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Enhanced hypoxic preconditioning by isoflurane: signaling gene expression and requirement of intracellular Ca²⁺ and inositol triphosphate receptors

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Abstract

Neurons preconditioned with non-injurious hypoxia or the anesthetic isoflurane express different genes but are equally protected against severe hypoxia/ischemia. We hypothesized that neuroprotection would be augmented when preconditioning with isoflurane and hypoxic preconditioning are combined. We also tested if preconditioning requires intracellular Ca²⁺ and the inositol triphosphate receptor, and if gene expression is similar in single agent and combined preconditioning. Hippocampal slice cultures prepared from 9 day-old rats were preconditioned with hypoxia (95% N₂, 5% CO₂ for 15 min, HPC), 1% isoflurane for 15 min (APC) or their combination (CPC) for 15 min. A day later cultures were deprived of O₂ and glucose (OGD) to produce neuronal injury. Cell death was assessed 48 hr after OGD. mRNA encoding 119 signal transduction genes was quantified with cDNA micro arrays. Intracellular Ca²⁺ in CA1 region was measured with fura-2 during preconditioning. The cell-permeable Ca²⁺ buffer BAPTA-AM, the IP₃ receptor antagonist Xestospongin C and RNA silencing were used to investigate preconditioning mechanisms. CPC decreased CA1, CA3 and dentate region death by 64–86% following OGD, more than HPC or APC alone (P<0.01). Gene expression following CPC was an amalgam of gene expression in HPC and APC, with simultaneous increases in growth/development and survival/apoptosis regulation genes. Intracellular Ca²⁺ chelation and RNA silencing of IP₃ receptors prevented preconditioning neuroprotection and gene responses. We conclude that combined isoflurane-hypoxia preconditioning augments neuroprotection compared to single agents in immature rat hippocampal slice cultures. The mechanism involves genes for growth, development, apoptosis regulation and cell survival as well as IP₃ receptors and intracellular Ca²⁺.

1. Introduction

Preconditioning is a potent method of inducing ischemic tolerance in the brain and other tissues that involves a non-injurious stress (mild hypoxia, brief ischemia, etc.) that induces resistance to injurious ischemia. Ischemic preconditioning can be induced by a wide variety of stimuli and has been demonstrated in both in intact animals and in cells and tissues in

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culture. Cerebral preconditioning was first demonstrated with hypoxia (Dahl and Balfour, 1964; Gidday et al., 1994) and later with heat stress, excitotoxins such as glutamate, bacterial endotoxins, oxidative stress, volatile anesthetics and neuromodulators such as adenosine and nerve growth factors (Chopp et al., 1989; Gidday, 2006; Shpargel et al., 2008; Simon et al., 2007). Preconditioning can be achieved in heart, kidneys, liver and other organs. Isoflurane preconditioning of the myocardium is effective in humans (Landoni et al., 2008) but cerebral protection with isoflurane or hypoxia remains an experimental procedure that has not yet been tested in human clinical trials.

Preconditioning involves changes in gene expression. The phenotype of preconditioned brain includes up-regulated stress response genes, intracellular signaling genes, hypoxia-inducible genes, and others (Shpargel et al., 2008; Simon et al., 2007). The specific pattern of gene expression depends on the type of preconditioning stimulus. For example, the volatile anesthetic isoflurane increases genes regulating growth and development, while hypoxic preconditioning increases expression of genes involved in the regulation of cell survival and apoptosis (Bickler and Fahlman, 2009). Differences in gene expression with isoflurane and ischemic preconditioning have also been described in the myocardium (da Silva et al., 2004).

Combinations of preconditioning agents may produce greater neuroprotection than single agents, have fewer side effects and require briefer periods for induction of preconditioning protection. However, few studies have tested combinations of preconditioning agents. Studies of combined hypoxic and anesthetic preconditioning have involved the heart but rarely the brain (Inoue et al., 2004; Inoue et al., 2006). The purpose of this study was to determine, in a rat hippocampal slice model, if combined hypoxic and isoflurane preconditioning is more neuroprotective than either type of preconditioning alone. We also wished to define differences in the pattern of gene expression in single-agent and combined preconditioning. Because increased intracellular calcium is an upstream signal common to different types of preconditioning (Bickler and Fahlman, 2004), we measured intracellular calcium ($[Ca^{2+}]_i$) in cells in the CA1 region during preconditioning. Further, we tested whether preventing increases in $[Ca^{2+}]_i$ with a cell permeable Ca^{2+} buffer (BAPTA-AM) prevents preconditioning neuroprotection in this cellular region. In addition, because endoplasmic reticulum IP_3 receptors are responsible for the increase in cytosolic Ca^{2+} required for induction of neuroprotection in both isoflurane and hypoxic preconditioning (Gray et al., 2005), we tested if this is also true for combined preconditioning by using siRNA against the IP_3 receptor.

2. Results

Survival and intracellular Ca^{2+} after preconditioning

The effects of preconditioning duration on cell death in CA1, CA3 and dentate regions was evaluated following hypoxic (HPC) and isoflurane preconditioning (APC). Fifteen min of either preconditioning agent did not increase cell death 24 hr later, but 30 min of APC increased death in all cell regions and 30 min of HPC increased death in CA3 and dentate (Fig. 1, panel A). Accordingly, a preconditioning duration of 15 min was used for the remaining studies.

Preconditioning with a combination of isoflurane and hypoxia (CPC) for 15 min yielded greater neuroprotection than did 15 min of HPC or APC alone (Fig. 1, Panel B). This protection was observed in all cell regions (CA1, CA3 and dentate) of the cultures.

Increases in intracellular Ca^{2+} in CA1 neurons of approximately 50 nM above pre-exposure baseline were observed with APC and HPC. A moderately larger increase (79 ± 13 nM,

$p < 0.05$ compared to APC and HPC) was found with combined preconditioning (Fig. 2 panel A). When added to the cultures 30 min before preconditioning, the cell-permeable Ca^{2+} buffer BAPTA-AM prevented the increase in $[\text{Ca}^{2+}]_i$.

Ca^{2+} and IP_3 receptors are involved in the mechanisms of combined preconditioning

Fig. 2, panel B shows that chelation of intracellular Ca^{2+} with BAPTA-AM and antagonism of IP_3 receptors with Xestospongin C during CPC prevents neuroprotection. The reagents used, BAPTA-AM and Xestospongin C, had no effect on survival when present during a mock preconditioning protocol. Further, slice cultures treated with RNA silencing reagents directed against IP_3 receptors were not protected against OGD by preconditioning with a combination of isoflurane and hypoxia (Fig. 3). The RNA silencing protocol resulted in a ~90% decrease in *ITPR1* mRNA levels at the time of preconditioning, as determined by quantitative PCR.

Patterns of gene expression following preconditioning

Table 1 presents the fold-changes in the expression of signal transduction genes 24 hr following HPC, APC, CPC and CPC with Ca^{2+} chelation pre-treatment. In this table, only genes exhibiting significant changes in expression (1.5 fold increase or decrease) following one of the types of preconditioning are included. A total of 40 of 119 genes on the array were significantly increased or decreased by at least one type of preconditioning. Of these, 30 genes were significantly changed following CPC, 23 by HPC and 25 by APC.

HPC induced genes regulating apoptosis and survival, whereas APC increased the expression of genes regulating growth and development. Of 12 apoptosis or cell survival-associated genes showing a significant change in expression following at least one type of preconditioning, HPC increased 10 genes compared to control. In contrast, APC increased the expression of only 2 genes in the apoptosis/survival regulation group (*Bax* and *Mdm2*), and decreases in one (*Birc3*).

Growth and development genes were preferentially increased by APC. Fourteen genes in this category were changed by preconditioning, with 12 of the 14 genes increasing following APC, and only 3 following HPC. Combined preconditioning increased both survival/apoptosis regulation and growth/development groups of genes.

Genes in the intracellular signaling and stress response groups were activated by both HPC and APC. Many of both gene groups were activated with CPC; that is, with CPC the pattern of gene response was generally the additive of HPC and APC expression patterns.

Treatment of slice cultures with BAPTA-AM before preconditioning prevented the increased expression of mRNA of almost all genes normally increased with preconditioning. Only *Ptgs2* and *Myc* were still increased in these cultures.

Quantitative PCR was used to validate the gene array methodology for a genes in several different groups. Table 2 demonstrates good general agreement between the qPCR and array methods.

3. Discussion

Combined preconditioning of rat hippocampal slice cultures with hypoxia and isoflurane increased survival compared to single agent preconditioning and involved a unique pattern of gene expression. The pattern of gene expression following combined preconditioning is a combination or amalgam of HPC and APC, in that both survival and apoptosis regulating genes are increased. Whereas hypoxia alone increased 10 survival/apoptosis genes, and

isoflurane increased 12 development/growth genes, the combination increased 19 genes from both groups. This pattern is seen in Table 1. We also found that combined preconditioning required the involvement of IP₃ receptors and moderate increases in intracellular Ca²⁺, the same as with hypoxic preconditioning (Bickler et al., 2009) or isoflurane preconditioning (Gray et al., 2005).

Differences in signal gene expression with similarly neuroprotective paradigms of isoflurane and hypoxic preconditioning were reported previously (Bickler and Fahlman, 2009). The present study involved a combination of hypoxia and isoflurane that increased the neuroprotective outcome and also involved a larger increase in intracellular Ca²⁺ during the preconditioning period. Because of this outcome, it is unknown whether the unique pattern of gene expression observed in this study was a result of the greater neuroprotective signaling or if it is related to the separate effects of each type of preconditioning. For example, it could be that a larger preconditioning “dose” (concentration or duration of isoflurane or hypoxia) would have produced gene changes, increases in [Ca²⁺]_i or survival benefits similar to the combined regimen studied here. This seems unlikely however, because longer preconditioning with either isoflurane or hypoxia increased cell death, probably from the inherent toxicity of hypoxia and anesthetics (Wei et al., 2008).

Changes in gene expression in hippocampal slice cultures during hypoxia or isoflurane preconditioning are similar to patterns in studies that have analyzed whole brain responses to preconditioning in intact animals. For example, increased expression of heat shock protein genes, cell signaling genes, and other stress response genes have been noted (Hamaya et al., 2000; Pan et al., 2006; Rampil et al., 2006; Sakamoto et al., 2005) and are similar to our general findings. The overall response of the brain transcriptome remains controversial, with recent reports (Stapels et al., 2010; Stenzel-Poore et al., 2003) suggesting that widespread transcriptional suppression is key to the conditioned state in post-ischemic brain. Our data would argue otherwise, although without genome-wide expression analysis, and analysis of gene expression after simulated ischemia, this cannot be verified. The genomic responses of the brain to anesthesia exposure also has been reported to vary substantially between studies, with some reports (e.g. (Rampil et al., 2006)) reporting widespread changes in multiple gene families (including some of the signaling genes found to be altered in this study), whereas other studies reported few changes (Sakamoto et al., 2005). The data in Table 1 would suggest relatively broad effects. This impression may be biased because although a small percentage of the total genome may be altered by anesthesia, many of the altered genes are in the signaling pathways examined in the present study.

Study limitations

It is important to note that this study is limited because only signal transduction genes were examined; i.e. we did not quantify expression of all genes in the rat hippocampus transcriptome. The main value of our study design is the generation of specific hypotheses concerning the mechanisms involved in preconditioning. While 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 is related mechanistically to neuroprotection. Our analysis did show that the pattern of gene expression normally seen after preconditioning was not present when cultures were treated with a cell permeable Ca²⁺ chelator, but because a broad collection of genes was affected by chelation, it remains uncertain which of the genes are key to the preconditioned phenotype. Another limitation of the study is that we measured gene response and not changes in the levels of proteins encoded by those genes. Because many signaling pathway proteins (e.g. protein kinases) are regulated by phosphorylation, a comprehensive understanding of the effects of altered gene expression on function would in addition require analysis of phosphorylation state and the functional effects of the activated

phospho-proteins. An analysis of the consequences of increased expression of specific genes is required to understand the neuroprotective basis of preconditioning. Blocking the expression of specific genes, for example with RNA interference, is one approach that could be used to obtain that information. Finally, our results may only apply to the immature brain. The effect of development and aging on the capacity of the brain to be preconditioned against ischemic damage is important, but little studied.

Although we did not analyze the entire genome's response to preconditioning, the 119 signal transduction genes is a sufficient sample to indicate broad patterns of responses, i.e. that both growth and development genes and apoptosis/survival regulation genes are part of the pattern of gene expression following combined isoflurane and hypoxic preconditioning. We suggest that measuring whole genome responses is unnecessary to define such important patterns in gene expression, especially when the focus is on a narrow question such differences in signaling gene activation between different types of preconditioning (Hess et al., 2001).

Role of Ca^{2+} in inducing gene expression changes

Differences in upstream initiators of gene expression during HPC, APC and CPC, except for the broadly important role of increases in intracellular Ca^{2+} , remain undefined. One may ask how a common upstream signal (increases in $[\text{Ca}^{2+}]_i$) can elaborate different gene responses depending on the stimulus. We speculate that Ca^{2+} signals arising during preconditioning with isoflurane and hypoxia are actually separate, as they arise from different mechanisms causing the increases in intracellular Ca^{2+} , and cause different patterns of gene expression. Hypoxia releases Ca^{2+} from the endoplasmic reticulum as does isoflurane (Bickler and Fahlman, 2004) but hypoxia involves changes in mitochondrial and cytosolic redox balance (Mayevsky and Rogatsky, 2006), resulting in a broad range of responses including cellular stasis such a spindle checkpoint arrest in development (Fischer et al., 2004) and a diverse group of cell defense mechanisms (Bickler and Donohoe, 2002). In contrast, signaling involving increases in intracellular Ca^{2+} produced by isoflurane preconditioning may be more similar to developmental signaling such as that following growth factor receptor activation, cell fate/differentiation decisions, and synaptic strengthening in the developing nervous system (Berglund and Augustine, 2008). It is also important to note that IP_3 receptor-mediated processes can have deleterious effects as well as beneficial ones. Several studies have shown that IP_3 receptor-mediated Ca^{2+} release can contribute to neuronal death following prolonged exposure to volatile anesthetics (Wei et al., 2008; Yang et al., 2008); this may be a function of exposure duration or anesthetic concentration.

Gene expression patterns in combined HPC and APC

Combined preconditioning increased the expression of numerous signaling genes. Combined preconditioning was associated larger numbers of genes expressed (40 of 119), rather than increases in the magnitude of gene expression compared to single agent preconditioning (data not presented). This increase in the total number of genes upregulated may be the basis for enhanced neuroprotection following combined preconditioning.

A greater number of cell survival and apoptosis regulating genes were increased following combined preconditioning than by single-agent preconditioning. These genes included Bcl2, Bcl2a1, Fn1 and p53. These responses are, on balance, consistent with pro-survival and anti-apoptosis signaling following preconditioning, even though p53 can be pro-apoptotic by up-regulating Bax. The relative levels of these proteins complexly influence survival or apoptosis (Basu and Haldar, 1998). Bcl2, p53 and Mdm2 were all increased 24 hr after CPC. Bcl2 is an important survival signal following hypoxic preconditioning (Meller et al., 2005). In intact rodents, isoflurane preconditioning increases Bcl2 levels (Li et al., 2008). The p53

gene product regulates apoptosis by interacting with a number of different proteins (Banasiak and Haddad, 1998). p53 mRNA increased following HPC and CPC, but not after APC. One of the genes induced by p53 is Bax, which is pro-apoptotic; translocation of Bax to mitochondria is a crucial step in p53-mediated apoptosis. Bax mRNA levels increased following APC, HPC and CPC. The pro-apoptotic actions of p53 and Bax must therefore be countered by the anti-apoptotic actions of other genes (e.g. Bcl2) or signals because, on balance, preconditioning enhances cell survival. Our preconditioning model could be used to determine the relative importance of these proteins in neuroprotection or injury.

Tank and Ppia were both increased by combined preconditioning. Tank is a scaffolding protein that binds TRAF proteins, and is a key activator of $\text{Nf}\kappa\text{B}$ (Bonif et al., 2006; Pomerantz and Baltimore, 1999), which promotes survival. Similarly, Ppia, which encodes a widely expressed scaffolding/protein folding gene (Colgan et al., 2000), was upregulated by HPC and CPC. This could suppress apoptosis following preconditioning, since deletion of unfolded of proteins is an adaptive response believed to increase cell survival following hypoxic endoplasmic reticulum stress (Feldman et al., 2005).

Isoflurane increased more genes associated with regulation of cell proliferation and development than did hypoxia, and CPC also increased the mRNA levels of these genes. Genes in this group included Egr1 (an early growth response gene), Pten (a tumor suppressor gene associated with developmental regulation) Rbp1 (retinol binding protein, an important developmental regulator), the Irf1 (interferon regulatory factor), Ccnd1 (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). However, bone morphogenic protein (Bmp4) was not increased following CPC.

All three types of preconditioning increased the expression of Myc, a gene regulating both growth and survival. The Myc-Max heterodimer binds to the promoter of ornithine decarboxylase (ODC1) a growth/cell metabolism gene (Dang, 1999; Grandori et al., 2000). ODC1 was increased by CPC. An unexplained finding was that Myc continued to be up-regulated (as was Ptg2) by preconditioning in Ca^{2+} -chelated cultures. This illustrates that important Ca^{2+} -independent processes probably are normally involved in preconditioning processes.

Expression of genes in the $\text{Nf}\kappa\text{B}$ pathway also showed greater quantitative expression following combined preconditioning. This may be significant for neuronal apoptotic/anti-apoptotic responses: $\text{Nf}\kappa\text{B}$ has both pro and anti apoptotic functions, activating genes with death-inducing properties like p53, c-myc, Fas and the survival genes Bcl-2, Bcl-x, and MnSOD. $\text{Nf}\kappa\text{B}$ induction of these survival genes may play a role in excitatory, chemical and ischemic preconditioning (Marini et al., 2007). In contrast, acutely inhibiting $\text{Nf}\kappa\text{B}$ delays p53 induced death. $\text{Nf}\kappa\text{B}$ therefore has a dual role, maintaining neuron survival under baseline conditions, but signaling death after DNA damage. The Jnk/JunD pathway works with $\text{Nf}\kappa\text{B}$ to increase expression of anti-apoptotic genes (Lamb et al., 2003). Gadd45 is involved in cellular response to DNA damage or oxidative stress. CPC increased Gadd45a mRNA.

Conclusions

Combining hypoxia with isoflurane in a preconditioning model with hippocampal slice cultures produces greater neuroprotection than either agent alone and a pattern of gene expression that is a unique amalgam of the two. Intracellular Ca^{2+} may be involved in the neuroprotective actions of this type of preconditioning. While the complete mechanistic basis for these observations was not revealed in this study, the findings have significant

implications for the long term effects of anesthesia and for the use of hypoxia or isoflurane as preconditioning agents against cerebral ischemia.

4. Experimental Procedures

The studies were approved by the University of California San Francisco Committee on Animal Research and conform to relevant National Institutes of Health guidelines for the use of animals in research.

Preparation of hippocampal slice cultures

Organotypic cultures of the hippocampus were prepared by standard methods (Laake et al., 1999; Stoppini et al., 1991) and slightly modified by our laboratory (Sullivan et al., 2002). Briefly, 7 to 9 day old Sprague Dawley rats (Charles River Laboratories, Hollister, CA) were anesthetized with 2–5% isoflurane until they did not move in response to a vigorous tail pinch. Following decapitation, the hippocampi were quickly removed and placed in 4 °C Gey's Balanced Salt Solution (GBSS) with 20 mM glucose. The isolated hippocampi were transversely sliced (400 µm thick) with a tissue slicer (Siskiyou Design Instruments, Grants Pass, OR), and returned to GBSS at room temperature. The slices were placed on 30-mm diameter membrane inserts (Millicell-CM, Millipore, Billerica, MA) in 6-well culture trays with 1.2 ml of slice culture medium per well. The slice culture medium consisted of 50% Eagle's Minimal Essential Medium, 25% Earle's balanced salt solution, 25% heat inactivated horse serum (all from the UCSF cell culture facility) with 6.5 mg/ml glucose and 5mM KCl. After 2 days in culture the serum concentration was decreased to 10%. 7–10 days later the slices were preconditioned.

Study design: preconditioning organotypic cultures of hippocampus

Slice cultures were preconditioned by placing them in a Billups-Rothenberg modular incubator chamber (Del Mar, CA) filled with humidified 95% N₂/5% CO₂ (HPC), or 1% isoflurane in humidified air and 5% CO₂ (APC) for 15 min. Combined preconditioning (CPC) was done similarly with 94% N₂, 5% CO₂ and 1% isoflurane, also for 15 min. RNA was extracted for gene array analysis 24 hr after preconditioning.

Simulation of ischemia with *in vitro* oxygen-glucose deprivation

Ischemia was simulated *in vitro* by one of 2 techniques: 1) immersion of cultures for 10 min into glucose-free media bubbled with 95% N₂/5% CO₂ (oxygen/glucose deprivation, OGD); 2) transfer of cultures to glucose free media and exposure to humidified 95% N₂/5% CO₂ for 30 min. The latter was used with cultures treated with RNA interference or BAPTA, as these reagents sometimes caused slices to float off the culture membranes. The temperature of the media was 37°C, measured with a thermocouple thermometer. The partial pressure of oxygen, measured with a polarographic oxygen electrode, was 0–0.2 mmHg. After this insult, the cultures were returned to standard slice culture media. The percentages of dead and living neurons in CA1, CA3 and dentate was measured 48 h after OGD.

Assessment of cell death in cultured hippocampal slice

Cell death was measured with propidium iodide (PI) fluorescence. PI, a highly polar dye, penetrates damaged plasma membranes and binds to DNA. 2.3 µM PI was added to the wells of the culture trays 30 min before imaging. After 30 minutes digital images of PI fluorescence (excitation light 520 nm, emission 600 nm) were taken with an inverted microscope and a SPOT Jr. Digital Camera (Diagnostic Instruments, Sterling Heights, MI). The camera sensitivity and the excitation light intensity were identical from day to day. Slices were discarded if they showed more than slight PI fluorescence in after 7–10 days in culture. PI fluorescence images were recorded in the dentate gyrus, CA1, and CA3 regions

of the hippocampal slices prior to OGD (signal assumed to represent 0 % cell death), and 48h following 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 (Sullivan et al., 2002). Serial measurements of PI fluorescence intensity were made in pre-defined areas (selecting CA1, CA3 and dentate separately) for each slice using NIH Image-J software (U.S. National Institutes of Health). After the measurement of PI fluorescence on the 2nd 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- μ M potassium cyanide and 2 mM sodium iodoacetate to the cultures for 20 minutes. One hr later, final images of PI fluorescence (equated to 100% cell death) were acquired. Percent of dead cells 48 hr after OGD were then calculated based on these values. PI fluorescence intensity is a linear function of cell death (Laake et al., 1999; Newell et al., 1995).

Statistical Analysis of Cell Death

The percentages of living and dead neurons in the different regions of the slices sometimes is not be normally distributed. Therefore, the Kruskal-Wallis test followed by the Mann-Whitney U-test (JMP, SAS Institute, Cary, N.C.) was used to compare the medians of different treatment groups. T-tests or ANOVA were used to compare the means of normally distributed data, and allowance was made for multiple comparisons (Tukey-Kramer multiple comparison or Dunnett's test). Differences were considered significant for $P < 0.05$.

Measurement of intracellular Ca^{2+} in CA1 neurons

$[\text{Ca}^{2+}]_i$ in CA1 was measured before, during and after preconditioning. Estimates of $[\text{Ca}^{2+}]_i$ in CA1 neurons in slice cultures were made using the indicator fura-2-AM and a dual excitation fluorescence spectrometer (Photon Technology International) coupled to a Nikon Diaphot inverted microscope. Slice cultures were incubated with 5–10 μ M fura-2 AM plus 1% pluronic acid for 30 min before measurements. Cultures for these measurements were grown on Nunc Anopore (Nalge Nunc) membranes because of their low ultraviolet fluorescence. 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 $[\text{Ca}^{2+}]_i$ was performed using the K_D of fura-2 determined *in vitro* with a Ca^{2+} buffer calibration kit (Invitrogen). 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 (Hyrz et al., 1997). Background fluorescence (i.e. fluorescence in the absence of fura) was subtracted from total fluorescence signals prior to calculation of $[\text{Ca}^{2+}]_i$ as described previously (Bickler and Hansen, 1998). Estimates of $[\text{Ca}^{2+}]_i$ with this technique are accurate to about ± 10 nM (Grynkiewicz et al., 1985). Measurements of $[\text{Ca}^{2+}]_i$ were made briefly at discrete periods during the preconditioning, to avoid photobleaching of fura-2. These were at baseline, at mid-point and termination of preconditioning, and after 10 min washout of preconditioning medium. Peak $[\text{Ca}^{2+}]_i$ always occurred at the end of the preconditioning period.

In some studies, the Ca^{2+} chelator BAPTA in the cell permeant form BAPTA-AM (10–25 μ M) was added to cultures 30 min before preconditioning and remained during preconditioning. In others, the IP_3 receptor antagonist Xestospongine C was included in the media during preconditioning. The cultures were transferred to fresh media after the preconditioning period.

RNA interference—Pools of interfering RNA (Custom smart pool RNAi, Dharmacon, Lafayette, CO) directed at the IP_3 receptor subtype 1 (ITPR1) were used to transfect slice cultures with the *Trans* IT-neural transfection reagent (Mirus Bio Corp, Madison, WI). Three treatment groups were studied: target RNAi, nonsense (nontargeting) RNAi, and

transfection reagents only. Preliminary studies defined time of maximal knockdown of IP₃ receptors; RT-PCR was used to quantify transcript levels at 12, 24 48, 72, and 96 hours after addition of transfection reagents to slice culture medium. Maximal reduction in mRNA and IP₃ receptor protein was at 72 hr after transfection in both cases. Slices were transfected on days 7, 8 and 9 in culture. To assess the effectiveness of target mRNA depletion, slices were transferred into -80°C RNAlater-ICE (Ambion, Austin, TX) and stored until RNA was extracted with Trizol (Invitrogen, Carlsbad, CA). DNA was synthesized using an OmniScript Reverse Transcriptase kit (Qiagen, Valencia, CA). Quantitative PCR was run using a QuantiTect SYBR Green PCR kit (Qiagen) and the following primers, created using Primer3 online software (Roizen and Skaletsky, 2000): IP3r1 Forward: tcgtgagtggttctacacaga, IP3r1 Reverse: agctgcttggtgtgtttat

(Product size: 112 bp, Accession number 1054962) and for β -actin forward: acagctgagagggaatcgt β -actin reverse: ttctccaggagggaagagg

(Product size: 107 bp, Accession number: 031144).

Microarray analysis

RNA for microarray analysis was extracted from slice cultures 24 hrs after mock preconditioning (control), hypoxic preconditioning, isoflurane preconditioning and combined 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 re-suspended in DEPC-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 μ l in RNASE-free water.

cDNA was synthesized using 0.1 to 2 μ g of total RNA, by means of the TrueLabeling™ LinearRNA Amplification Kit (SuperArray). From this cDNA an amplified Biotin-Labeled cRNA was synthesized. Biotinylated UTP was obtained from Roche Applied Science. The cDNA 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 260nm and 280nm 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 output 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 there was a minimum 1.5-fold increase or decrease compared to the control level.

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Figure 1.

Panel A Effects of hypoxic and isoflurane preconditioning duration on cell death 24 hrs later. HPC is hypoxic preconditioning 15 min, APC-10 is 1% isoflurane for 15 min and HPC-30 and APC-30 are 30 min duration preconditioning treatments. * indicates significant increase in PI fluorescence compared to mock PC group. *Panel B.* The combination of hypoxic preconditioning (HPC) and anesthetic preconditioning with 1% isoflurane (APC) decreases cell death in hippocampal slice cultures exposed to oxygen/glucose deprivation (OGD) in CA1, CA3 and dentate more than HPC or APC alone. * indicates significant difference compared to HPC and APC groups, with 12–18 observations in each group. Images of PI fluorescence in mock preconditioned and combination preconditioned slice cultures are also presented.

Figure 2.

Panel A Intracellular Ca^{2+} concentration in CA1 region cells at baseline and at the completion of preconditioning. * indicates significant increase compared to baseline, # indicates significant difference compared to HPC and APC groups. N=6–8 observations in each group. *Panel B*. Chelation of intracellular Ca^{2+} changes and antagonism of IP_3 receptors with Xestospongin C (XestoC) during combined hypoxia and isoflurane preconditioning (CPC) prevents neuroprotection. Slice cultures were treated with BAPTA-AM (25 μM) or Xestospongin C (10 μM) for 30 min before and during the CPC. Cell death was assessed 24 hours after oxygen glucose deprivation, which was 24 hr after the preconditioning. * indicates significant reduction in cell death compared to mock preconditioning groups.

Figure 3.

RNA interference against IP_3 receptor gene expression prevents a combination of hypoxia and isoflurane from preconditioning hippocampal slice cultures. Each group represents mean PI fluorescence intensity (a measure of cell death) and SDs for 12–16 cultures. Examples of PI fluorescence from the different treatment groups are also shown.

Table 1

Changes in expression of signal transduction genes following hypoxic (HPC), isoflurane (APC) and combined preconditioning in hippocampal slice cultures. + or – indicates at least 1.5 fold increase or decrease in gene expression compared to mock preconditioned cultures. The fold increase was calculated as the mean fold change in 3–5 separate array experiments. The CPC + BAPTA column refers to cultures pre-treated with the cell-permeant Ca^{2+} chelator BAPTA-AM before combined preconditioning with hypoxia and isoflurane.

	HPC	APC	Combined (CPC)	CPC+BAPTA
Survival and Apoptosis				
Bax	+	+	+	
Bcl2	+		+	
Bcl2a1			+	
Bcl2L1			–	
Cebpb	+		+	
Fos	+			
Fn1	+		+	
Mdm2	+	+	+	
P53	+		+	
Birc3	+	–	+	
Ppia	+		+	
Tank	+		+	
Growth and Development				
Atf2	–			
Egr	–	+	+	
Pten	–	+	+	
Rpl32	+	+		
Cdkn1a	+		+	
Ccnd1		+	+	
Igfbp3		+	+	
Myc	+	+	+	+
Bmp4		+		
Gadd45a		+	+	
Rbp1		+		
Rbp2		+		
Irf1		+	+	
Egfr		+	+	
Signal Transduction				
Ccl2	+	+	+	
Prkcb1	+	+		
Pkce	+	+	+	
Il4r	+	+	+	
Jun		+		
Vcam	+	+		
Odc1		+	+	

	HPC	APC	Combined (CPC)	CPC+BAPTA
Stress				
Response				
Nfkb1	+	+	+	
Nfkb1a			+	
Ptgs2	+		+	+
Hsf1	+	+	+	
Hspb1	+		+	
Hspca	+	+		
Hspcal3	+	+	+	

Table 2

Comparison of microarray and quantitative polymerase chain reaction (PCR) data. Data are fold-changes (means $\pm$ SE) in mRNA levels in gene arrays (n=6 data sets) and in quantitative PCR (n=4) of genes from apoptosis regulation (Birc-3), cell division and differentiation pathways (Myc) and cell activation signaling (Jun). Quantitative PCR fold-change is expressed relative to actin gene mRNA, with fold change calculated as $2^{\Delta\Delta Ct}$.

Gene	Fold Change in Microarray			Fold Change in qPCR		
	<u>Hypoxia</u>	<u>Isoflurane</u>	<u>Combined</u>	<u>Hypoxia</u>	<u>Isoflurane</u>	<u>Combined</u>
Birc-3	3.7 $\pm$ 1.9	-5.2 $\pm$ 6.1	4.3 $\pm$ 0.9	6.1 $\pm$ 2.5	-1.2 $\pm$ 0.5	3.3 $\pm$ 3.0
Myc	3.9 $\pm$ 2.8	2.5 $\pm$ 1.4	4.3 $\pm$ 2.0	2.1 $\pm$ 1.0	2.95 $\pm$ 2.1	1.9 $\pm$ 0.8
Jun	1.0 $\pm$ 0.4	4.2 $\pm$ 3.5	1.2 $\pm$ 0.9	0.52 $\pm$ 0.45	3.4 $\pm$ 2.1	1.4 $\pm$ 1.7