Published in final edited form as:

Neuroscience. 2009 April 21; 160(1): 51–60. doi:10.1016/j.neuroscience.2009.02.013.

Inositol 1,4,5-triphosphate receptors and NAD(P)H mediate Ca^{2+} signaling required for hypoxic preconditioning of hippocampal neurons

Philip E. Bickler, MD, PhD, Christian S. Fahlman, PhD, Jonathan Gray, BS, William McKleroy, BS, and Daniel E. Warren, PhD

Department of Anesthesia, University of California at San Francisco, San Francisco, CA 94143-0542

Abstract

Exposure of neurons to a non-lethal hypoxic stress greatly reduces cell death during subsequent severe ischemia (hypoxic preconditioning, HPC). In organotypic cultures of rat hippocampus, we demonstrate that HPC requires inositol triphosphate (IP_3) receptor-dependent Ca^{2+} release from the endoplasmic reticulum (ER) triggered by increased cytosolic NAD(P)H. Ca^{2+} chelation with intracellular BAPTA, ER Ca^{2+} store depletion with thapsigargin, IP_3 receptor block with xestospongine, and RNA interference against subtype 1 of the IP_3 receptor all blunted the moderate increases in $[\text{Ca}^{2+}]_i$ (50–100 nM) required for tolerance induction. Increases in $[\text{Ca}^{2+}]_i$ during HPC and neuroprotection following HPC was not prevented with NMDA receptor block or by removing Ca^{2+} from the bathing medium. Increased NAD(P)H fluorescence in CA1 neurons during hypoxia and demonstration that NADH manipulation increases $[\text{Ca}^{2+}]_i$ in an IP_3R -dependent manner revealed a primary role of cellular redox state in liberation of Ca^{2+} from the ER. Blockade of IP_3Rs and intracellular Ca^{2+} chelation prevented phosphorylation of known HPC signaling targets, including MAPK p42/44 (ERK), protein kinase B (Akt) and CREB. We conclude that the endoplasmic reticulum, acting via redox/NADH-dependent intracellular Ca^{2+} store release, is an important mediator of the neuroprotective response to hypoxic stress.

Introduction

Enhancing the capacity of neurons to adapt to hypoxic stress has implications for improving the survival of neurons during lethal insults from diseases such as stroke and hypoxic encephalopathy. We propose that the endoplasmic reticulum (ER) is involved in the phenomenon of hypoxic preconditioning, in which a prior exposure to non-injurious hypoxia induces tolerance to subsequent severe hypoxic or ischemic stress (Dirnagl et al., 2003). Increasing evidence points to the ER as the critical organelle in the transduction of various degrees of cellular stress into cell defense/survival or apoptosis decisions, depending on the severity and duration of stress (Lin et al., 2007). The ER unfolded protein response (UPR) is a set of protein signaling pathways and transcription factors that control apoptosis after severe oxidative stress or in neurodegenerative diseases (Lin et al., 2008) and in neuronal preconditioning (Hayashi et al., 2003, Hayashi et al., 2005). In this study we test the hypothesis

Correspondence to: Philip Bickler, UCSF Dept. of Anesthesia, 513 Parnassus Avenue, Sciences 255, Box 0542, San Francisco, CA 94143-0542. Phone: (415) 476-1411, fax (415) 476-8841, email: E-mail: bicklerp@anesthesia.ucsf.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

that a measured release of Ca^{2+} from the ER is another mechanism by which the ER controls cell survival or death following hypoxic/ischemic stress. At one extreme, excessive release of Ca^{2+} from the ER can play a role in neurodegenerative processes associated with diseases such as Alzheimer's dementia and brain ischemia (Mattson, 2007), while more moderate Ca^{2+} release may promote cell survival responses mediated by the Bcl-2 family of proteins (White et al., 2005, Li et al., 2007). In this study, we show that a moderate response of the endoplasmic reticulum, involving 50–100 nM increases of intracellular Ca^{2+} , underlie induction of ischemic tolerance following hypoxic preconditioning.

A growing body of evidence shows that a moderate/non-injurious increase in $[\text{Ca}^{2+}]_i$ plays a critical role in neuronal hypoxic preconditioning. Moderate increases in $[\text{Ca}^{2+}]_i$ are known to act through a number of neuroprotective signaling pathways, including the MAP kinase ERK pathway (Strohm et al., 2000, Hardingham et al., 2001, Mottet et al., 2003, Lange-Asschenfeldt et al., 2004), the nitric oxide pathway (Huang, 2004), and through transcription factors related to neuroprotective gene expression (Tauskela et al., 2003). Although Ca^{2+} -related ischemic neuronal tolerance can be induced with activation of NMDA receptors (Gonzalez-Zulueta et al., 2000), activation of voltage-gated Ca^{2+} channels, and application of low concentrations of Ca^{2+} ionophores (Bickler and Fahlman, 2004), these mechanisms are normally involved only with more severe excitotoxic or ischemic stress. The source of Ca^{2+} involved in non-excitotoxic cellular adaptation to hypoxia and nature of the signals involved in generating this Ca^{2+} response is thus still undefined.

In this study, in addition to showing that release of Ca^{2+} from the endoplasmic reticulum is critical to the neuroprotective preconditioning response, we identify the mechanism by which the Ca^{2+} release occurs. This mechanism is shown to involve changes in cytosolic redox balance during hypoxia, specifically hypoxia-induced increases in [NADH] or [NADPH] (Mayevsky and Rogatsky, 2007), that catalyze the release of Ca^{2+} from the ER via an inositol-triphosphate-receptor dependent mechanism (Kaplin et al., 1996). The mechanism of NADH production requires the enzyme GAPDH (Patterson et al., 2005), which suggests that cytosolic rather than mitochondrial NADH is the initiating signal in the release of Ca^{2+} from the endoplasmic reticulum.

Materials and Methods

Study design: preconditioning and simulated ischemia in organotypic cultures of hippocampus

Hypoxic preconditioning (HPC) was achieved by immersing slice cultures of hippocampus in medium bubbled with 95% N_2 /5% CO_2 gas for 5 min. Twenty-four hr after HPC, slices were subjected to simulated ischemia with 10 min immersion in media bubbled with 95% N_2 /5% CO_2 and lacking glucose (oxygen/glucose deprivation, OGD). The PO_2 of these solutions was 5 ± 2 mmHg, tested with a Clark-type oxygen electrode and the temperature was 37 ± 0.5 °C. The duration of preconditioning and OGD were selected based on preliminary studies designed to find durations of hypoxia and OGD that would produce injury and protection that would involve significant injury with OGD alone (60–80% cell death) and significant protection (about 50% reduction in death) so that further mechanism-based studies could be done. The percentages of dead and living neurons remaining in CA1, CA3 and dentate regions of the slices were assessed 48 hr after the OGD. Measurements of $[\text{Ca}^{2+}]_i$ or intracellular [NADH] in CA1 neurons within the cultures were made in separate groups of slices during preconditioning conditions. Samples to determine expression and phosphorylation state of MAP kinases p42/44, the anti-apoptotic protein Akt and CREB, or for RNA extraction for gene array analysis were obtained 24 hr after preconditioning.

Preparation of hippocampal slice cultures

Organotypic cultures of the hippocampus were prepared by standard methods (Stoppini et al., 1991, Laake et al., 1999) as modified by our laboratory (Sullivan et al., 2002). Animal care was approved by the University of California San Francisco Committee on Animal Research and conforms to relevant National Institutes of Health guidelines. Briefly, Sprague Dawley rats (9 days old, Charles River Laboratories) were anesthetized with 3–5% isoflurane. This anesthetic dissipates rapidly from the hippocampal slices. The pups were decapitated and the hippocampi were quickly removed and placed in 4 °C Gey's Balanced Salt Solution (GBSS). Next, the hippocampi were transversely sliced (400 µm thick) with a tissue slicer (Siskiyou Design Instruments), and stored in GBSS at 4 °C for one hour. The slices were then transferred onto 30-mm diameter membrane inserts (Millicell-CM, Millipore or Anopore, Nunc), 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, UCSF Cell Culture Facility), 25% Earle's balanced salt solution, 25% heat inactivated horse serum (UCSF cell culture facility) with 10 mM glucose and 5mM KCl. Seven to 10 days elapsed between slice preparations and preconditioning.

Assessment of cell death in cultured hippocampal slices

Cell viability was assessed by propidium iodide (PI) imaging. 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 minutes the slices were examined with a Nikon Diaphot 200 inverted microscope and digital images were taken using a SPOT Jr. Digital Camera (Diagnostic Instruments Inc.). Excitation light wavelength was 520 nm and the fluorescence emission filter 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 prior to OGD (signal assumed to represent 0 % cell death), and after 2 and 3 days 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 (manually outlining CA1, CA3 and dentate separately during analysis) for each slice using NIH Image-J software (U.S. National Institutes of Health). Thus, cell death was followed in the same regions of each slice following simulated ischemia. After the measurement of PI fluorescence on the 3rd 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. Adding 100µM potassium cyanide and 2 mM sodium iodoacetate to the cultures for 20–30 minutes did this. One hr later, final images of PI fluorescence (equated to 100% cell death) were acquired. Longer periods of time did not appreciably increase PI uptake. Percentages of dead cells at 0, 2 and 3 days post OGD were then calculated based on these values. PI fluorescence intensity is a linear function of cell death (Newell et al., 1995, Laake et al., 1999). This was validated in our preparation by comparing PI cell death assessment with histologic assessment of cell death. We compared cell death estimated by the PI method with that of counting damaged/missing CA1 neurons in fixed sections of slices stained with hematoxylin and eosin.

Measurements of $[Ca^{2+}]_i$

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 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

μM fura-2 AM plus 1% pleuronic acid for 30 min before measurements. Cultures for these measurements were grown on Nunc Anopore (Nalge Nunc) culture tray inserts because of their low auto-fluorescence 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 $[\text{Ca}^{2+}]_i$ was done by 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).

In studies to delineate the cause of increases in $[\text{Ca}^{2+}]_i$ during hypoxia, antagonists of NMDA receptors (APV) and phospholipase C (U73122) were used. To determine the minimal effective concentration of APV, measurements of $[\text{Ca}^{2+}]_i$ in CA1 neurons were made during 5–10 sec application of 100 μM NMDA via perfusion. Ten μM APV, but not 1 μM , completely blocked increases in $[\text{Ca}^{2+}]_i$. The concentration of U73122 (1 μM) was chosen based on studies with cultured cortical neurons and hippocampal slice cultures in which we demonstrated blockage of phospho-lipase-C mediated increases in $[\text{Ca}^{2+}]_i$ resulting from a variety of stimuli (Donohoe et al., 2001, Bickler et al., 2004).

Measurements of NAD⁺/NADH and NAD(P)H fluorescence in hippocampal slice cultures

Fluoro NAD/NADH Reagents from Cell Technology Inc (Mt. View, CA) were used to measure [NADH] levels in extracts from hippocampal slice cultures. Standards and slice culture extracts were assayed on a Molecular Devices fluorescence plate reader according to instructions supplied with the NAD/NADH assay kit and expressed as nmoles NADH/slice, since slice size is uniform. Measurement of changes in the intensity of emitted 450 nm light during excitation with 366 nm light was used as a regional index of labile NAD(P)H levels in the slice cultures (Mayevsky and Rogatsky, 2007). As with measurements of $[\text{Ca}^{2+}]_i$, we sampled fluorescence only from a small area of CA1 cell body region. A Sutter Instruments DG-5 xenon light source with narrow pass 360 nm excitation and 450 emission filters (Chroma Technology) was used in these experiments along with a Photometrics ES2 camera.

Measurements of lactate and pyruvate

Measurements of slice lactate and pyruvate concentrations during hypoxia involved washing slice culture inserts with HBSS ($3 \times 2\text{mL}$) and placing them into 50 ml flasks containing 7 ml of HBSS previously bubbled for 1 h with water-saturated air/5% CO_2 (normoxia) or N_2 /5% CO_2 (hypoxia). The flask was sealed and flushed with the normoxic or hypoxic humidified gas mixtures while the media was continuously bubbled. After 5 (hypoxia) or 10 (hypoxia and normoxia) min, inserts were quickly removed from the flasks, immersed in liquid nitrogen, and lyophilized for 24 h. Individual slices were cut from each insert with a scalpel and placed into bullet tubes with 100 μl of ice-cold 0.6N perchloric acid. For each replicate, 3 inserts containing 4–5 slices each were pooled. The tubes were shaken continuously for 1 h at 4 degrees to allow complete extraction of the metabolites, after which 50 μl of 0.6 M KHCO_3 was added. The tubes were vortexed and centrifuged at maximum speed for 5 min. Lactate was measured on 50 μl of the resultant supernatant using a lactic oxidase-based colorimetric assay (Trinity Biotech, 735-10). Pyruvate was measured similarly except with an assay employing lactate dehydrogenase and NADH. Disappearance of NADH fluorescence (366 excitation, 450 nm emission) was measured with a Hitachi F-2000 fluorometer.

Immunostaining and 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. Protein content in each sample was measured (Bradford protein reagent, Biorad) and adjusted so that equal amounts of protein were applied to each lane. Protein bands were visualized after incubation with horseradish peroxidase-linked secondary antibodies followed by an enhanced chemiluminescence (Amersham) or SuperSignal (Pierce) chemiluminescent reagent followed by film exposure. Immunostaining intensity was analyzed by scanning the photographic images and using image analysis software (NIH Image). Antibodies to p-Akt (Ser 473 phosphorylation) and those to MAPK p42/44 (Thr 202/204 phosphorylation) were obtained from Cell Signaling Technology.

RNA interference

Pools of interfering RNA (Custom smart pool RNAi, Dharmacon, Lafayette, CO) directed at the IP₃ receptor subtype 1 (IPTR1) and the *TransIT*-neural transfection reagent (Mirus Bio Corp, Madison, WI) were used to transfect slice cultures. Three treatment groups were studied: target RNAi, nonsense 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 and 72 hours after addition of transfection reagents to slice culture medium. The greatest reduction in mRNA and protein was at 72 hr after transfection. 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): IPTR1 Forward: tcgtggatgttctacacaga, IPTR1 Reverse: agctgcttggtgtgtttat (Product size: 112 bp, Accession number 1054962) and for β -actin forward: acagctgagagggaatcgt and β -actin reverse: ttctccaggaggagaagag (Product size: 107 bp, Accession number: 031144).

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) was used to compare the means 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 $P < 0.05$.

RESULTS

Hypoxic preconditioning produces robust neuroprotection in hippocampal slice cultures

Preconditioning with 5 min hypoxia 24 hr before severe oxygen and glucose deprivation (OGD) substantially reduced subsequent cell death in CA1 neurons. Protection was observed 48 and 72 hr after the OGD (Figs. 1A and 1B).

The propidium iodide fluorescence method of cell death assessment was compared to standard histologic methods (hematoxylin/eosin staining). Regression analysis showed that the propidium iodide method reports about 7% more death than the histologic analysis, but the correlation was quite robust (cell death by PI = 0.949 X cell death by histology + 0.068, $r^2 = 0.93$, $n = 13$ comparisons).

Intracellular Ca^{2+} changes during hypoxic preconditioning and OGD

At the end of the 5 min HPC average $[\text{Ca}^{2+}]_i$ in CA1 increased from 75 ± 18 nM to 125 ± 30 nM ($p < 0.01$, $n = 13$). Twenty-four hr after HPC, $[\text{Ca}^{2+}]_i$ was not different between preconditioned cultures and sham-preconditioned controls. Importantly, the increase in $[\text{Ca}^{2+}]_i$ in CA1 neurons that occurred during subsequent OGD was reduced about 5-fold by preconditioning (Fig. 1C).

Small increases in $[\text{Ca}^{2+}]_i$ during preconditioning are required for neuroprotection

To determine if the moderate increases in $[\text{Ca}^{2+}]_i$ observed during preconditioning hypoxia are required for the induction of ischemic tolerance, we loaded hippocampal slice cultures with the cell-permeable Ca^{2+} chelator BAPTA-AM. $[\text{Ca}^{2+}]_i$ levels in control and BAPTA-AM-treated slices during preconditioning are shown in Fig. 2A, demonstrating that BAPTA-AM prevented the increase in $[\text{Ca}^{2+}]_i$ during preconditioning, but did not alter $[\text{Ca}^{2+}]_i$ significantly in the control (baseline) state. BAPTA-AM present during preconditioning blocked ischemic tolerance/neuroprotection in CA1 neurons (Fig. 2B); cell death in the slice cultures treated with BAPTA during preconditioning was similar to cultures treated with mock preconditioning. Representative images of slices from each group are shown in Fig. 2C, with bright areas in the images indicating PI fluorescence.

Causes of Ca^{2+} increase in HPC

We next carried out a series of studies to delineate the source of the moderately increased $[\text{Ca}^{2+}]_i$ in CA1 neurons during preconditioning. Fig. 3A illustrates that removing Ca^{2+} from the extracellular medium or antagonizing NMDA receptors with APV did not alter the increase in $[\text{Ca}^{2+}]_i$ during preconditioning. Similarly, inhibition of phospholipase C with U73122, which prevents increases in $[\text{Ca}^{2+}]_i$ initiated by several types of cell surface signals, also did not affect the rise in $[\text{Ca}^{2+}]_i$ during preconditioning. However, inhibition of the Ca^{2+} release complex on the endoplasmic reticulum with the selective IP_3 receptor antagonist xestospongine C completely prevented the increase in $[\text{Ca}^{2+}]_i$ during hypoxia. Xestospongine did not change $[\text{Ca}^{2+}]_i$ in basal conditions, suggesting that in hippocampal slice cultures xestospongine C does not inhibit the ER Ca^{2+} uptake pump or deplete intracellular Ca^{2+} stores. Consistent with the endoplasmic reticulum being the chief source of Ca^{2+} increase during HPC, treatment of slice cultures with 10 μM thapsigargin, which inhibits ER Ca^{2+} uptake pumps and thereby depletes ER Ca^{2+} stores, substantially limited the increase in $[\text{Ca}^{2+}]_i$ during hypoxia (Fig. 3B). To further investigate whether IP_3 receptors are involved in the increase in $[\text{Ca}^{2+}]_i$ in CA1 during hypoxia, we used siRNA directed against subtype 1 of the IP_3 receptor, the subtype most abundant in rat CA1 hippocampal neurons (Nicolay et al., 2007). Quantitative PCR demonstrated a 60–80% reduction in mRNA for this target ($\Delta\Delta\text{Ct} > 1.3$, > 2.4 fold reduction $n = 4$). Figure 4 shows that increases in $[\text{Ca}^{2+}]_i$ during hypoxia do not occur following siRNA against IP_3 R1. Taken together, the data in Figs. 3 and 4 indicate that IP_3 receptors on the endoplasmic reticulum are the most likely cause of cytosolic Ca^{2+} increase during hypoxic preconditioning in slice cultures.

NAD(P)H increases during hypoxic preconditioning and may cause an increase in $[\text{Ca}^{2+}]_i$

During hypoxia, electron flow through the mitochondrial electron transport chain is decreased, shifting the $\text{NAD}^+/\text{NAD(P)H}$ redox balance towards NAD(P)H in mitochondria and cytosol. NAD(P)H triggers Ca^{2+} release from the ER via the IP_3 receptor- Ca^{2+} release complex (Kaplin et al., 1996). To determine if this mechanism contributes to the rise in $[\text{Ca}^{2+}]_i$ during preconditioning, we measured NAD(P)H fluorescence in CA1 neurons. The time-course of the change in 450 nm fluorescence (360 $\pm$ 20 nm excitation) during a 5-min preconditioning period is shown in Fig. 5A, indicating an average increase in basal fluorescence of about 50%. Further, using a fluorescent detection reagent highly selective for NADH, we found that after 5 min of hypoxia there was about a 20% increase ($P < 0.05$) in total-cell extract levels of NADH (Fig.

5B). Because NADH fluorescence is derived partly from cytosolic and partly from mitochondrial sources (Mayevsky and Rogatsky, 2007), we needed to demonstrate that cytosolic NADH can increase $[Ca^{2+}]_i$ in CA1 neurons. To do this, we perfusion-applied NADH to slices and observed a substantial increase in 450 nm fluorescence in the CA1 subfield. The fluorescence persisted after perfusate washout (data not shown). An increase in the lactate/pyruvate ratio in extracts from slices after 5 min of hypoxia also indicates that cytosolic redox balance shifted to greater NADH levels during hypoxia (Fig. 5B). In addition, when NADH was applied to slices pre-treated with the Ca^{2+} indicator fura-2 AM, increases in $[Ca^{2+}]_i$ were observed (Fig. 5C) and the increase in $[Ca^{2+}]_i$ produced by NADH application was blocked by antagonizing IP_3 receptors with xestospongine C. Taken together, the results show that during hypoxia, increases in cytosolic NADH are very likely linked to IP_3 R-mediated release of Ca^{2+} from the endoplasmic reticulum.

IP_3 Rs are required for hypoxic preconditioning neuroprotection

Blockade of IP_3 Rs by the selective antagonist xestospongine C reveals that hypoxic preconditioning-induced tolerance requires IP_3 receptors. In Fig. 6, the percentages of dead neurons 48 hr after OGD with or without prior hypoxic preconditioning are presented. While HPC reduced cell death by over 75% compared to sham HPC controls, xestospongine C in the medium before and during HPC prevented this neuroprotective effect. Neuroprotection was still observed with the NMDA antagonist MK-801 in the medium and when medium $[Ca^{2+}]$ was reduced to <0.1 mM during the HPC period, showing that protection does not require Ca^{2+} influx from the extracellular environment. In agreement with the conjecture that the IP_3 receptor is critical to preconditioning, RNA interference directed against subtype 1 of the IP_3 receptor abrogated preconditioning neuroprotection (Fig. 6B).

Ca^{2+} -dependent phosphorylation of signaling intermediates during preconditioning

Preconditioning of hippocampal slice cultures was associated with increased phosphorylation of ERK (MAP kinase p42/44), Akt (serine 308), and CREB (serine 133) (Fig. 7A–C) and with increased p-CREB in the nuclear fraction (Fig. 7A). Phosphorylation of Akt was prevented by incubation of cultures with xestospongine C during preconditioning (Figs. 7B and 7C).

DISCUSSION

Preconditioning requires NAD(P)H- a redox signal coupled to Ca^{2+} via the ER

We have found that Ca^{2+} released from the endoplasmic reticulum is key to the induction of hypoxic preconditioning neuroprotection in rat hippocampal neurons. We suggest that the central event in tolerance induction by hypoxia is the IP_3 -receptor dependent increase in $[Ca^{2+}]_i$ produced by alterations in redox balance, with corresponding increases in cytosolic NAD(P)H concentration. This is consistent with both increased mitochondrial NADH (Mayevsky and Rogatsky, 2007) and an increase in cytosolic NAD(P)H level. The lactate/pyruvate ratio, a redox-coupled indicator of cytosolic oxidation/reduction balance (Ying, 2007), increases during HPC, suggesting an approximate doubling of cytosolic NADH levels, although direct measurements of total slice NADH and CA1 NADH fluorescence suggest increases of 20–50%. NADH shuttles in the mitochondrion directly link increases in mitochondrial $[NADH]$ to increases in cytosolic $[NADH]$ (Ying, 2008). Shifts in brain cytosolic redox balance toward greater $[NAD(P)H]$ within just minutes of non-injurious hypoxia have been clearly identified both *in vivo* (Zoremba et al., 2007) and *in vitro* with brain slices and NMR spectroscopy (Espanol et al., 1996). In the hippocampal slice, measurements of cytosolic NADH with 2-photon spectroscopy also have revealed increases in NADH/ NAD^+ during hypoxia (Kasischke et al., 2004). A coupling between hypoxia-generated NADH and Ca^{2+} signaling includes the observation that exogenously applied NADH can produce a similar rise in $[Ca^{2+}]_i$ as that occurring during hypoxia (Fig. 5B). Although

extracellular pyridine nucleotides can have a range of significant biological effects (Ying, 2008), this response was antagonized by the IP₃ receptor blocker xestospongine, arguing that an intracellular translocation of NADH was of sufficient magnitude to allow it to interact with IP₃Rs in the intracellular Ca²⁺ release mechanism. With both hypoxia-induced and experimentally-applied increases in [NADH], the increase in [Ca²⁺]_i is eliminated by an IP₃ receptor antagonist and RNAi against the IP₃ receptor. We believe that NADH and not IP₃ is the agent leading to IP₃R activation and Ca²⁺ release during hypoxia. This was established by showing that inhibition of phospholipase C, which has been associated with IP₃ generation during injurious degrees of hypoxia (Bickler and Fahlman, 2006), does not alter the rise in [Ca²⁺]_i during hypoxia. We also do not think that activation of other receptors, such as metabotropic glutamate receptors, could be responsible for the increases in [Ca²⁺]_i during preconditioning. This is because the brief duration of hypoxia does not lead to glutamate release, based on evidence that NMDA receptors do not contribute to the rise in [Ca²⁺]_i during hypoxia. Our data are also consistent with those in studies by Kaplan and Patterson et al. showing that changes in cytosolic redox balance during hypoxia are linked to Ca²⁺ release from the endoplasmic reticulum (Kaplan et al., 1996, Patterson et al., 2004, Patterson et al., 2005). Therefore, we conclude that moderate increases in [Ca²⁺]_i during preconditioning appear to be mostly derived from NAD(P)H-dependent release of Ca²⁺ from internal stores.

Role of the endoplasmic reticulum in adaptation of neurons to hypoxic stress

Our study showed that the endoplasmic reticulum in neurons is an important organelle in the cellular defense response in adaptation to hypoxia and resisting cell injury and death following ischemia. This result is consistent with emerging evidence that the ER participates in regulating cellular fate decisions between apoptosis versus survival/proliferation both via the unfolded protein response that follows oxidative stress (Lin et al., 2007), and in mediating Ca²⁺-overload apoptosis in a variety of neurodegenerative conditions (Mattson, 2006). It has been recently shown that the pro-survival functions of the Bcl-2 family of proteins involve modulation/enhancement of IP₃ receptor-mediated Ca²⁺ signaling (White et al., 2005, Li et al., 2007). The response of the ER to cellular stress is a continuum, with mild stress such as hypoxia leading to adaptive responses protecting the integrity of protein synthesis and other cellular functions, and more massive or overwhelming damage triggering apoptosis with the probable involvement of other parts of the cell death pathway such as the mitochondrion.

Ca²⁺ and neuronal preconditioning

Demonstrating that neuronal preconditioning with non-injurious hypoxia primarily involves Ca²⁺ release from the intracellular compartment does not rule out that Ca²⁺ from other sources can induce ischemic tolerance under other conditions. For example, NMDA receptor activation with exogenous glutamate or NMDA (Gonzalez-Zulueta et al., 2000) or even application of low levels of a Ca²⁺-selective ionophore (Bickler and Fahlman, 2004) can precondition hippocampal neurons to minimize injury following otherwise lethal ischemia. Similarly, the volatile anesthetic isoflurane can produce tolerance-inducing increases in intracellular Ca²⁺ via phospholipase C (Bickler et al., 2005). A variety of Ca²⁺ sources, specific to the preconditioning stimulus, apparently can participate in signaling leading to a neuroprotective cellular phenotype.

Ca²⁺-dependent “downstream” signals in neuronal preconditioning

Our work also defines some of the more downstream signaling events that may be required for the development of ischemic tolerance. Nuclear CREB translocation was associated with preconditioning (Fig. 7 A). Another significant finding related to the importance of upstream Ca²⁺ signaling and survival responses was that block of IP₃ receptors with xestospongine prevented the phosphorylation of the survival kinase Akt (Figs. 7B and C), a phosphorylation

event required for preconditioning-induced ischemic neuronal tolerance (Yin et al., 2007). These findings are consistent with many other studies showing that survival kinases, including Akt and a number of MAP kinases are involved with various types of preconditioning (for review see (Perez-Pinzon, 2007) and Ran and Sharp ((Sharp et al., 2004,Ran et al., 2005). The novelty here is that we show that upstream of these signals, Ca^{2+} release from the ER is apparently an early triggering event in the cascade that leads from decreases in molecular oxygen to neuroprotective responses.

Conclusions

In summary, we show that changes in cellular oxidation/reduction balance during hypoxia increase cytosolic NAD(P)H, and that this triggers the release of Ca^{2+} from the endoplasmic reticulum via activation of IP_3 receptors. This Ca^{2+} release, in turn, activates signaling pathways involved in survival signaling. Ca^{2+} release from the endoplasmic reticulum is therefore a key determinant of the adaptive or neuroprotective response of neurons to hypoxic stress.

Acknowledgments

Supported by NIH GM52212 to P. Bickler

References

- Bickler PE, Fahlman CS. Moderate increases in intracellular calcium activate neuroprotective signals in hippocampal neurons. *Neuroscience* 2004;127:673–683. [PubMed: 15283966]
- Bickler PE, Fahlman CS. The inhaled anesthetic, isoflurane, enhances Ca^{2+} -dependent survival signaling in cortical neurons and modulates MAP kinases, apoptosis proteins and transcription factors during hypoxia. *Anesth Analg* 2006;103:419–429. [PubMed: 16861427]table of contents
- Bickler PE, Fahlman CS, Ferriero DM. Hypoxia increases calcium flux through cortical neuron glutamate receptors via protein kinase C. *J Neurochem* 2004;88:878–884. [PubMed: 14756808]
- Bickler PE, Hansen BM. Hypoxia-tolerant neonatal CA1 neurons: Relationship of survival to evoked glutamate release and glutamate receptor-mediated calcium changes in hippocampal slices. *Dev Brain Res* 1998;106:57–69. [PubMed: 9554954]
- Bickler PE, Zhan X, Fahlman CS. Isoflurane preconditions hippocampal neurons against oxygen-glucose deprivation: role of intracellular Ca^{2+} and mitogen-activated protein kinase signaling. *Anesthesiology* 2005;103:532–539. [PubMed: 16129978]
- Dirnagl U, Simon RP, Hallenbeck JM. Ischemic tolerance and endogenous neuroprotection. *Trends Neurosci* 2003;26:248–254. [PubMed: 12744841]
- Donohoe PH, Fahlman CS, Bickler PE, Vexler ZS, Gregory GA. Neuroprotection and intracellular Ca^{2+} modulation with fructose-1,6-bisphosphate during in vitro hypoxia-ischemia involves phospholipase C-dependent signaling. *Brain Research* 2001;917:158–166. [PubMed: 11640901]
- Espanol MT, Litt L, Chang LH, James TL, Weinstein PR, Chan PH. Adult rat brain-slice preparation for nuclear magnetic resonance spectroscopy studies of hypoxia. *Anesthesiology* 1996;84:201–210. [PubMed: 8572333]
- Gonzalez-Zulueta M, Feldman A, Klesse L, Kalb R, Dillman J, Parada L, Dawson T, Dawson V. Requirement for nitric oxide activation of p21(ras)/extracellular regulated kinase in neuronal ischemic preconditioning. *Proc Natl Acad Sci U S A* 2000;97:436–441. [PubMed: 10618436]
- Gryniewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985;260:3440–3450. [PubMed: 3838314]
- Hardingham GE, Arnold FJL, Bading H. A calcium microdomain near NMDA receptors: on switch for ERK-dependent synapse-to-nucleus communication. *Nature Neuroscience* 2001;4:565–566.
- Hayashi T, Saito A, Okuno S, Ferrand-Drake M, Chan PH. Induction of GRP78 by ischemic preconditioning reduces endoplasmic reticulum stress and prevents delayed neuronal cell death. *J Cereb Blood Flow Metab* 2003;23:949–961. [PubMed: 12902839]

- Hayashi T, Saito A, Okuno S, Ferrand-Drake M, Dodd RL, Chan PH. Damage to the endoplasmic reticulum and activation of apoptotic machinery by oxidative stress in ischemic neurons. *J Cereb Blood Flow Metab* 2005;25:41–53. [PubMed: 15678111]
- Huang PL. Nitric oxide and cerebral ischemic preconditioning. *Cell Calcium* 2004;36:323–329. [PubMed: 15261488]
- Hyrz K, Handran DS, Rothman SM, Goldberg MP. Ionized intracellular calcium concentration predicts excitotoxic neuronal death: observations with low affinity fluorescent calcium indicators. *J Neurosci* 1997;17:6669–6677. [PubMed: 9254679]
- Kaplin AI, Snyder SH, Linden DJ. Reduced nicotinamide adenine dinucleotide-selective stimulation of inositol 1,4,5-trisphosphate receptors mediates hypoxic mobilization of calcium. *J Neurosci* 1996;16:2002–2011. [PubMed: 8604044]
- Kasischke KA, Vishwasrao HD, Fisher PJ, Zipfel WR, Webb WW. Neural activity triggers neuronal oxidative metabolism followed by astrocytic glycolysis. *Science* 2004;305:99–103. [PubMed: 15232110]
- Laake JH, Haug F-M, Weiloch T, Ottersen OP. A simple *in vitro* model of ischemia based on hippocampal slice cultures and propidium iodide fluorescence. *Brain Research Protocols* 1999;4:173–184. [PubMed: 10446412]
- Lange-Asschenfeldt C, Raval A, Dave Mochly-Rosen D, Sick TJ, Perez-Pinzon MA. Epsilon protein kinase C mediated ischemic tolerance requires activation of the extracellular regulated kinase pathway in the organotypic hippocampal slice. *J Cereb Blood Flow Metab* 2004;24:636–645. [PubMed: 15181371]
- Li C, Wang X, Vais H, Thompson CB, Foscett JK, White C. Apoptosis regulation by Bcl-x(L) modulation of mammalian inositol 1,4,5-trisphosphate receptor channel isoform gating. *Proc Natl Acad Sci U S A* 2007;104:12565–12570. [PubMed: 17636122]
- Lin JH, Li H, Yasumura D, Cohen HR, Zhang C, Panning B, Shokat KM, Lavail MM, Walter P. IRE1 signaling affects cell fate during the unfolded protein response. *Science* 2007;318:944–949. [PubMed: 17991856]
- Lin JH, Walter P, Yen TS. Endoplasmic reticulum stress in disease pathogenesis. *Annu Rev Pathol* 2008;3:399–425. [PubMed: 18039139]
- Mattson MP. Neuronal life-and-death signaling, apoptosis, and neurodegenerative disorders. *Antioxid Redox Signal* 2006;8:1997–2006. [PubMed: 17034345]
- Mattson MP. Calcium and neurodegeneration. *Aging Cell* 2007;6:337–350. [PubMed: 17328689]
- Mayevsky A, Rogatsky G. Mitochondrial function *in vivo* evaluated by NADH fluorescence: From animal models to human studies. *Am J Physiol Cell Physiol* 2007;292:C615–640. [PubMed: 16943239]
- Mottet D, Michel G, Renard P, Ninane N, Raes M, Michiels C. Role of ERK and calcium in the hypoxia-induced activation of HIF-1. *J Cell Physiol* 2003;194:30–44. [PubMed: 12447987]
- Newell DW, Barth A, Papermaster V, Malouf AT. Glutamate and non-glutamate receptor mediated toxicity caused by oxygen and glucose deprivation in organotypic hippocampal cultures. *J Neuroscience* 1995;15:7702–7711.
- Nicolay NH, Hertle D, Boehmerle W, Heidrich FM, Yeckel M, Ehrlich BE. Inositol 1,4,5 trisphosphate receptor and chromogranin B are concentrated in different regions of the hippocampus. *J Neurosci Res* 2007;85:2026–2036. [PubMed: 17471556]
- Patterson RL, Boehning D, Snyder SH. Inositol 1,4,5-trisphosphate receptors as signal integrators. *Annu Rev Biochem* 2004;73:437–465. [PubMed: 15189149]
- Patterson RL, van Rossum DB, Kaplin AI, Barrow RK, Snyder SH. Inositol 1,4,5-trisphosphate receptor/GAPDH complex augments Ca^{2+} release via locally derived NADH. *Proc Natl Acad Sci U S A* 2005;102:1357–1359. [PubMed: 15677321]
- Perez-Pinzon MA. Mechanisms of neuroprotection during ischemic preconditioning: lessons from anoxic tolerance. *Comp Biochem Physiol A Mol Integr Physiol* 2007;147:291–299. [PubMed: 17045830]
- Ran R, Xu H, Lu A, Bernaudin M, Sharp FR. Hypoxia preconditioning in the brain. *Dev Neurosci* 2005;27:87–92. [PubMed: 16046841]

- Roizen, S.; Skaletsky, HJ. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz, S.; Misener, S., editors. *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press; Totowa, N.J.: 2000. p. 365-366.
- Sharp FR, Ran R, Lu A, Tang Y, Strauss KI, Glass T, Ardizzone T, Bernaudin M. Hypoxic preconditioning protects against ischemic brain injury. *NeuroRx* 2004;1:26–35. [PubMed: 15717005]
- Stoppini L, Buchs PA, Muller D. A simple method for organotypic cultures of nervous tissue. *J Neurosci Methods* 1991;37:173–182. [PubMed: 1715499]
- Strohm C, Barancik T, Bruhl ML, Kilian SA, Schaper W. Inhibition of the ER-kinase cascade by PD98059 and UO126 counteracts ischemic preconditioning in pig myocardium. *J Cardiovasc Pharmacol* 2000;36:218–229. [PubMed: 10942164]
- Sullivan BS, Leu D, Taylor DM, Fahlman CS, Bickler PE. Isoflurane prevents delayed cell death in an organotypic slice culture model of cerebral ischemia. *Anesthesiology* 2002;96:189–195. [PubMed: 11753020]
- Tauskela JS, Brunette E, Monette R, Comas T, Morley P. Preconditioning of cortical neurons by oxygen-glucose deprivation: tolerance induction through abbreviated neurotoxic signaling. *Am J Physiol Cell Physiol* 2003;285:C899–911. [PubMed: 12814913]
- White C, Li C, Yang J, Petrenko NB, Madesh M, Thompson CB, Foscett JK. The endoplasmic reticulum gateway to apoptosis by Bcl-X(L) modulation of the InsP3R. *Nat Cell Biol* 2005;7:1021–1028. [PubMed: 16179951]
- Yin W, Signore AP, Iwai M, Cao G, Gao Y, Johnnides MJ, Hickey RW, Chen J. Preconditioning suppresses inflammation in neonatal hypoxic ischemia via Akt activation. *Stroke* 2007;38:1017–1024. [PubMed: 17272774]
- Ying W. NAD⁺ and NADH in brain functions, brain diseases and brain aging. *Front Biosci* 2007;12:1863–1888. [PubMed: 17127427]
- Ying W. NAD⁺/NADH and NADP⁺/NADPH in cellular functions and cell death: regulation and biological consequences. *Antioxid Redox Signal* 2008;10:179–206. [PubMed: 18020963]
- Zoremba N, Homola A, Rossaint R, Sykova E. Brain metabolism and extracellular space diffusion parameters during and after transient global hypoxia in the rat cortex. *Exp Neurol* 2007;203:34–41. [PubMed: 16956608]

Fig. 1.

Hypoxic preconditioning (HPC) with 5 min hypoxia creates tolerance to oxygen and glucose deprivation (OGD). *A and B.* Propidium iodide fluorescence (bright regions=PI positive) images and percentage of dead neurons 48 and 72 hr after OGD. * significant increase in cell death compared to mock PC. ($P < 0.05$ for $n = 8$). *C.* $[Ca^{2+}]_i$ in CA1 neurons (means $\pm$ SE, $n = 6$) immediately after sham or hypoxic preconditioning, 24 h after HPC, and immediately after 10 min of OGD ** $P < 0.01$, *** $P < 0.001$.

Fig. 2.

Small increases in $[Ca^{2+}]_i$ are required for hypoxia to induce ischemic tolerance in hippocampal slice cultures. **A.** BAPTA-AM buffers increases in $[Ca^{2+}]_i$ during hypoxic preconditioning. * significant increase in $[Ca^{2+}]_i$ ($P<0.05$) compared to baseline; numbers indicate number of observations. **B.** BAPTA-AM present during preconditioning eliminates induction of tolerance to oxygen and glucose deprivation (OGD). * indicates significant reduction in cell death following HPC ($P<0.05$). **C.** Representative propidium iodide fluorescence images from slices at baseline, after OGD (no preconditioning), in preconditioned slices 48 hr after OGD, and in a slice treated with BAPTA-AM before preconditioning.

Fig. 3.

Source of increased $[\text{Ca}^{2+}]_i$ during preconditioning hypoxia is intracellular stores. **A.** $[\text{Ca}^{2+}]_i$ in CA1 neurons during hypoxic preconditioning in untreated control slices, and in slices pretreated with a phospholipase C inhibitor (U73122, 1 μM), an IP_3 receptor antagonist xestospongins C (xesto C, 1 μM), the NMDA receptor antagonist α -phosphonovalerate (APV, 10 μM) or low extracellular Ca^{2+} (<0.1 mM). Data are means $\pm$ SEM in 8–10 slices. **B.** Depletion of $[\text{Ca}^{2+}]_i$ stores with thapsigargin (10 μM) decreases the magnitude of the $[\text{Ca}^{2+}]_i$ rise in CA1 during hypoxia. * indicates significant ($P < 0.05$) increase in $[\text{Ca}^{2+}]_i$ compared to hypoxia alone group. Data are means $\pm$ SD for 6 experiments.

Fig. 4.

RNA interference directed against IP₃ receptor type 1 in hippocampal slice cultures eliminates the moderate increase in $[Ca^{2+}]_i$ that occurs during preconditioning hypoxia. **A.** Mean peak increase in $[Ca^{2+}]_i$ in different treatment groups (n=12–13). **B.** Average $[Ca^{2+}]_i$ in nonsense control and RNAi groups at baseline, during hypoxia and after return to normal oxygenation. Error bars indicate means $\pm$ SEM in 12–13 slices and * indicates significant difference compared to nonsense controls.

Fig. 5. NADH fluorescence in CA1 neurons during hypoxic preconditioning. **A.** Normalized changes in NADH fluorescence in CA1 cells during perfusion in normally oxygenated or hypoxic medium (means $\pm$ SE, n=7). **B.** Total slice NADH content and [lactate]/[pyruvate] ratios before and after preconditioning hypoxia (n=4 separate experiments, * P<0.05). **C.** Mean $[Ca^{2+}]_i$ in CA1 cells exposed to 1 mM NADH (n=6) with or without IP₃R antagonist (xestospongine-C, 10 μ M) pre-treatment (n=5).

Fig. 6.

Induction of OGD tolerance requires IP₃ receptors. A. Effects of xestospongine-C (Xesto, 1 μ M), MK-801 (10 μ M) or low Ca²⁺ (<0.01 mM) medium during preconditioning on cell death 48 hr after OGD in CA1 neurons. B. RNA interference directed against ITPR1 blocks preconditioning neuroprotection in CA1 neurons. HPC= hypoxic preconditioned, nsRNAi= nonsense (control) RNAi. Cell death was measured 48 hr after oxygen/glucose deprivation.

Fig. 7. Hypoxic preconditioning in hippocampal slice cultures is associated with increased nuclear CREB translocation, phosphorylation of survival kinases and requires the calcium-calmodulin dependent kinase II α . **A.** Western blots of phospho CREB and p42/44 in cytosolic and nuclear fractions (nuclear/protein extraction kit, BioVision, Inc) from mock-preconditioned and hypoxia-preconditioned slice cultures. * $P<0.01$ compared to control, with $n=7$. **B** and **C.** Western blots and densitometry of Akt levels ($n=5$).