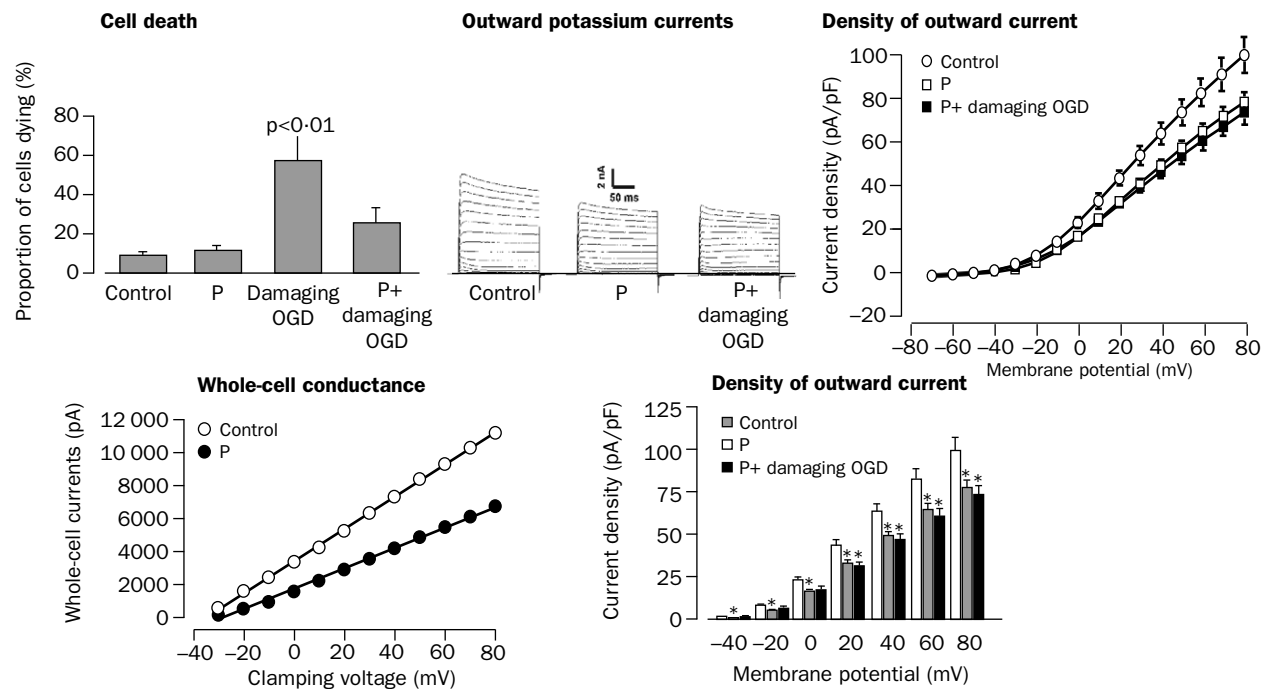


Figure 3: Effect of preconditioning (P) and oxygen and glucose deprivation (OGD)

Rat cortical neuronal cells were exposed to oxygen and glucose deprivation for 30 min (preconditioning), 120 min (damaging OGD), or 30 min followed 24 h later by damaging OGD. For cell death, values are mean and SE (n=6); p value by one-way ANOVA with Bonferroni multiple comparison post-hoc analysis. For density of outward currents at various membrane potentials, *p<0.05 by Student's t test (unpaired) to compare points on different curves, but activated by stepping to the same potential.

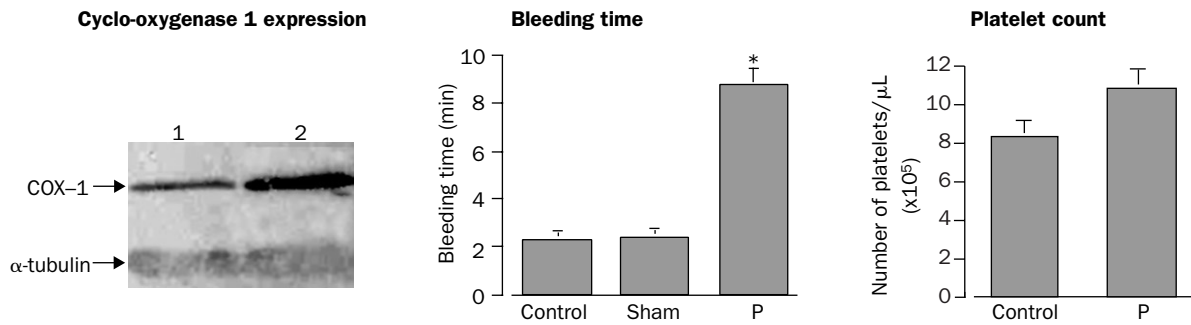


Figure 4: **Ischaemic preconditioning (P), bleeding time, and expression of cyclo-oxygenase 1 (COX-1)**

Western blot of cyclo-oxygenase 1 expression in preconditioned mice. Lane 1=non-ischaemic hemisphere; lane 2=ischaemic hemisphere. α -tubulin was included as loading control. Bleeding times and platelet counts are group mean and SE; * $p < 0.05$ by one-way ANOVA with Student-Newman-Keuls post-hoc analysis.

inhibition of channel function (referred to as “CHANNEL ARREST”), immunosuppression, and hypocoagulation.^{7,8,13}

We found that preconditioning elicited changes in a substantial number of genes (table 6), the functions of which can also lead to a similar state of controlled cellular arrest to that found in hibernation or tolerance of hypoxia. Many changes are seen in genes that would be predicted to induce metabolic suppression, which is consistent with the fact that oxygen limitation leads to lower energy utilisation due partly to decreased demand.⁸ Several genes were suppressed that would be expected to influence cellular energetics via changes in protein utilisation, ion permeability, and energy metabolism. There was also suppression of several genes involved in protein processing; thus, the energy demands of protein turnover could be reduced after preconditioning.

We used an in-vitro neuronal culture system of oxygen and glucose deprivation to model ischaemia/hypoxia. Preconditioning attenuated cell death caused by hypoxia of long duration by about 50% (figure 3). This protective effect in vitro was blocked by inhibition of new protein synthesis (data not shown), which is similar to results reported in vivo⁴ and suggests that gene expression must be activated to confer protection against ischaemic challenge.

To assess ion-channel function, we used patch-clamp techniques to measure voltage-gated potassium currents and whole-cell conductance in cells subjected to preconditioning oxygen and glucose deprivation (figure 3). In most neurons that have been recorded, membrane depolarisations activate outward current that consists of a slow-activating sustained component (probably through delayed rectifier potassium channels) and fast-decaying transient current (probably through A-type potassium channels). There was a significant decrease in whole-cell potassium currents and in current density in neurons 24 h after the preconditioning oxygen and glucose deprivation compared with control cells. Potassium current was significantly lower across membrane potentials from -40 mV to 80 mV. At 0 mV, for example, the density of potassium current was decreased from 22.8 pA/pF (SE 2.02) in control neurons ($n=21$) to 16.0 pA/pF (1.41) in preconditioned neurons ($n=19$, $p < 0.05$). Similarly, cells that received a preconditioning stimulus followed by injurious hypoxia also showed decreased outward potassium currents.

Whole-cell conductance in both control and preconditioned neurons was measured by linear regression of the current-voltage relation between -30 mV and 80 mV. The slope conductance was 82.9 nS (4.6) in control neurons and 69.1 nS (4.9) in neurons subjected to preconditioning oxygen and glucose deprivation ($p < 0.05$). Together, our findings show that potassium-channel activity was suppressed after brief non-injurious

oxygen and glucose deprivation, similar to adaptations found in cortical neurons of hypoxia-tolerant species. Our microarray analyses showed that expression of several genes involved in ion transport, including the voltage-gated potassium channel subunit, Kv1.5, was lower in brains given preconditioning followed by injurious ischaemia (table 6)—a finding consistent with our results that preconditioning lowers whole-cell conductance and potassium-channel activity in vitro.

Hibernation is accompanied by a pronounced (about 90%) decrease in blood flow,¹⁴ similar to that seen in ischaemia. Typically, states of low blood flow increase the risk of thrombus formation due to platelet aggregation and fibrin deposition; however, longer blood clotting times during hibernation keep this risk to a minimum, providing a neuroprotective strategy during these periods of low blood flow.¹³ Prolongation of clotting can be achieved through various means, including decreased numbers of platelets¹⁴ and changes in the intrinsic clotting cascades.¹⁵ Our microarray analysis showed that cyclo-oxygenase 1, which is known to increase vasodilation and inhibit platelet aggregation via its involvement in the synthesis of prostacyclin,^{16,17} is upregulated after preconditioning. Furthermore, we found that concentrations of cyclo-oxygenase 1 protein increased after preconditioning (figure 4). This finding links hypocoagulation with preconditioning. Aspirin, a known modulator of the cyclo-oxygenase pathways, has been implicated as a chemical preconditioner protecting against hypoxia.¹⁸

We tested this possibility in preconditioned mice; 72 h after the preconditioning, bleeding times were about five times longer than in untreated controls (figure 4). The long coagulation times were not due to a reduction in platelet numbers. Aggregation of platelets is inhibited by prostacyclin,¹⁷ a downstream mediator of the successive actions of the two enzymes, cyclo-oxygenase 1 and prostacyclin synthase. With real-time PCR, we found that expression of prostacyclin synthase was increased during preconditioning (two fold, data not shown), similar to induction of cyclo-oxygenase 1 expression. These results suggested that increased expression of cyclo-oxygenase 1 and prostacyclin synthase lead to increased synthesis of prostacyclin, which, in turn, could influence platelet aggregation and prolong blood coagulation in mice exposed to preconditioning.

Discussion

We found that in three related conditions, brain ischaemia induced distinct gene-response patterns. Furthermore, preconditioning caused pronounced suppression of gene expression in response to a duration of ischaemia that is ordinarily injurious. Such suppression contrasted sharply with the upregulation of mRNA in ischaemic injury alone

(figure 2). Preconditioning reprogrammes the gene response to ischaemia, as shown by the induction of unique gene sets by ischaemia in the presence or absence of preconditioning. Specifically, we found that the genetic response to injurious ischaemia was characterised by upregulation of a large number of genes related to immune defence strategies. After preconditioning, however, the transcriptional response to ischaemic injury was largely suppressed. We were surprised to find that this change was not simply one of lack of response, but rather a reprogramming of the genetic response to ischaemia, whereby downregulated genes control metabolism, cell-cycle regulation, and ion-channel activity. These features mimic specific adaptive neuroprotective strategies seen in hibernation and other hypoxia-tolerant states. Thus, as in hibernation, preconditioning elicits endogenous genetic adaptations that confer tolerance to the injurious effects of oxygen deprivation. Although hypothermia (which is also neuroprotective) can be associated with hibernation, the neuroprotective effect of hibernation is not solely dependent on low brain temperature.¹⁹ Studies on brain slices from hibernating ground squirrels showed striking tolerance to hypoxia at low temperatures, but this protective state remained even in the absence of hypothermia.

The finding that exposure to a brief ischaemic event (preconditioning) causes a complex reprogramming of the cellular responses to ischaemia may reflect a fundamental regulatory mechanism for governing host responses to harmful stimuli. A similar process occurs in the setting of endotoxin exposure. Macrophages exposed to a small dose of endotoxin before a large dose respond differently from macrophages not so primed. This reprogramming capacity is viewed as protective to the host because the outcome is a finely controlled shift in the balance of proinflammatory and anti-inflammatory cytokines.^{20,21} Similarly, preconditioning by exposure to a brief period of oxygen deprivation elicits a state refractory to subsequent injurious ischaemia by a complex reprogramming of gene expression. This adaptive process emphasises the importance of physiological responses that are shaped by previous signals and thereby governed by the context in which they are generated.

Tolerance to oxygen deprivation is a highly evolved defence strategy that suppresses energy turnover through downregulation of pathways that control ATP supply and demand, electrochemical activities, and protein utilisation. Energy turnover is suppressed to about a tenth of normal in some organisms during periods of oxygen limitation.⁸ Hypoxia is associated with depressed protein synthesis, mediated in part via translational mechanisms that affect both initiation and elongation.^{22,23} We found suppression of several genes involved in protein processing, which suggests that the energy demands of protein turnover may be reduced after preconditioning (table 6). The 26S proteasome subunit, ATPase 3, was downregulated, which would be expected to decrease degradation of ubiquitinated proteins. Given the energy requirements of proteasome activity, decreased expression of this subunit might lessen ATP usage. In addition, downregulation of two ubiquitin-system enzymes, ubiquitin-protein ligase 3 and ubiquitin carboxy-terminal hydrolase might reduce protein turnover by decreasing ubiquitinylation of certain proteins required before degradation and limiting regeneration of free ubiquitin, respectively. Decreased expression of these genes could lengthen the half-life of various proteins and lower energy requirements via a decrease in ATP demands, which are characteristically high for this process.

Our microarray analyses also suggest that neuroprotection induced by preconditioning may depend on strategies to suppress energy turnover because expression of many genes

that regulate energy metabolism and protein turnover was decreased. Those genes with known functions that involve energy metabolism lie in enzymatic pathways that use various substrates including aminoacids, carbohydrates, and lipids. Suppression of these genes could lower energy demands and conserve energy. For example, suppression of aldo-keto reductase and alcohol dehydrogenase, enzymes that require a pyridine coenzyme (NAD or NADP) for their function, would lead to decreased energy demand. Suppression of the glycosidase α -N-acetylgalactosaminidase might also limit energy utilisation owing to its putative involvement in neuroaxonal transport.²⁴ Deficiency of this enzyme in human beings has been associated with impaired cerebral glucose metabolism,²⁴ which suggests that decreased expression of this gene in preconditioning could lead to a similar reduction in glucose metabolism. Inhibition of carnitine palmitoyltransferase I (involved in fatty-acid oxidation) is protective in myocardial ischaemia.²⁵ Suppression of this enzyme after preconditioning could lower fatty-acid metabolism, thus lessening demand for ATP—an effect that would influence astrocytes directly since they use fatty acids preferentially for energy production.²⁶ Metabolism in neurons, which relies more heavily on glucose oxidation, could be affected indirectly by suppression of carnitine palmitoyltransferase I, since astrocytes provide energy substrates to neighbouring neurons.

A similar adaptation to ischaemia, referred to as myocardial hibernation, develops in the heart; cardiomyocyte contractile function is downregulated to match oxygen availability in an effort to preserve myocardial viability.²⁷ Thus, suppression of cellular functions as a means of matching oxygen demand with supply may be an adaptation that occurs in response to limited oxygen supply during periods of hibernation and ischaemia.

We speculate that adaptations such as suppressed ion-channel function, which exist in neurons from hypoxia-tolerant species,¹⁹ occur in mammalian neurons after preconditioning. One important means of stabilising cells at much lower than normal rates of ATP turnover occurs through suppression of ion pumping or “channel arrest”, resulting in decreased membrane permeability. It in turn is accompanied by a reduction in the amount of ATP required to maintain ionic homeostasis.²⁸ Studies show that regulated suppression of membrane ion channels and ion pumps supports energy conservation during oxygen limitation^{28,29} and promotes tolerance to hypoxia. We found a significant reduction in the ion permeability of the plasma membrane of neurons made tolerant to ischaemia by preconditioning. Whole-cell conductance was lower and voltage-gated potassium-channel activity was significantly lower in preconditioned cells *in vitro*, supporting the notion that altered channel activity may serve as a neuroprotective mechanism of ischaemic tolerance. Also, we found that several genes encoding receptors or channels that regulate parts of ion transport are downregulated by preconditioning, such as ionotropic glutamate receptors (kainate 2 and delta 2), G-protein coupled inward rectifying K⁺ channel 2c, ion transport regulator 7 (FXD7), and a cation transport ATPase family member (Vc49a08.r1; table 6). Changes in the density of these ion-transport molecules probably modulate electrical gradients across the cell membrane to control excitability and consequently restrict ATP turnover, although we have not yet tested this idea directly.

Decreased blood flow during hibernation is accompanied by neuroprotective adaptations to this “low flow” state, such as reduced numbers of circulating leucocytes and platelets as well as increased clotting times. Such changes could offer protection by suppressing thrombus formation.¹⁹ Our finding

that in the absence of ischaemic injury, preconditioning prolongs bleeding times suggests that this hypocoagulable state mirrors that seen in hibernation. The mechanism of the prolonged clotting time induced by preconditioning is unclear, although alterations in platelet aggregation are probably involved. We have found that preconditioning increases expression of cyclo-oxygenase 1 and prostacyclin synthase; these enzymes act successively to produce prostacyclin, which in turn, inhibits platelet aggregation and vasoconstriction.¹⁷ A beneficial role of cyclo-oxygenase 1 in ischaemic injury is suggested by the finding that mice lacking the enzyme show greater ischaemic brain injury than normal mice, which is attributed to more severe reduction in blood flow during ischaemia.¹⁶ Moreover, the reciprocal outcome occurs with adenovirus-mediated overexpression of cyclo-oxygenase 1 and prostacyclin synthase, which blunts ischaemic injury, presumably by increased production of prostacyclin and subsequent inhibition of platelet aggregation and vasoconstriction.³⁰ Collectively, these studies suggest that cyclo-oxygenase 1 and prostacyclin synthase have inhibitory effects on coagulation that may contribute to neuroprotection in preconditioning and ischaemic injury, and support a link between preconditioning and adaptation to hibernation.

Our studies with microarray analyses contribute to an emerging view of ischaemic tolerance. Preconditioning reprogrammes the response to ischaemia, which leads to a new state refractory to cellular damage. The genetic profile of ischaemic tolerance is characterised by dampened expression of a large number of genes, the functions of which influence glucose metabolism, protein turnover, cell-cycle regulation, and ion-channel abundance. These features, together with increased expression of genes involved in hypocoagulation, mimic hibernation and hypoxia tolerance, which suggests the existence of a conserved endogenous genomic programme of physiological adaptations to oxygen limitation that improve survival. The microarray findings here are in keeping with previous studies of individual genes and pathways proposed to be effectors of ischaemic tolerance.^{3,15} Further inroads to understanding and defining the strategies that lead to reversible cellular arrest may yield novel clues to therapeutic strategies for effective treatment of stroke injury.

Contributors

Mary Stenzel-Poore, Susan Stevens, and Roger Simon devised the studies, and were responsible for design, execution, and preparation of the report. Zhigang Xiong and Xiangping Chu did the patch clamping and neurophysiology studies, Nikola Lessov the mouse surgical procedures, Holly Rosenzweig the bleeding time experiments, Christina Harrington the microarray analysis, and Eric Tobar real-time PCR. Robert Meller produced the in-vitro tolerance model. Tatyana E Shaw made the putative assignment of gene function. Motomi Mori was responsible for statistics.

Conflict of interest statement

CAH has a significant financial interest in Affymetrix Inc.

Acknowledgments

We thank Yi-Ching Hsieh for work on statistical analysis, Martha Johnson for insightful discussion throughout the project, and Clara Schindler for technical assistance. Microarray assays were done in the Affymetrix Microarray Core of the OHSU Gene Microarray Shared Resource; data analysis was provided through the Bioinformatics and Biostatistics Core. This work was supported by National Institutes of Health grants NS39492 (MS-P), NS35965 (RPS), and NS24728 (RPS).

References

- 1 Tasaki K, Ruetzler CA, Ohtsuki T, Martin D, Nawashiro H, Hallenbeck JM. Lipopolysaccharide pre-treatment induces resistance against subsequent focal cerebral ischemic damage in spontaneously hypertensive rats. *Brain Res* 1997; **748**: 267–70.
- 2 Kersten JR, Schmeling TJ, Pagel PS, Gross GJ, Warltier DC. Isoflurane mimics ischemic preconditioning via activation of K(ATP) channels:

reduction of myocardial infarct size with an acute memory phase. *Anesthesiology* 1997; **87**: 361–70.

- 3 Dirnagl U, Simon RP, Hallenbeck JM. Ischemic tolerance and endogenous neuroprotection. *Trends Neurosci* (in press).
- 4 Barone FC, White RF, Spera PA, et al. Ischemic preconditioning and brain tolerance. Temporal histological and functional outcomes, protein synthesis requirement, and interleukin-1 receptor antagonist and early gene expression. *Stroke* 1998; **29**: 1937–51.
- 5 Simon RP, Niuro M, Gwinn R. Prior ischemic stress protects against experimental stroke. *Neurosci Lett* 1993; **163**: 135–37.
- 6 Weih M, Kallenberg K, Dirnagl U, Harms L, Wernecke KD, Einhaupl KM. Attenuated stroke severity after prodromal TIA: a role for ischemic tolerance in the brain? *Stroke* 1999; **30**: 1851–54.
- 7 Frerichs KU. Neuroprotective strategies in nature: novel clues for the treatment of stroke and trauma. *Acta Neurochir* 1999; **73**: 57–61.
- 8 Hochachka PW, Buck LT, Doll CJ, Land SC. Unifying theory of hypoxia tolerance: Molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proc Natl Acad Sci USA* 1996; **93**: 9493–98.
- 9 Hill J, Gunion-Rinker L, Kulhanek D, et al. Temporal modulation of cytokine expression following focal cerebral ischemia in mice. *Brain Res* 1999; **820**: 45–54.
- 10 Franklin K, Paxinos G. The mouse brain in stereotaxic coordinates. San Diego: Academic Press, 1997.
- 11 Xiong Z-G, Lu W-Y, MacDonald JF. Extracellular calcium sensed by a novel cation channel in hippocampal neurons. *Proc Natl Acad Sci USA* 1997; **94**: 7012–17.
- 12 Storey KB, Storey JM. Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation. *Q Rev Biol* 1990; **65**: 145–74.
- 13 Drew KL, Rice ME, Kuhn TB, Smith MA. Neuroprotective adaptations in hibernation: therapeutic implications for ischemia-reperfusion, traumatic brain injury and neurodegenerative diseases. *Free Radic Biol Med* 2001; **31**: 563–73.
- 14 Frerichs KU, Kennedy C, Sokoloff L, Hallenbeck JM. Local cerebral blood flow during hibernation, a model of natural tolerance to "cerebral ischemia". *J Cereb Blood Flow Metab* 1994; **14**: 193–205.
- 15 Srere H, Belke D, Wang LC, Martin SL. Alpha 2-macroglobulin gene expression during hibernation in ground squirrels is independent of acute phase response. *Am J Physiol* 1995; **268**: R1507–12.
- 16 Iadecola C, Sugimoto K, Niwa K, Kazama K, Ross ME. Increased susceptibility to ischemic brain injury in cyclooxygenase-1-deficient mice. *J Cereb Blood Flow Metab* 2001; **21**: 1436–41.
- 17 Moncada S. Biology and therapeutic potential of prostacyclin. *Stroke* 1983; **14**: 157–68.
- 18 Kasischke K, Huber R, Li H, Timmler M, Riepe MW. Primary hypoxic tolerance and chemical preconditioning during estrus cycle in mice. *Stroke* 1999; **30**: 1256–62.
- 19 Frerichs KU, Hallenbeck JM. Hibernation in ground squirrels induces state and species-specific tolerance to hypoxia and aglycemia: an *in vitro* study in hippocampal slices. *J Cereb Blood Flow Metab* 1998; **18**: 168–75.
- 20 Haslberger A, Sayers T, Reiter H, Chung J, Schutze E. Reduced release of TNF and PCA from macrophages of tolerant mice. *Circ Shock* 1988; **26**: 185–92.
- 21 Shnyra A, Brewington R, Alipio A, Amura C, Morrison DC. Reprogramming of lipopolysaccharide-primed macrophages is controlled by a counterbalanced production of IL-10 and IL-12. *J Immunol* 1998; **160**: 3729–36.
- 22 Frerichs KU, Smith CB, Brenner M, et al. Suppression of protein synthesis in brain during hibernation involves inhibition of protein initiation and elongation. *Proc Natl Acad Sci USA* 1998; **95**: 14511–16.
- 23 Chen Y, Matsushita M, Naim AC, et al. Mechanisms for increased levels of phosphorylation of elongation factor-2 during hibernation in ground squirrels. *Biochemistry* 2001; **40**: 11565–70.
- 24 Rudolf J, Grond M, Schindler D, Heiss W, Desnick RJ. Cerebral glucose metabolism in type I α -N-acetylgalactosaminidase deficiency: an infantile neuroaxonal dystrophy. *J Child Neurol* 1999; **14**: 543–47.
- 25 Lopaschuk GD, Spafford M. Response of isolated working hearts to fatty acids and carnitine palmitoyltransferase I inhibition during recovery of coronary flow in acutely and chronically diabetic rats. *Circ Res* 1989; **65**: 378–87.
- 26 Esfandiari A, Soifyoudine D, Paturneau-Jouas M. Inhibition of fatty acid beta-oxidation in rat brain cultured astrocytes exposed to the neurotoxin 3-nitropropionic acid. *Dev Neurosci* 1997; **19**: 312–20.
- 27 Heusch G, Schulz R. The biology of myocardial hibernation. *Trends Cardiovasc Med* 2000; **10**: 108–14.
- 28 Buck LT, Hochachka PW. Anoxic suppression of Na⁺-K⁺-ATPase and constant membrane potential in hepatocytes: support for channel arrest. *Am J Physiol* 1993; **265**: R1020–25.
- 29 Ghai HS, Buck LT. Acute reduction in whole cell conductance in anoxic turtle brain. *Am J Physiol* 1999; **277**: R887–93.
- 30 Lin H, Lin T-N, Cheung W-M, et al. Cyclooxygenase-1 and bicentric cyclooxygenase-1/prostacyclin synthase gene transfer protect against ischemic cerebral infarction. *Circulation* 2002; **105**: 1962–69.