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Expression of Signal Transduction Genes Differs after Hypoxic or Isoflurane Preconditioning of Rat Hippocampal Slice Cultures

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Background: **Preconditioning neurons with noninjurious hypoxia (hypoxic preconditioning, HPC) or the anesthetic isoflurane (APC) induces tolerance of severe ischemic stress. The mechanisms of both types of preconditioning in the hippocampus require moderate increases in intracellular Ca^{2+} and activation of protein kinase signaling. The authors hypothesized that the expression of signal transduction genes would be similar after APC and HPC.**

Methods: **Hippocampal slice cultures prepared from 9-day-old rats were preconditioned with hypoxia (5 min of 95% nitrogen/5% carbon dioxide) or 1% isoflurane in air/5% carbon dioxide for 1 h. A day later, cultures were subjected to 10 min oxygen and glucose deprivation (simulated ischemia). Intracellular Ca^{2+}, measured in CA1 neurons at the completion of preconditioning, and cell death in CA1, CA3, and dentate regions was assessed 48 h after simulated ischemia. Message RNA encoding 119 signal transduction genes was quantified with rat complimentary DNA microarrays from pre–oxygen-glucose deprivation samples.**

Results: **Both APC and HPC increased intracellular Ca^{2+} approximately 50 nM and decreased CA1, CA3, and dentate neuron death by about 50% after simulated ischemia. Many signaling genes were increased after preconditioning, with hypoxia increasing more apoptosis/survival genes (8 of 10) than isoflurane (0 of 10). In contrast, isoflurane increased more cell cycle/development/growth genes than did hypoxia (8 of 14 genes, *vs.* 1 of 14).**

Conclusions: **Despite sharing similar upstream signaling and neuroprotective outcomes, the genomic response to APC and HPC is different. Increased expression of antiapoptosis genes after HPC and cell development genes after APC has implications both for neuroprotection and long-term effects of anesthetics.**

PRECONDITIONING the nervous system to tolerate otherwise damaging ischemia has been demonstrated with a wide variety of preconditioning stimuli, with various species of experimental animals, and with different types of ischemic stress. First demonstrated in the brain with noninjurious exposure to hypoxia,[1,2] preconditioning can be induced by thermal stress, excitotoxins such as glutamate, bacterial endotoxins, oxidative stress, neuromodulators, and volatile anesthetics.[3–6] A variety of signals have been associated with preconditioning neuroprotection, particularly mitogen-activated protein kinase signaling pathways (reviewed by Perez-Pinzon[7] and Ran and Sharp[8,9]). Isoflurane preconditioning of the heart is effective in humans,[10] but cerebral protection with isoflurane (APC) or hypoxic preconditioning (HPC) remains an experimental procedure that has not yet been tested in human clinical trials.

It remains unclear if all types of cerebral preconditioning involve common signal transduction and genomic responses. This is a relevant question because it may be possible to elicit the preconditioned phenotypes with more efficacy and lower risk if specific key signals in the preconditioning process are identified. On the basis of work with isolated cortical neurons and hippocampal slice cultures, we have proposed that moderate and noninjurious increases in intracellular Ca^{2+} may be a universal upstream signal in the process of neuroprotective adaptation to preconditioning and gene expression that forms the neuroprotective phenotype.[11] Specifically, we have found similar neuroprotective survival benefit and mitogen-activated protein kinase pathway activation after 50- to 100-nM increases in $[Ca^{2+}]_i$, after preconditioning neurons in hippocampal slice cultures with 1% isoflurane, noninjurious hypoxia, or with low levels of calcium ionophores. In each, blocking the increase in $[Ca^{2+}]_i$ or blocking Ca^{2+}-dependent signaling pathways abrogates preconditioning neuroprotection.[12,13] However, whether the downstream signaling responses during HPC or APC are identical has not been explored. Although both anesthetic and hypoxic preconditioning involves moderate increases in $[Ca^{2+}]_i$, the mechanisms involved in producing the increase in Ca^{2+} are not identical, with hypoxia increasing cytosolic nicotinamide adenine dinucleotide triggering Ca^{2+} liberation from the endoplasmic reticulum[14] and isoflurane activating the intraperitoneal$_3$ receptor or increasing intraperitoneal$_3$ levels in the cell.[11]

The purpose of this study is to test the hypothesis that preconditioning with hypoxia or isoflurane involves similar alterations in the expression of signal transduction genes. This study was designed as a preliminary survey of differences in gene expression to guide further studies that can test specific hypotheses relevant to gene expression and the mechanisms of preconditioning. Rather than examining the entire genome's response to preconditioning, we have focused on signal transduction genes to provide insights into one aspect of the mechanistic differences between the two types of preconditioning neuroprotection and because signal transduction genes have broad effects *via* a number of signaling pathways.

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Materials and Methods

All studies were approved by the University of California San Francisco Committee on Animal Research and conform to relevant National Institutes of Health guidelines.

Preparation of Hippocampal Slice Cultures

Organotypic cultures of the hippocampus were prepared by standard methods[15,16] modified by our laboratory.[17] Briefly, Sprague-Dawley rats (9 days old; Charles River Laboratories, Hollister, CA) were anesthetized with 2–5% isoflurane. The pups were decapitated, and the hippocampi were quickly removed and placed in 4°C Gey's Balanced Salt Solution. Next, the hippocampi were transversely sliced (400-μm-thick) with a tissue slicer (Siskiyou Design Instruments, Grants Pass, OR), and stored in Gey's Balanced Salt Solution at 4°C for 10 min. The slices were then transferred onto 30-mm-diameter membrane inserts (Millicell-CM; Millipore, Billerica, MA), and put into 6-well culture trays with 1.2 ml of slice culture medium per well. The slice culture medium consisted of 50% Minimal Essential Medium (Eagle's with Earle's balanced salt solution), 25% Earle's balanced salt solution, 25% heat-inactivated horse serum (all media were from the University of California at San Francisco cell culture facility) with 6.5 mg/ml glucose and 5 mM KCl. Slices were kept in culture for 7–10 days before preconditioning.

Study Design: Preconditioning Organotypic Cultures of Hippocampus

Preconditioning involved immersing slice cultures of hippocampi in medium bubbled with 95% N_2/5% CO_2 gas for 5 min (HPC) or for 1 h in 1% isoflurane in air 5% CO_2 (APC). The percentages of dead and living neurons remaining in CA1 was assessed 48 h after the simulated ischemia. Twenty-four hours after preconditioning, RNA was extracted for gene array analysis.

Simulation of Ischemia with in vitro *Oxygen–Glucose Deprivation*

In vitro ischemia was simulated by immersion of cultures into glucose-free media bubbled with 95% N_2/5% CO_2 (oxygen/glucose deprivation, OGD). The temperature of the media was 37°C, measured with a thermocouple thermometer. The partial pressure of oxygen, measured with a Clark-type oxygen electrode, was approximately 0–0.2 mmHg. After this insult, the cultures were returned to standard slice culture media.

Measurement of Intracellular Calcium in CA1 Neurons

In separate groups of slices, $[Ca^{2+}]_i$ was measured before, during, and after preconditioning. Estimates of $[Ca^{2+}]_i$ in CA1 neurons in slice cultures were made by using the indicator fura-2-AM and a dual excitation fluorescence spectrometer (Photon Technology International, Birmingham, NJ) coupled to a Nikon (Tokyo, Japan) Diaphot inverted microscope. Slice cultures were incubated with 5–10 μM fura-2-AM plus 1% pleuronic acid for 30 min before measurements. Cultures for these measurements were grown on Nunc Anopore (Nalge Nunc, Rochester, NY) culture tray inserts because of their low autofluorescence at fura-2 excitation wavelengths. Slit apertures in the emission light path were adjusted to restrict measurement of light signals to those coming from the CA1 cell body region. Calibration of $[Ca^{2+}]_i$ was done by using the K_D of fura-2 determined *in vitro* with a Ca^{2+} buffer calibration kit (Invitrogen, Carlsbad, CA). The calibration process involved using the same light source, optical path, and filters as used with the slice culture measurements. The K_D for fura-2 was 311 nM, similar to published values.[18] Background fluorescence (*i.e.*, fluorescence in the absence of fura) was subtracted from total fluorescence signals before calculation of $[Ca^{2+}]_I$, as described previously.[19] Estimates of $[Ca^{2+}]_i$ with this technique are accurate to about $\pm$ 10 nM.[20] Measurements of $[Ca^{2+}]_i$ were made briefly at discrete periods during the preconditioning to avoid photobleaching of fura-2. These were at baseline, at midpoint and termination of preconditioning, and after 10 min washout of preconditioning medium. Peak $[Ca^{2+}]_i$ always occurred at the end of the preconditioning period.

Assessment of Cell Death in Cultured Hippocampal Slice

Cell viability was assessed fluorometrically with propidium iodide (PI) uptake. PI, a highly polar fluorescent dye, penetrates damaged plasma membranes and binds to DNA. Before imaging, slice culture media containing 2.3 μM PI was added to the wells of the culture trays. After 30 min, the slices were examined with a Nikon Diaphot 200 inverted microscope, and fluorescent digital images were taken using a SPOT Jr. Digital Camera (Diagnostic Instruments, Sterling Heights, MI). Excitation light wavelength was 520 nm, and emission was 600 nm. The camera sensitivity and the excitation light intensity were standardized to be identical from day to day. PI fluorescence was measured in the dentate gyrus, CA1, and CA3 regions of the hippocampal slices. Slices were discarded if they showed more than slight PI fluorescence in these regions after 7–10 days in culture. Slices were imaged before OGD (signal assumed to represent 0% cell death) and 2 days after OGD. In previous studies, we found that maximum post-OGD death consistently occurs at about day 2 or 3 and declines over the next 11 days.[17] Serial measurements of PI fluorescence intensity were made in predefined areas (manually outlining CA1, CA3, and dentate separately) for each slice using NIH Image-J software (U.S. National Institutes of

Health, Washington, DC). Thus, cell death was occurred in the same regions of each slice after simulated ischemia. After the measurement of PI fluorescence on the secnd post-OGD day, all the neurons in the slice were killed to produce a fluorescence signal equal to 100% neuron death in the regions of interest. This was done by adding 100 μM potassium cyanide and 2 mM sodium iodoacetate to the cultures for at least 20 min. One hour later, final images of PI fluorescence (equated to 100% cell death) were acquired. Percent of dead cells 48 h after OGD were then calculated on the basis of these values. PI fluorescence intensity is a linear function of cell death.[16,21]

Cell Death Statistical Analysis

The percentage survival of neurons in the different regions of the slices may not be normally distributed. Therefore, the Kruskal-Wallis test followed by the Mann–Whitney U-test (JMP; SAS Institute, Cary, NC) was used to compare the medians of different treatment groups. *t* Tests or ANOVA were used to compare other group means, and allowance was made for multiple comparisons (Tukey-Kramer multiple comparison or Dunnett's test). Differences were considered significant for $P < 0.05$.

Microarray Analysis

RNA for microarray analysis was extracted from slice cultures 24 h after mock preconditioning (control), hypoxic preconditioning, and isoflurane preconditioning as follows. Pooled tissue slices (12–18) were homogenized in 1 ml of TriZol reagent. The RNA was precipitated from the aqueous phase with isopropyl alcohol, rinsed with 75% ethanol, and then resuspended in diethyl-pyrocarbonate–treated water. RNA was further purified by means of the ArrayGrade Total RNA isolation kit (SuperArray; SA Biosciences, Frederick, MD) and concentrated down to a final volume of 50 μl in RNASE-free water.

Complementary DNA was synthesized using 0.1 to 2 μg of total RNA by means of the TrueLabeling LinearRNA Amplification Kit (SuperArray). From this complementary DNA, an amplified Biotin-Labeled cRNA was synthesized. Biotinylated URIDINE TRIPHOSPHATE was obtained from Roche Applied Science (Indianapolis, IN). The complementary DNA synthesis reaction was incubated overnight at 37°C. The cRNA was then purified using spin columns from SuperArray's cRNA Cleanup Kit. Quality and concentration of cRNA was determined by absorbance of 260 nm and 280 nm light.

The cRNA was hybridized onto Oligo GEArrays at 60°C overnight with continuous agitation. The arrays used were Rat Signal Transduction Pathway Finder Microarrays ORN-14, ORN-14.2, and Rat Apoptosis Microarray ORN-12 from SuperArray. Table 1 contains a listing of all the genes on the arrays. After rinsing in wash buffers, the arrays were probed using a chemiluminescence method. Arrays were exposed to high performance chemiluminescence film (Hyperfilm; ECL, Amersham, South San Francisco, CA) and developed in a mechanical darkroom developer. Films were scanned at the highest pixel density (1200 dpi ouridine triphosphateut resolution) for analysis.

Statistical Analysis of Array Data

Array scans were analyzed using the Internet-based GEArray Expression Analysis Suite provided by SuperArray. All genes were normalized to a series of "housekeeping" gene expression levels and a group of synthetic control sequences included on the array by the manufacturer. For background normalization, a pair of blank spots and local background correction for each tetra spot was employed. Gene expression was considered significant if

Table 1. List of Genes on the ORN-14 Microarray

Mitogenic pathway	Egr1 (egr-1), Fos, Jun (c-jun), Nab2
Wnt pathway	Cdh1, Ccnd1 (cyclin D1), Fgf4, Jun, Lef1, Myc (c-myc), Pparg, Tcf7, Vegf, Vegfc, Wisp1, Wisp2
Hedgehog pathway	Bmp2, Bmp4, Mo-En-1 (engrailed), Foxa2, Hhip, Ptch, Ptch1, Wnt1, Wnt2, Wsb1
TGFβ pathway	Cdkn1a (p21^{Waf1}, p21^{Cip1}), Cdkn1b (p27), Cdkn1c (p57^{Kip2}), Cdkn2a (p16^{Ink4}), Cdkn2b (p15^{Ink2b}), Cdkn2c (p18, cdk4 inhibitor), Cdkn2d (p19)
Survival pathway	PI3 Kinase/AKT Pathway: Bcl2, Ccnd1, Fn1 (fibronectin), Jun, Mmp7 (matrilysin), Myc, Pten; Jak/Src Pathway: Bcl2, Bcl2l1.NFκB Pathway: Bcl2a1, Birc1b, Birc3, Birc7, Tert
p53 pathway	Bax, Cdkn1a, Ei24 (Pig8), Gadd45a, Igfbp3, Mdm2, Tnfrsf10b (TrailR/DR5), Tnfrsf6
Stress pathway	Atf2, Fos, Hsf1 (tcf5), Hspb1 (hsp25), Hspca, Hspcal3, Myc, Tp53 (p53)
NFκB pathway	Ccl20, Cxcl1, Icam1, Ikbkb, Il1a, Il2, Lta (TNFβ), Nfκb1 (NFκB), Nfκbia (IkBα), Nos2 (iNOS), Pecam, Tank, Tnf (TNFα), Vcam1
NFAT pathway	Cd5, Il2, Tnfsf6 (FasL)
CREB pathway	Cyp19a1 (aromatase p450), Egr1, Fos
Jak-Stat pathway	Csn2 (β-casein), Cxcl9 (Mig), Il4, Il4r, Irf1, Mmp10 (stromelysin-2), Nos2 (iNOS), Pzp
Estrogen pathway	Bcl2 (Bcl-2), Brca1, Ctsd (cathepsin D), Egfr, Igfbp4, Pgr (PR), Trim25
Androgen pathway	Cdk2, Cdkn1a (p21^{Waf1}/p21^{Cip1}), Egfr, Klk3 (Klkb1), Ngfg, Tmepai (N4wbp4), TMPRSS9
Calcium and protein kinase C pathways	Csf2 (GM-CSF), Fos, Il2, Il2ra, Jun, Myc, Odc1, Prkca, Prkcb1, Prkce, Tfrc
Insulin pathway	Cebpb, Fasn, Gys1, Gys2, Hk2, Lep (Ob)
LDL pathway	Ccl2 (Scya2/mcp-1), Csf2, Sele (ELAM-1), Selp (P-selectin), Vcam1
Retinoic acid pathway	Ctsd, Mo-En-1, Hoxa1, Hoxb1, Rbp1 (CRBPI), Rbp2 (CRABPII), Stra6

CREB = cyclic adenosine monophosphate response element beta; LDL = low-denisity lipoprotein; NFAT = nuclear factor of activated T cells; NF-κB = nuclear factor κB; TGFβ = tumor necrosis factor β.

there was a minimum 1.5-fold increase or decrease over the control tissue level.

Quantitative Polymerase Chain Reaction Analysis

RNA was extracted from pooled (12–18) hippocampal slices with the trizol/chloroform method, precipitated with isopropanol, washed with 75% ethanol in diethyl-pyrocarbonate–treated water, and resuspended in volumes of 23 or 40 μl in diethyl-pyrocarbonate–treated water. RNA samples were treated with DNAse I (Invitrogen, Carlsbad, CA) for 15 min (room temperature), heat inactivated for 10 min at 65°C in 25 mM EDTA. Reverse transcription for complimentary DNA was done using the Omniscript RT reagent (Quiagen, Valencia, CA). Quantitative polymerase chain reaction (qPCR) was done after labeling the nucleotides with SYBR Green (QuantiTect, Qiagen). A total volume of 25.0 μl of SYBR/RNAse–free water, primers, and template was used in each qPCR. SA Biosciences (Frederick, MD) supplied primers for Birc3 (PPR06459A-200), cJun (PPR53221A), and cMyc (PPR45580A-200). The "housekeeping" genes used for normalizing gene expression was GAPDH or α-actin. The polymerase chain reaction was performed in a Stratagene (La Jolla, CA) Mx300 thermocycler. The thermal profile used was: 95°C for 10 min, 95° for 15 s, and 60°C for 1 min for 40 cycles.

Western Blots

Western blots of proteins from culture homogenates were performed with standard methods. Five to eight slices were pooled for each assay, and each study was repeated 3–4 times. Samples were obtained 24 h after preconditioning. Protein content in each sample was measured (Bradford protein assay with Coomassie blue) and adjusted so that equal amounts of protein were applied to each lane. Protein bands were visualized after incubation with biotinylated secondary antibodies followed by an enhanced chemiluminescence assay. The intensity of immunostaining was analyzed by scanning the photographic images and using image analysis software (NIH Image) to quantify the staining intensity. Antibodies to Birc-3, c-Jun, c-Myc, and p53 were obtained from Cell Signaling Technology (Beverly, MA).

Results

Survival and Intracellular Calcium after Preconditioning

The methods for isoflurane preconditioning (APC) and hypoxic preconditioning (HPC) yielded similar reductions in cell death after simulated ischemia (oxygen/glucose deprivation, OGD) (fig. 1A). After HPC, reductions in cell loss were seen in CA1, CA3, and dentate. With APC, cell death was reduced in CA1 and dentate but not significantly in the CA3 region ($P = 0.065$). Examples

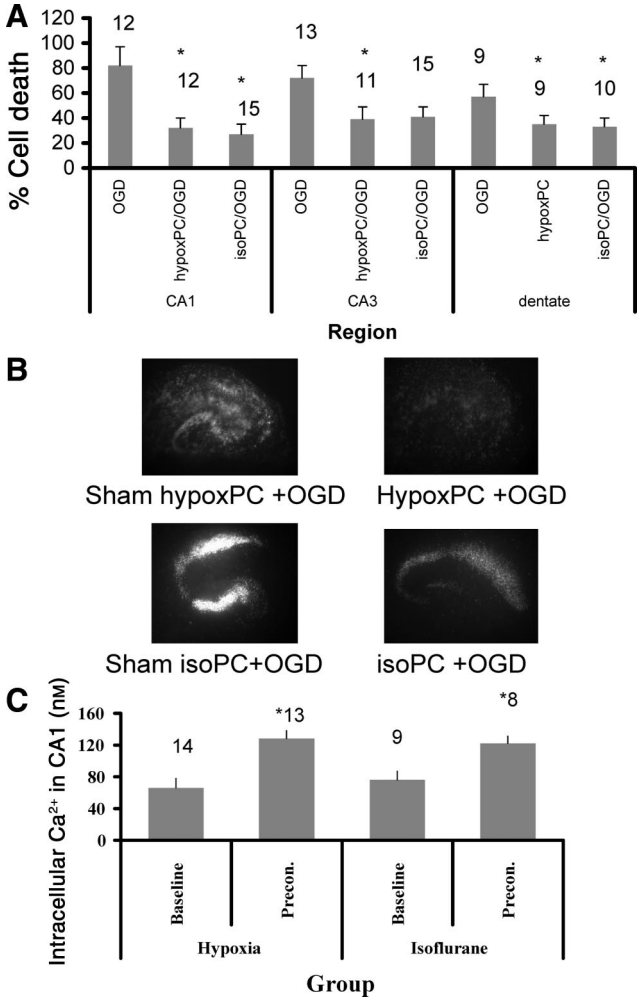

Fig. 1. Hypoxic and isoflurane preconditioning results in similar reduction in cell death after oxygen/glucose deprivation (OGD) and similar increases in intracellular Ca^{2+} during preconditioning. (*A*) Percent dead cells in CA1, CA3, and dentate cell regions in hippocampal slice cultures exposed to OGD after mock preconditioning, OGD after isoflurane preconditioning, and OGD after hypoxic preconditioning. Data are medians ± interquartile range. * = Significant difference compared to OGD group. (*B*) Examples of propidium iodide fluorescence in hippocampal slice cultures. (*C*) Intracellular Ca^{2+} concentration in CA1 neurons in hippocampal slice cultures at the end of 5 min of hypoxic or 1 h of isoflurane preconditioning. Data are means ± SE. * = Significant differences from baseline.

of propidium iodide fluorescence images used for analysis of cell death are shown in figure 1B.

Shown in figure 1C are measurements of peak $[Ca^{2+}]_i$ in CA1 neurons during preconditioning with 5 min of hypoxia or isoflurane. Increases of $[Ca^{2+}]_i$ of about 50 nM were observed during both types of preconditioning. The increase in $[Ca^{2+}]_i$ during APC remained stable over the subsequent 30–60 min; therefore, the data shown in figure 1 C are representative of $[Ca^{2+}]_i$ during the entire preconditioning stimulus.

Patterns of Gene Expression after Preconditioning

Figure 2 presents the fold-changes in the expression of apoptosis and survival-associated genes 24 h after HPC

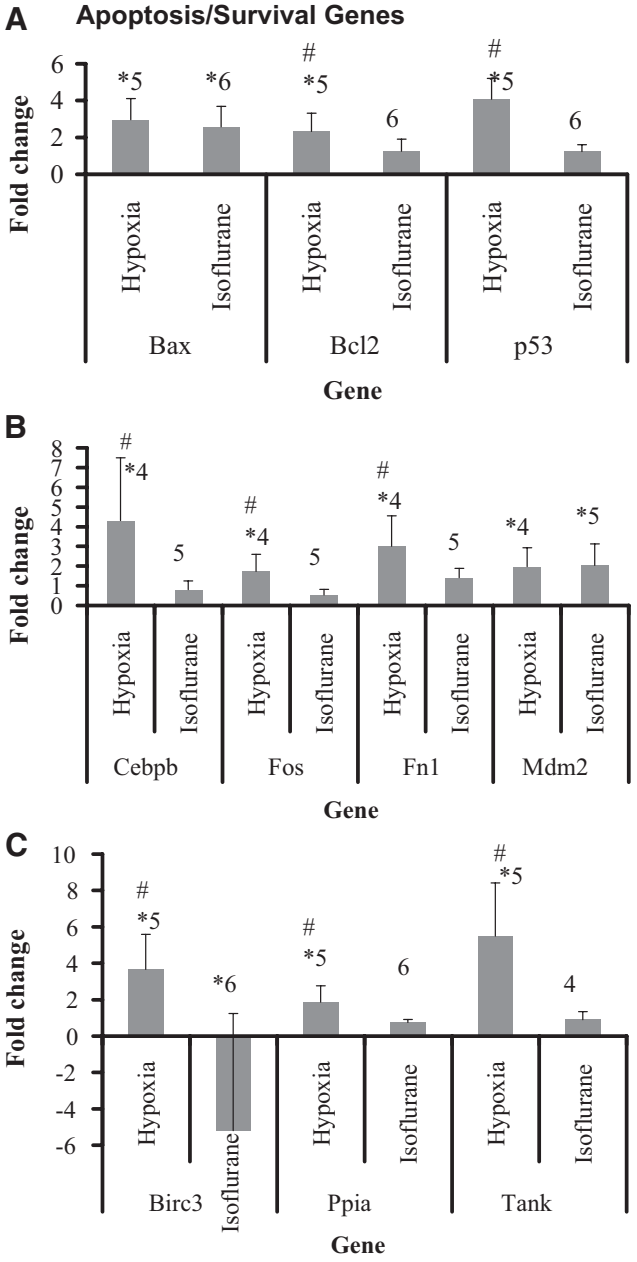

Fig. 2. Fold changes in survival-associated and apoptosis regulating genes after hypoxic or isoflurane preconditioning. Bax = *Bcl*II-associated X protein; *Bcl*II = B-cell lymphoma protein, type 2; p53 = tumor protein 53; Cebpb = CCAAT/enhancer binding protein beta; Fos = transcription factor activator protein-1; Fn1 = fibronectin 1; Mdm2 = murine double minute protein; Birc3 = baculoviral IAP repeat-containing protein 3; Ppia = peptidylproyl isomerase A; Tank = TRAF family member-associated nuclear factor (NF)-κB activator. *Numbers* = number of separate preconditioning experiments; *error bars* = standard errors; * = at least a ±1.5-fold change; # = significant difference between hypoxia and isoflurane. *Numbers above bars* = number of independent preconditioning studies.

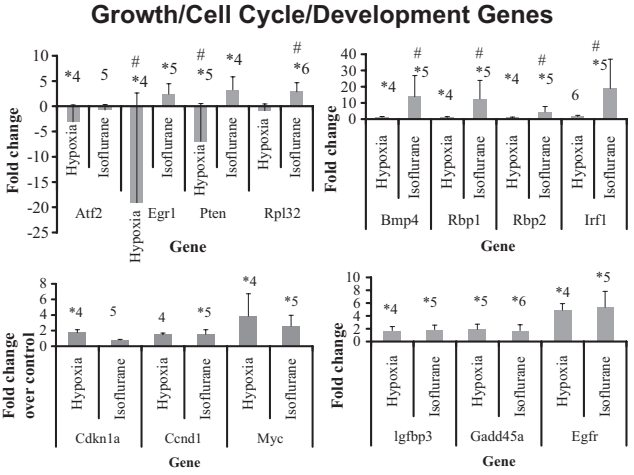

Fig. 3. Fold-changes in cell cycle and development-regulating genes after hypoxic and isoflurane preconditioning. ATf2 = activating transcription factor 2; Egr1 = early growth response protein 1; Pten = phosphatase and tensin homolog; Rpl32 = ribosomal protein L32; Bmp4 = bone marrow morphogenetic protein 4; Rbp1 = retinol binding protein 1; Rbp2 = retinol binding protein 2; Irf1 = interferon regulatory factor 1; Cdkn1a = cyclin-dependent kinase inhibitor 1A; Ccnd1 = cyclin d1; Mcc = mutated in colorectal cancer gene; Igfbp3 = insulin-like growth factor receptor binding protein 3; Gadd45a = growth arrest and DNA damage-inducible gene, alpha; Egfr = epidermal growth factor receptor. *Numbers* = indicate number of separate preconditioning experiments; *error bars* = standard errors; * = at least a ±1.5-fold change; # = significant difference between hypoxia and isoflurane.

or APC. Only genes exhibiting significant changes in expression (±1.5 fold change in expression) after one or both types of preconditioning are presented in this and the other figures. A total of 37 genes on the array were significantly increased or decreased by one or both types of preconditioning. Table 1 contains a complete list of genes on the array.

Of 10 apoptosis or cell survival–associated genes showing a significant change in expression after either type of preconditioning, HPC increased all 10 genes compared to control. In eight of these, the increase after HPC was greater than with APC. In contrast, APC increased the expression of only two of these genes (Bax and Mdm2), and the increase was smaller than with HPC.

Major differences in gene expression after APC and HPC were also seen for genes related to growth, differentiation, and cell cycle regulation (fig. 3). Fourteen genes in this category were increased after preconditioning, with eight of the genes showing greater increases after APC. Greater increase by HPC was only seen in one gene (Cdkn1a, cyclin-dependent kinase inhibitor 1a).

Both APC and HPC increased the expression of genes in the stress-response pathways (*e.g.*, heat shock proteins and the nuclear factor-κB [NF-κB]) and in cell signaling pathways that are involved in diverse signaling processes (figs. 4 and 5). There was no obvious differentiation of response between the two types of preconditioning with respect to the distribution of genes that were significantly increased above controls.

qPCR was used to confirm array data for selected genes in the apoptosis, signaling, and differentiation pathways. Table 2 compares fold changes in gene expression measured with the array and polymerase chain reaction for

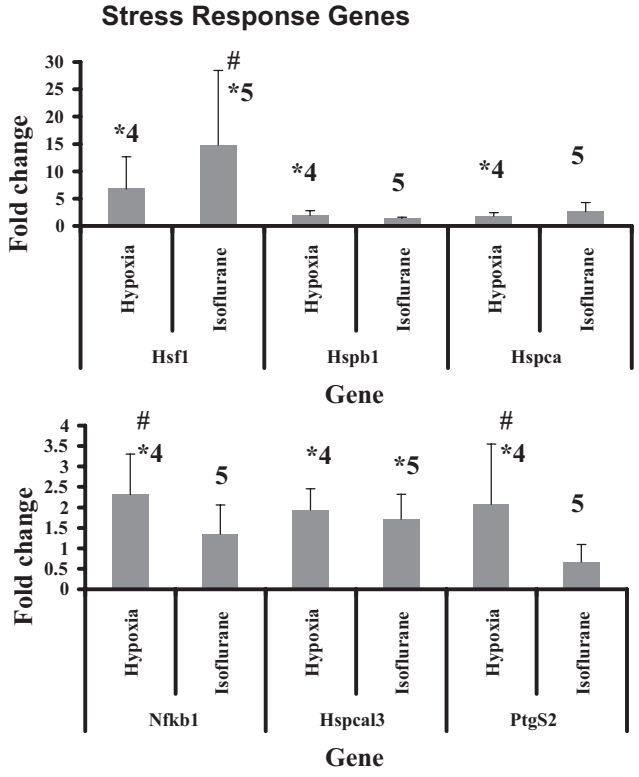

Fig. 4. Fold changes in stress response gene mRNA after hypoxic or isoflurane preconditioning. Hsf1 = hear shock factor 1; Hspb1 = heat-shock protein b1; Hspca = heat shock protein a (cytosolic); Nfkb1 = nuclear factor kappa b-1; Hspcal3 = heat-shock protein 90; PtgS2 = prostaglandin-endoperoxide synthase 2. *Numbers* = indicate number of separate preconditioning experiments; *error bars* = standard errors; * = at least a ±1.5-fold change; # = significant difference between hypoxia and isoflurane.

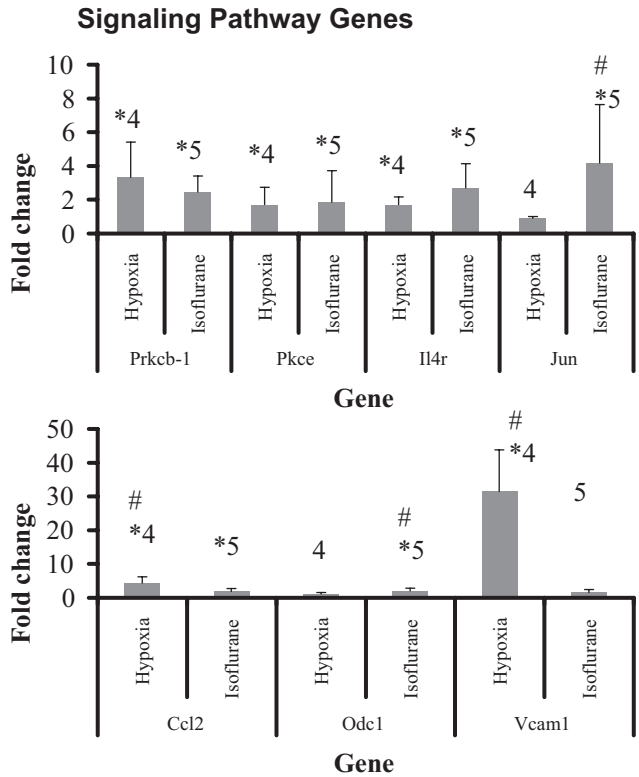

Fig. 5. Fold changes in signaling pathway genes after hypoxic or isoflurane preconditioning. Prkcb1 = protein kinase C beta 1; Pkce = protein kinase C epsilon; Il4r = interleukin 4 receptor; Jun = proto-oncogene jun; Ccl2 = chemokine C-C-motif ligand 2; Odc1 = ornithine decarboxylase 1; Vcam1 = vascular cell adhesion molecule-1. *Numbers* = indicate number of separate preconditioning experiments; *error bars* = standard errors; * = at least a ±1.5-fold change; # = significant difference between hypoxia and isoflurane.

Birc3, c-Myc, and c-Jun. Good correspondence between the array and polymerase chain reaction methodologies was found.

To investigate the significance of changes in mRNA levels, we performed Western blots on protein extracts obtained from the same preconditioning studies in which the gene array analysis was done. In figure 6, we show that changes in protein levels for Birc-3, c-Myc, c-Jun, and p53 were in the same direction as in polymerase chain reaction and/or the arrays (Table 2 and figs. 2, 3, and 5).

Discussion

We have compared similarly neuroprotective protocols of APC and HPC and found significantly different patterns of expression within a sample of 119 signal transduction genes. Whereas hypoxia generally increased the expression of pro-survival genes, isoflurane increased expression of genes related to development, cell cycle, and proliferation. For example, hypoxia increased the pro-survival gene Birc3, and isoflurane decreased its expression. Isoflurane increased expression of cell cycle/development genes Egr and Pten, whereas hypoxia decreased them substantially (figs. 2 and 3). Although there were increases in a number of the same signal transduction pathway genes in both types of preconditioning, the results indicate that different signals are ultimately involved in hypoxic and isoflurane preconditioning, despite similarity in upstream signaling involving increases in intracellular Ca^{2+} and phosphorylation of mitogen-activated protein kinases.[12]

Relatively little work has been done to directly compare the mechanisms underlying different and equipo-

Table 2. Comparison of Microarray and qPCR Data

	Fold Change in Microarray		Fold Change in qPCR	
	Hypoxia	Isoflurane	Hypoxia	Isoflurane
Birc-3	3.65 ± 1.9	–5.2 ± 6.1	6.1 ± 2.5	–1.2 ± 0.5
Jun	0.91 ± 0.1	4.2 ± 3.5	0.52 ± 0.45	3.4 ± 2.1
Myc	3.85 ± 2.8	2.5 ± 1.4	2.1 ± 1.0	2.95 ± 2.1

Data are fold changes in mRNA levels in gene arrays (n = 7 data sets, means ± SE) and in quantitative polymerase chain reaction (qPCR) (n=10) of genes from apoptosis regulation (Birc-3), cell signal (Jun), and cell division/differentiation (Myc) pathways. qPCR fold change is relative to actin gene mRNA, with fold change calculated as $2^{\Delta\Delta Ct}$.

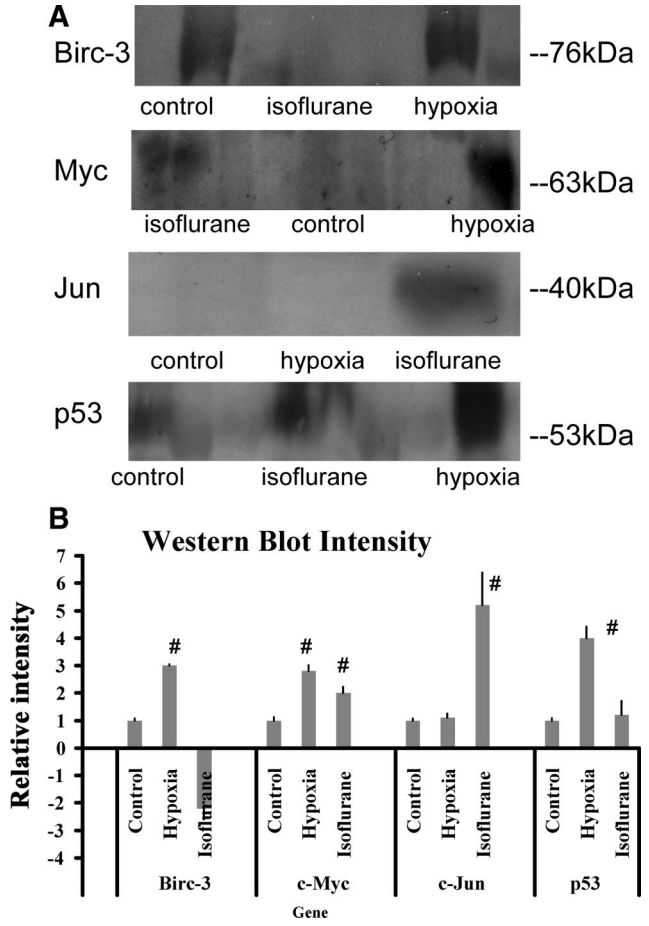

Fig. 6. Western blots of protein extracts from preconditioning studies. (*A*) Images from blots; (*B*) Average band intensity from four blots, normalized to control. # = Significant difference from control.

tent preconditioning stimuli in the same tissue. One exception is the study by de Silva *et al.* in the heart,[22] in which the entire genomic response to isoflurane and ischemic preconditioning was compared. As in our study, there was a divergence of the gene clusters or groups elicited by each type of preconditioning, with only 25% sharing of altered genes. Previous studies with cerebral preconditioning with hypoxic or ischemic generally have revealed patterns of gene expression similar to those we have seen in our hippocampal slice model with both hypoxia or isoflurane. These genes include heat shock proteins (Hspb1, Hspca, Hspcal3; fig. 4), trophic/growth factors (figs. 3 and 5), survival proteins (fig. 2), and signaling pathway genes (fig. 5). Similar patterns have been observed in intact animal models of hypoxic preconditioning with a variety of stimuli, including oxidative stress, heat, toxins, and volatile anesthetics.[9,23]

It is important to point out that this study is limited by the survey nature of the assessment in gene function and serves as a hypothesis-generating mechanism rather than a definitive assessment of the entire genomic response to preconditioning. Further, although we have described correlations between gene expression and selected changes in gene expression during preconditioning, it was beyond the scope of the study to prove that changes in any gene or group of genes are related mechanistically to neuroprotection. Additional studies, for example with RNA interference to block expression of specific genes, are required to demonstrate this link. Another limitation of this study is that multiple significance tests were conducted to identify significant changes in gene expression without adjusting the overall error rate to the desired 0.05 level.

Although we did not analyze the entire genome's response to HPC or APC, the 119 signal transduction genes represent a sample sufficient, we believe, to accurately indicate broad patterns of responses. We argue, as have others, that measuring whole genome responses is unnecessary to find important changes in gene expression, especially when the focus is on a narrower question such as signaling gene activation.[24] There are limitations with respect to categorizing genes as regulating growth, mediating survival, or other functions. The categories we have used are those generally accepted as the main function of the genes, although overlaps certainly occur.

The divergent gene responses observed between APC and HPC are probably related to important differences in signals generated during and after the preconditioning. Hypoxia increases intracellular Ca^{2+} *via* the endoplasmic reticulum, as does isoflurane,[12] but hypoxia involves changes in mitochondrial and cytosolic redox balance.[25] Hypoxia can create cellular stasis such a spindle checkpoint arrest in development,[26] at the same time activating cell defense mechanisms.[27] In contrast, signaling involving increases in intracellular Ca^{2+} produced by isoflurane preconditioning may be similar to developmental signaling induced by growth factor receptor activation, cell fate/differentiation decisions, and synaptic strengthening in the developing nervous system.[28] Additional work is required to prove this suggested distinction between the mechanisms involved in neuroprotective signaling with hypoxic and isoflurane preconditioning.

Apoptosis/Cell Survival Genes

Changes in the levels of the genes *Bcl*II, Birc3, p53, Mdm2, and Bax after hypoxic preconditioning are, on balance, consistent with pro-survival and antiapoptosis signaling after preconditioning. The relative levels of these proteins complexly influence survival or apoptosis.[29] *Bcl*II, p53, and Mdm2 were all increased 24 h after HPC. *Bcl*II is an important survival signal after preconditioning.[30] Because isoflurane did not alter the levels of this apoptosis regulator, other survival pathways in APC must be activated as well. Increased expression of *Bcl*II has been reported in preconditioning with hypoxia.[3] Isoflurane also had no effect on the related proteins Bcl2a1 and Bcl2l1, whereas hypoxia decreased the levels

of both. In intact rodents, isoflurane preconditioning increases *Bcl*II levels.[31]

The p53 gene product regulates apoptosis by interacting with a number of different proteins, with p53 levels correlated with the severity and duration of hypoxia.[32] We found that p53 mRNA increased after hypoxic preconditioning but not after isoflurane preconditioning. This increase in p53 mRNA after HPC is similar to that seen after cyanide exposure.[33] One of the genes induced by p53 is the pro-apoptotic Bax. Translocation of Bax to mitochondria is a crucial step in p53-mediated apoptosis. Bax mRNA levels increased after both isoflurane and hypoxia preconditioning. The pro-apoptotic actions of p53 and Bax must therefore be countered by the anti-apoptotic actions of other genes or signals because, on balance, preconditioning enhances survival.

Hypoxic preconditioning produced twice the increase in p53 mRNA as seen with Mdm2. In the regulation of cell survival or apoptosis, the levels of p53 and Mdm2 oscillate out of phase with Mdm2 opposing the proapoptotic actions of p53.[34,35] Recently, it was shown that Mdm2 and p53 proteins are components of an autoregulatory loop in which the Mdm2 gene is transactivated by p53. Isoflurane did not increase p53 mRNA, but it increased Mdm2, which would result in suppression of p53 action, which would inhibit p53-mediated effects, such as apoptosis.

The Birc3 protein regulates apoptosis by suppressing the expression and action of proteins in the tumor necrosis factor family. HPC increased Birc3 mRNA levels, consistent with neuroprotection. However, isoflurane substantially depressed Birc3 levels, a difference confirmed with qPCR (table 2).

Other growth-regulating and cell survival response genes were differentially affected by APC and HPC. Tank is a scaffolding protein that binds TRAF proteins, and it is a key activator of NF-κB,[36,37] thereby playing a role in cell survival regulation. Whereas hypoxic preconditioning increased Tank, expression was unchanged after isoflurane. Similarly, Ppia, which encodes a widely expressed scaffolding/protein folding gene,[38] was upregulated by HPC but not APC. This could have significance in the suppression of apoptosis after HPC; unfolding of proteins is an adaptive response activated during hypoxia, believed to increase cell survival during endoplasmic reticulum stress.[39]

Growth/Cell Cycle/Development Genes

Isoflurane increased more genes associated with regulation of cell proliferation and development than did hypoxia. Genes in this group included Egr1 (an early growth response gene), Pten (a tumor suppressor gene associated with developmental regulation), Bmp4 (a morphogenetic protein found in many tissues), Rbp1 (retinol binding protein, an important developmental regulator), the Irf1 (interferon regulatory factor), Ccnd1-(the cell cycle protein cyclin d1), Egfr (epidermal growth factor receptor), Igfbp3 insulin like growth factor receptor and Cdkn1 (cyclin dependent kinase inhibitor, significant because it *decreased* after isoflurane). Of note, several of these and related genes are upregulated by isoflurane in neuronal progenitor cells isolated from the neonatal rat hippocampus (Dr. Jeffrey Sall, MD, PhD, Assistant Professor, Department of Anesthesia, University of California, San Francisco, CA; personal verbal communication, September 2008).

Both HPC and APC increased the expression of Myc, a gene predominately affecting growth but also playing a role in regulating survival. The Myc-Max heterodimer binds to the promoter of ornithine decarboxylase (ornithine decarboxylase 1) a growth/cell metabolism gene.[40,41] Although ornithine decarboxylase 1 was unchanged during HPC (fig. 5), it was significantly increased by APC.

Stress Response Genes

A variety of stress response genes were increased after both APC and HPC, with responses variable between the two. The c-Fos gene is expressed after a variety of stresses, including hypoxia, oxidative stress, and excitotoxicity.[42] HPC induced an increase in c-Fos mRNA, whereas isoflurane caused a depression of that gene's mRNA levels.

Expression of genes in the NF-κB pathway also varied between HPC and APC. HPC increased NF-κB1, whereas APC did not. These differences in NF-κB1 expression may have significant ramifications for neuronal apoptotic/antiapoptotic responses; NF-κB has both proapoptotic and antiapoptotic functions, activating genes with death-inducing properties like p53, c-myc, Fas, and the survival genes Bcl-2, Bcl-x, and MnSOD. NF-κB induction of these survival genes may play a role in excitatory, chemical, and ischemic preconditioning.[43] In contrast, acutely inhibiting NF-κB delays p53-induced death. Thus, NF-κB has a dual role, maintaining neuron survival under normal conditions and signaling death after DNA damage. The Jnk/JunD pathway interacts with NF-κB to increase expression of antiapoptotic genes.[44] Inhibiting NF-κB enhances the stability of Gadd45a mRNA, thereby upregulating expression of Gadd45a posttranscriptionally.[45] Gadd45 is a gene involved in cellular response to DNA damage or oxidative stress. Both APC and HPC increased Gadd45a mRNA.

Conclusions

Multiple signal pathway genes (37 in a sample of 119) are significantly upregulated or downregulated 24 h after preconditioning with isoflurane or hypoxia. Despite similar effects on cell survival and on intracellular Ca^{2+}, the gene expression responses are not identical, with hypoxia generally having more effects on cell survival genes

and isoflurane increasing genes associated with development/proliferation. Although the mechanistic differences between these divergent responses are not yet apparent, they may have significant implications for the long-term effects of anesthesia and for the use of hypoxia or isoflurane as preconditioning agents.

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