INOSITOL 1,4,5-TRIPHOSPHATE RECEPTORS AND NAD(P)H MEDIATE Ca\(^{2+}\) SIGNALING REQUIRED FOR HYPOXIC PRECONDITIONING OF HIPPOCAMPAL NEURONS

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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 xestospongin, and RNA interference against subtype 1 of the IP\(_3\) receptor all blunted the moderate increases in [Ca\(^{2+}\)]\(_i\) (50–100 nM) required for tolerance induction. Increases in [Ca\(^{2+}\)]\(_i\) during HPC and neuroprotection following HPC were 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 [Ca\(^{2+}\)]\(_i\), in an IP\(_3\)-R-dependent manner revealed a primary role of cellular redox state in liberation of Ca\(^{2+}\) from the ER. Blockade of IP\(_3\)Rs 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. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: ischemic tolerance, endoplasmic reticulum, neuroprotection, intracellular calcium, inositol triphosphate receptors.

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 (HPC), in which a prior exposure to non-injurious hypoxia induces tolerance to subsequent severe hypoxic or ischemic stress (Dimagi 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, 2005). In this study we test the hypothesis 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 ER, involving 50–100 nM increases of intracellular Ca\(^{2+}\), underlies induction of ischemic tolerance following HPC.

A growing body of evidence shows that a moderate/ non-injurious increase in [Ca\(^{2+}\)]\(_i\) plays a critical role in neuronal HPC. Moderate increases in [Ca\(^{2+}\)]\(_i\) are known to act though 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 Fahman, 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 ER 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., 1998). 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 ER.

**EXPERIMENTAL PROCEDURES**

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

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 hours 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 mm Hg, tested with a Clark-type oxygen electrode and the temperature was 37 ± 0.5 °C. The duration of preconditioning and OGD was 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 h after the OGD. Measurements of [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 of neurons within the cultures were made in separate groups of slices.

**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. Use of multiple brain slices from each animal reduced the number of animals sacrificed. Briefly, Sprague–Dawley rats (9 days old, Charles River Laboratories, Hollister, CA) were anesthetized with 3%–5% isoflurane. This anesthetic dissolved rapidly from the hippocampal slices. The pups were decapitated and the hippocampus 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 1 h. The slices were then transferred onto 30-mm diameter membrane plates (Siskiyou Design Instruments), and stored in GBSS at 4 °C for 1 h. The slices were then transferred onto 30-mm diameter membrane plates (Siskiyou Design Instruments), and stored in GBSS at 4 °C for 1 h. The slices were then transferred onto 30-mm diameter membrane plates (Siskiyou Design Instruments), and stored in GBSS at 4 °C for 1 h.

**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 medium 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 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 (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 100 μM potassium cyanide and 2 mM sodium iodoacetate to the cultures for 20–30 min did this. One hour later, final images of PI fluorescence (equaled 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% pleuro nic 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. Silt 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). 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 (Hyrc et al., 1997). Background fluorescence (i.e. fluorescence in the absence of fura) was subtracted from total fluorescence signals prior to calculation of [Ca^{2+}]_i, as described previously (Bickler and Hansen, 1998). Estimates of [Ca^{2+}]_i with this technique are accurate to about ±10 nM (Gryniewicz et al., 1985).

In studies to delineate the cause of increases in [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 [Ca^{2+}]_i in CA1 neurons were made during 5–10 s application of 100 μM NMDA via perfusion. Ten micromolar APV, but not 1 μM. Completely blocked increases in [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 phospholipase-C mediated increases in [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, USA) 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 [Ca²⁺], 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 nm emission filters (Chroma Technology) was used in these experiments along with a Photometrics EScamera.

Measurements of lactate and pyruvate

Measurements of slice lactate and pyruvate concentrations during hypoxia involved washing slice culture inserts with HBSS (3×2 ml) and placing them into 50 ml flasks containing 7 ml of HBSS previously bubbled for 1 h with water-saturated air/5% CO₂ (normoxia) or N₂/5% CO₂ (hypoxia). The flask was sealed and flushed with the normoxic or hypoxic humidified gas mixtures while the medium 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 0.6 N perchloric acid. For each replicate, three inserts containing four to five slices each were pooled. The tubes were shaken continuously for 1 h at 4 °C to allow complete extraction of the metabolites, after which 50 μl of 0.6 M KHCO₃ 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 nm 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 three to four times. Protein content in each sample was measured (Bradford protein reagent, Bio-Rad) and adjusted so that equal amounts of protein

Fig. 1. HPC with 5 min hypoxia creates tolerance to OGD. (A, B) PI fluorescence (bright regions = PI positive) images and percentage of dead neurons 48 and 72 h after OGD. * Significant increase in cell death compared to mock P.C. (P<0.05 for n=8). (C) [Ca²⁺] in CA1 neurons (means±SE, n=6) immediately after sham or HPC, 24 h after HPC, and immediately after 10 min of OGD ** P<0.01, *** P<0.001.
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, USA) directed at the IP₃ receptor subtype 1 (ITPR1) and the TransIT-neural transfection reagent (Mirus Bio Corp., Madison, WI, USA) 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 and 72 h after addition of transfection reagents to slice culture medium. The greatest reduction in mRNA and protein was at 72 h 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, USA) and stored until RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA). DNA was synthesized using an OmniScript Reverse transcriptase kit (Qiagen, Valencia, CA, USA). 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: agctgcttggtgtgttttat (product size: 112 bp, accession number 1054962) and for /H9252–actin forward: acagctgagagggaatcgt and /H9252–actin reverse: ttctccagggaggaagag (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

HPC produces robust neuroprotection in hippocampal slice cultures

Preconditioning with 5 min hypoxia 24 h before severe OGD substantially reduced subsequent cell death in CA1 neurons. Protection was observed 48 and 72 h after the OGD (Fig. 1A, 1B).

The PI fluorescence method of cell death assessment was compared to standard histologic methods (hematoxylin/eosin staining). Regression analysis showed that the PI method reports about 7% more death than the histologic analysis, but the correlation was quite robust (cell death by PI = 0.949, cell death by histology = 0.068, r² = 0.93, n = 13 comparisons).

Intracellular Ca²⁺ changes during HPC and OGD

At the end of the 5 min HPC average [Ca²⁺], in CA1 increased from 75±18 to 125±30 nM (P<0.01, n = 13). Twenty-four hours after HPC, [Ca²⁺] was not different

Fig. 2. Small increases in [Ca²⁺], are required for hypoxia to induce ischemic tolerance in hippocampal slice cultures. (A) BAPTA-AM buffers increases in [Ca²⁺] during HPC. * Significant increase in [Ca²⁺] (P<0.05) compared to baseline; numbers indicate number of observations. (B) BAPTA-AM present during preconditioning eliminates induction of tolerance to OGD. * Indicates significant reduction in cell death following HPC (P<0.05). (C) Representative PI fluorescence images from slices at baseline, after OGD (no preconditioning), in preconditioned slices 48 h after OGD, and in a slice treated with BAPTA-AM before preconditioning.
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 fivefold by preconditioning (Fig. 1C).

**Small increases in [Ca\(^{2+}\)]_i during preconditioning are required for neuroprotection**

To determine if the moderate increases in [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. [Ca\(^{2+}\)], levels in control and BAPTA-AM-treated slices during preconditioning are shown in Fig. 2A, demonstrating that BAPTA-AM prevented the increase in [Ca\(^{2+}\)] during preconditioning, but did not alter [Ca\(^{2+}\)], 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 [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 [Ca\(^{2+}\)]_i during preconditioning. Similarly, inhibition of phospholipase C with U73122, which prevents increases in [Ca\(^{2+}\)]_i initiated by several types of cell surface signals, also did not affect the rise in [Ca\(^{2+}\)]_i during preconditioning. However, inhibition of the Ca\(^{2+}\) release complex on the ER with the selective IP\(_3\) receptor antagonist xestospongin C completely prevented the increase in [Ca\(^{2+}\)]_i during hypoxia. Xestospongin did not change [Ca\(^{2+}\)], in basal conditions, suggesting that in hippocampal slice cultures xestospongin C does not inhibit the ER Ca\(^{2+}\) uptake pump or deplete intracellular Ca\(^{2+}\) stores. Consistent with

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**Fig. 3.** Source of increased [Ca\(^{2+}\)]_i during preconditioning hypoxia is intracellular stores. (A) [Ca\(^{2+}\)]_i in CA1 neurons during HPC in untreated control slices, and in slices pre-treated with a phospholipase C inhibitor (U73122, 1 \(\mu\)M), an IP\(_3\) receptor antagonist xestospongin C (xesto C; 1 \(\mu\)M), the NMDA receptor antagonist \(N\)-phosphonovalerate (APV, 10 \(\mu\)M) or low extracellular Ca\(^{2+}\) (<0.1 mM). Data are means±SEM in 8 to 10 slices. (B) Depletion of [Ca\(^{2+}\)]_i stores with thapsigargin (10 \(\mu\)M) decreases the magnitude of the [Ca\(^{2+}\)]_i rise in CA1 during hypoxia. * Indicates significant \((P<0.05)\) increase in [Ca\(^{2+}\)]_i compared to hypoxia alone group. Data are means±SD for six experiments.
the ER being the chief source of Ca\(^{2+}\) increase during HPC, treatment of slice cultures with 10 \(\mu\)M thapsigargin, which inhibits ER Ca\(^{2+}\) uptake pumps and thereby depletes ER Ca\(^{2+}\) stores, substantially limited the increase in [Ca\(^{2+}\)] during hypoxia (Fig. 3B). To further investigate whether IP\(_3\) receptors are involved in the increase in [Ca\(^{2+}\)], 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 \(n=4\)). Fig. 4 shows that increases in [Ca\(^{2+}\)], 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 ER are the most likely cause of cytosolic Ca\(^{2+}\) increase during HPC in slice cultures.

**NAD(P)H increases during HPC and may cause an increase in [Ca\(^{2+}\)]**

During hypoxia, electron flow through the mitochondrial electron transport chain is decreased, shifting the NAD\(^+\)/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 [Ca\(^{2+}\)], during preconditioning, we measured NAD(P)H fluorescence in CA1 neurons. The time course of the change in 450 nm fluorescence (360±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\(^{2+}\)], 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\(^{2+}\)], were observed (Fig. 5C) and the increase in [Ca\(^{2+}\)], produced by NADH application was blocked by antagonizing IP\(_3\) receptors with xestospongin 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 ER.

**IP\(_3\)Rs are required for HPC neuroprotection**

Blockade of IP\(_3\)Rs by the selective antagonist xestospongin C reveals that HPC-induced tolerance requires IP\(_3\) receptors. In Fig. 6, the percentages of dead neurons 48 h after OGD with or without prior HPC are presented. While HPC reduced cell death by over 75% compared to sham HPC controls, xestospongin 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 xestospongin C during preconditioning (Fig. 7B, 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 ER is key to the induction of HPC neuroprotection in rat hippocampal neurons. We suggest that the central event in tolerance induction by hypoxia is the IP\(_3\)-receptor dependent increase in \([\text{Ca}^{2+}]_i\) 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] (Mayevsky and Rogatsky, 2007) to increases in cytosolic [NADH] (Ying, 2008). Shifts in brain cytosolic redox balance toward greater [NAD(P)H] within just minutes of non-injurious hyp-
of xestospongin-C (Xesto, 1 μM), MK-801 (10 μM) or low Ca²⁺ (<0.01 mM) medium during preconditioning on cell death 48 h 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 h after OGD.

Fig. 6. Induction of OGD tolerance requires IP₃ receptors. (A) Effects of xestospongin-C (Xesto, 1 μM), MK-801 (10 μM) or low Ca²⁺ (<0.01 mM) medium during preconditioning on cell death 48 h 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 h after OGD.

Evidence that NMDA receptors do not contribute to the rise in [Ca²⁺] during hypoxia. Our data are also consistent with those in studies by Kaplan et al. (1996) and Patterson et al. (2004, 2005) showing that changes in cytosolic redox balance during hypoxia are linked to Ca²⁺ release from the ER. Therefore, we conclude that moderate increases in [Ca²⁺] during preconditioning appear to be mostly derived from NAD(P)H-dependent release of Ca²⁺ from internal stores.

Role of the ER in adaptation of neurons to hypoxic stress

Our study showed that the ER 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. 7A). Another significant finding related to the importance of upstream Ca²⁺ signaling and survival responses was that block of IP₃ receptors with xestospongin prevented the phosphoryla-
tion of the survival kinase Akt (Fig. 7B, 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 Sharp et al., 2004; Ran et al., 2005).

The novelty here is that we show that upstream of these signals, Ca\(^{2+}\)/H\(^{1+}\) 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 ER via activation of IP\(_3\) receptors. This Ca\(^{2+}\) release, in turn, activates signaling pathways involved in survival signaling. Ca\(^{2+}\) release from the ER is therefore a key determinant of the adaptive or neuroprotective response of neurons to hypoxic stress.

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Fig. 7. HPC in hippocampal slice cultures is associated with increased nuclear CREB translocation and phosphorylation of survival kinases and requires the calcium-calmodulin dependent kinase IIa. (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, C) Western blots and densitometry of Akt levels (n=5).


