Preconditioning of cortical neurons by oxygen-glucose deprivation: tolerance induction through abbreviated neurotoxic signaling

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Preconditioning of cortical neurons by oxygen-glucose deprivation: tolerance induction through abbreviated neurotoxic signaling. Am J Physiol Cell Physiol 285: C899–C911, 2003. First published June 18, 2003; 10.1152/ajpcel.00110.2003.—Transient exposure of rat cortical cultures to nonlethal oxygen-glucose deprivation (OGD preconditioning) induces tolerance to otherwise lethal oxygen-glucose deprivation (OGD) or N-methyl-d-aspartate 24 h later. This study evaluates the role of cytosolic and mitochondrial Ca2+-dependent cellular signaling. Mechanistic findings are placed in context with other models of ischemic preconditioning or known neurotoxic pathways within cortical neurons. Tolerance to otherwise lethal OGD is suppressed by performing OGD preconditioning in the presence of the broad-spectrum catalytic antioxidants Mn(III)tetra(4-carboxyphenyl)porphyrin (MnTBAP) or Zn(II)tetra(4-carboxyphenyl)porphyrin (Zn(II)TBAP), but not by a less active analog, Mn(III)tetra(4-sulfonatophenyl)porphyrin, or a potent superoxide scavenger, Mn(III)tetra(N-ethyl-2-pyridyl)porphyrin chloride. Inhibitors of adenosine A1 receptors, nitric oxide synthase, mitogen-activated protein kinase, and poly(ADP-ribose) polymerase fail to suppress OGD preconditioning despite possible links with reactive oxygen species in other models of ischemic preconditioning. Preconditioning is suppressed by 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), which has been ascribed elsewhere to inhibition of superoxide transport to the cytosol through mitochondrial anion channels. However, although it induces mitochondrial Ca2+-uptake, neuronal preconditioning is largely insensitive to mitochondrial uncoupling with carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone or 2,4-dinitrophenol. Uncouplers will prevent production of mitochondrial reactive oxygen species, implying nonmitochondrial targets by MnTBAP, Zn(II)TBAP, and DIDS. Emphasizing the importance of an increase in cytosolic Ca2+ during preconditioning, a Ca2+/calmodulin-dependent protein kinase II inhibitor, KN-62, suppresses development of subsequent tolerance. Summarizing, only those cellular transduction pathways that have the potential to be neurotoxic may be activated by preconditioning in cortical neurons. Finally, a marked decrease in extracellular glutamate is observed during otherwise lethal OGD in preconditioned cultures, suggesting that this end effector may represent a point of convergence across different preconditioning models.

N-methyl-d-aspartate; Ca2+; antioxidants; mitochondria

ISCHEMIC PRECONDITIONING is a ubiquitous phenomenon, having been successfully applied in many species, organs, and isolated preparations (27, 45). This implies that cellular receptors/signaling common to all models may be activated. Ultimately, identification of similarities in signal transduction pathways in the heart and brain, for instance, suggests that novel therapeutic approaches may be developed to protect both organs. However, numerous signaling pathways appear to be implicated for ischemic preconditioning (for instance, see Refs. 27, 45).

Cultured neurons exposed to tolerance-inducing oxygen-glucose deprivation (OGD) preconditioning can be used to investigate neuron-specific stimuli that might mediate cerebral ischemic preconditioning (6). An initial attempt to correlate cellular signaling between the heart and brain revealed diverse pathways (63). Hence, an additional framework was sought to study the mechanism of neuronal OGD preconditioning.

Ischemic preconditioning protocols that induce tolerance are also capable of producing injury if too intensive or persistent. As for cerebral ischemic preconditioning, the threshold for OGD preconditioning and for neuronal injury may not be that far apart. The preconditioning paradigm employed in this study requires subjecting cortical cultures to as great an OGD stress as possible without causing toxicity. Thus we now consider the possibility that preconditioning initiates an endogenous neuroprotective response against OGD toxicity, primarily through cellular signaling that has the potential to be neurotoxic if of sufficient intensity. In cortical neurons, OGD of sufficient duration results in neuronal N-methyl-d-aspartate (NMDA) receptor-mediated Ca2+ influx, which may induce the formation of toxic reactive oxygen species. The feasibility of such a concept has already been demonstrated at the receptor level: transient activation of the neuronal NMDA receptor alone may precondition or kill cortical neurons, depending on the intensity of the stimulus (20).

Cerebral ischemic or excitotoxic generation of reactive oxygen species has been the subject of intense investigation. Intermediate between physiological and pathological activity, reactive oxygen species may also

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serve as activators of redox-sensitive signal transduction pathways during ischemic preconditioning. Mitochondrial Ca\textsuperscript{2+} uptake upregulates energy metabolism, resulting in increased generation of reactive oxygen species, although the mechanism (70) and temporal pattern of oxidant production by excitotoxicity (69) remains uncertain. The antioxidant MnTBAP \([\text{Mn(III)tetra(4-carboxyphenyl)porphyrin}]\) suppresses ischemic tolerance generated by anion channels (68). Reactive oxygen species have been implicated in ischemic tolerance generated by mitochondrial Ca\textsuperscript{2+} channels (66). Reactive oxygen species-dependent hypoxic preconditioning in cardiomyocytes is suppressed by an anion channel blocker, 4,4'-disothiocyanostilbene-2,2'-disulfonic acid (DIDS), implicating superoxide transfer to the cytosol via mitochondrial anion channels (68). Reactive oxygen species have been implicated in ischemic tolerance generated by "chemical" preconditioning of the brain (for example, see Ref. 73). Superoxide radicals and H\textsubscript{2}O\textsubscript{2} alone can "precondition" neurons against glutamate toxicity (48, 49) or ischemia (40).

In the current study, Ca\textsuperscript{2+}-dependent neuronal signaling activated by NMDA receptors during OGD preconditioning is evaluated. Specifically, signaling pathways implicated in other models of ischemic preconditioning, as well as those linked to neurotoxic signaling activated by lethal OGD in cortical neurons, are investigated.

**MATERIALS AND METHODS**

**Materials**

Tissue culture plates were purchased from either DuPont-Life Technologies (Burlington, ON, Canada) or VWR Canlab (Mississauga, ON, Canada), and 12-mm glass coverslips were purchased from Belco Glass (Vineland). Fetal bovine serum and modified Eagle’s medium (MEM) were obtained from Wisent Canadian Laboratories (St-Bruno, QC, Canada). Horse serum was acquired from Hydclone Laboratories (Logan). Fluoro-4, Am, rufa red-AM, Fluor-4F, and DIDS were bought from Molecular Probes (Eugene, OR). 4,5-Diaminofluorescein diacetate (DAF-2) was purchased from Calbiochem (San Diego, CA). Diethylamine (DEA)/NONOate was purchased from Cedarlane Laboratories (Hornby, ON, Canada). MnTBAP and KN-62 were purchased from Alexis Biochemicals (San Diego, CA). Zn(II)tetrakis(4-carboxyphenyl)porphyrin \([\text{Zn(II)TBAP}], \text{Mn(III)tetra(4-sulfonatophenyl)porphyrin} [\text{Mn(III)TPPS}], \text{and Mn(III)tetra(N-ethyl-2-pyridyl)}\) porphyrin chloride \([\text{Mn(III)TE-PyP2}], \text{1,5-isoquinolinediol (IQL)}, \text{8-cyclopentylthiophylline (8-CTP)}, \text{8-p-sulfonylethylthiophylline (8-SPT)}, \text{8-cyclopentyl-1,3-dipropylxanthine (DPCPX)}, \text{N}\textsuperscript{\text{6}}R-phenylisopropyladenosine (R-PIA)}, \text{carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP)}, \text{U0126}, \text{2,4-dinitrophenol (DNP)}, \text{tetrodotoxin (TTX)}, \text{and all other reagents were purchased from Sigma (St. Louis, MO).}

**Preparation of Rat Cortical Cultures**

Cultures of E18 rat cortical neurons were prepared as described previously (64). Timed-pregnant Sprague-Dawley rats (Charles River Canada, St. Constant, QC, Canada) were anesthetized with halothane and killed by cervical dislocation. After dissection of the cortical region of the fetal brain, cortical neurons were dispersed by trituration, centrifuged at 250 g for 5 min at 4°C, and suspended in a medium containing MEM supplemented to 25 mM glucose, 10% fetal bovine serum, 10% horse serum, and 2 mM glutamine. Cells were plated at 1.0 × 10\textsuperscript{6} cells/ml on poly-L-lysine-coated, glass coverslips (for confocal measurements) or at 1.7 × 10\textsuperscript{6} cells/ml on tissue culture plates. Cultures were treated with 15 μg/ml 5-fluoro-2’-deoxyuridine and 35 μg/ml uridine after 4 days in vitro to minimize glial growth. At 7 days in vitro, one-half of the medium was replaced with medium consisting of MEM and 10% horse serum. Experiments were performed on cultures from 14–18 days in vitro. All experiments were approved by the Institute’s Animal Care Committee.

**Neurotoxic Insults**

**OGD.** OGD was performed as previously described (64) by placing cultures in a 37°C culture chamber housed in an anaerobic glovebox (Forma Scientific, Marietta, OH). Cultures were washed twice in a glucose-free balanced salt solution (BSS) at 25°C with the following composition (in mM): 140 NaCl, 5 KCl, 2 CaCl\textsubscript{2}, 20 HEPEs, and 0.03% glucose and maintained at pH 7.4. Cultures were then subjected to an anaerobic environment of 95% N\textsubscript{2}-5% CO\textsubscript{2} for 75–90 min, producing an O\textsubscript{2} partial pressure equal to 10–15 Torr, as measured with an oxygen microelecorder (Microelecorders, Londonberry, NH). OGD was terminated by replacement of stored medium and by returning the cultures to a standard incubator maintained at 37°C in 95% O\textsubscript{2}-5% CO\textsubscript{2}. Control cultures were exposed to BSS that contained 3 mM p-glucose and maintained in the standard incubator. Durations of OGD employed were 60–70 min for OGD preconditioning or 75–90 min for lethal OGD. As described previously (64), constant durations for each condition were not employed due to variability in the susceptibility of cultures to OGD between, but not within, platings. **NMDA exposure.** After being washed in glucose-containing BSS, cultures were exposed to 15–25 μM NMDA or 30–40 μM NMDA to precondition or to kill neurons, respectively, in glucose-containing BSS for 15–25 min at room temperature. After washout, the stored medium was returned to cultures.

**Drug application.** Drugs were either coapplied with OGD or NMDA treatment or applied 0.5–2 h before, during, and 24 h post-OGD or -NMDA treatment. For each 12-well plate subject to OGD, 3 to 6 wells were devoted to drug-free OGD to account for variability arising between plates or platings of cells. All experiments were performed on 2–6 different platings of cells, with 3–6 wells devoted to each condition.

**Assessment of Neuronal Injury**

Neuronal injury was assessed 24 h after treatments by exposing cells to PI measurement of fluorescence intensities with a platereadere (64). Briefly, media in 12-well plates were replaced with 0.5 ml/well of BSS containing glucose and 4.5 μM PI and incubated at 37°C for 30 min. The fluorescence intensity (Ex = 530 ± 20 nm; Em = 645 ± 20 nm) from four locations within each well was then measured on a Cytofluor 2350 plateradeere (Millipore, Bedford, MA). The %PI uptake was determined by subtracting the fluorescence measured in untreated sister cultures containing PI and then normalizing values to the fluorescence representing 100% neuronal death, which was obtained by exposing sister cultures to 100 μM NMDA for 15 min. Thus the %PI uptake is equivalent to the percentage of dead cells above control levels (death in untreated control wells was <5%).

**Preparation of Rat Cortical Cultures**

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Mitochondrial Ca\(^{2+}\) loading was evaluated by using a modified published procedure (8). Briefly, the uptake of mitochondrial Ca\(^{2+}\) after transient NMDA exposure was defined as the Ca\(^{2+}\) detected by cytosolic fluorescence indicators after mitochondrial depolarization induced by the protonophore FCCP. After coloading of the cultures with the Ca\(^{2+}\)-sensitive fluorescence dyes, 5 \(\mu\)M fluo 3-AM and 10 \(\mu\)M fura red-AM, coverslips were placed in a microperfusion system housed on the stage of an LSM-410 Zeiss (Carl Zeiss, Thornwood, NY) inverted laser scanning microscope equipped with an argon-krypton ion laser and with a Fluar \(\times\) 40 1.3 oil-immersion objective. After a region of interest was chosen, fluorophores were excited with a 488-nm laser line, selected by a band-pass filter 485/20 and attenuated by a neutral density filter (to \(<1\)%). Emitted fluorescence from both fluorophores was separated at a cut-off point of 560 nm by dichroic beam splitters (FT560). Fluo-3 and fura red fluorescence were simultaneously detected after filtration through a 515- to 540-nm band-pass and a long-pass LP590 filter, respectively. Images of 256 \(\times\) 256 pixels were acquired at a rate of one image/10 s, and images were averaged over 8 scans. Images were acquired with 50% of the light emitted from an optical slice 5 \(\mu\)m thick, with a confocal aperture adjusted to a full-width half maximum of 5 \(\mu\)m for both emitted wavelengths. Ratios of Flu-3 to fura red intensities were evaluated for each image.

Measurement of Cytosolic Intracellular Ca\(^{2+}\)

Cytosolic Ca\(^{2+}\) concentrations were determined by using temperature-controlled platereader measurements of fluorescence intensity from 12-well plates containing cultures loaded with a Ca\(^{2+}\)-sensitive low-affinity fluorescent dye, Fluo-4FF-AM \((K_a = 9.7\ \mu\)M; Ex = 485 \(\pm\) 20 nm; Em = 530 \(\pm\) 20 nm). Cultures were washed in glucose-containing BSS and then loaded with 4.5 \(\mu\)M Fluo-4FF in BSS for 45 min at 37°C, washed twice, and allowed to equilibrate for 15 min at 22°C. Before treatment, cultures were washed once in BSS and fluorescence intensity was measured from four locations within each well in the platereader at 25°C. Fluorescence was again measured during a 15- to 20-min exposure to NMDA: ± inhibitor in BSS (the increase in fluorescence intensity for NMDA was typically 4 times that of the initial signal). The \%\_increase in \([\text{Ca}^{2+}]_i\) was calculated by subtracting the initial reading from the final reading, divided by the initial reading. Values were normalized relative to 0\% for control wash and 100\% for NMDA alone.

Measurement of Extracellular Glutamate Concentration

Immediately after exposure of cultures to OGD, control wash, or glutamate, buffer solutions were collected and stored at \(-80\)°C. Glutamate concentrations were determined by using a commercially available kit (Molecular Probes), in which glutamate is enzymatically converted to H\(_2\)O\(_2\), which in turn reacts with Amplex Red reagent to produce the fluorescent product resorufin, and were detected using the fluorescent platereader.

Statistics

Three to four wells were investigated per condition from a minimum of three different platings. Data are represented as means \(\pm\) SE. Statistical comparisons were made by analysis of variance (ANOVA). When significant differences were observed, Tukey’s test was employed for multiple comparisons. Statistical significance was inferred at \(P < 0.05\).

RESULTS

Three related preconditioning protocols were employed to investigate the mechanism of OGD preconditioning. The first pathway investigated was OGD preconditioning-induced tolerance to otherwise lethal OGD. The second pathway investigated was NMDA preconditioning-induced tolerance to otherwise lethal OGD. We have verified (19) that OGD preconditioning activates NMDA receptors (63). Moreover, transient activation of NMDA receptors (NMDA preconditioning) also renders cortical cultures profoundly OGD tolerant (19). Hence, investigating this second pathway allows a determination of whether signaling identified in OGD preconditioning exerts its actions upstream or downstream of the NMDA receptor. The third pathway investigated was NMDA preconditioning-induced tolerance to otherwise lethal NMDA. We have previously suggested that NMDA preconditioning can induce tolerance to otherwise lethal NMDA, which may represent an important subpathway in OGD-preconditioning induced tolerance to OGD (64).

Role of Reactive Oxygen Species in OGD and NMDA Preconditioning

MnTBAP. Preconditioning by exposing cultures to OGD for 60–70 min rendered neurons significantly resistant to 75–90 min of otherwise lethal OGD 24 h later, as evaluated using %PI uptake 24 h later. Coapplication of 200 \(\mu\)M MnTBAP during OGD preconditioning significantly suppressed tolerance to otherwise lethal OGD (Fig. 1A). Preconditioning by exposing cultures to 15–25 \(\mu\)M NMDA for 15–25 min rendered neurons significantly resistant to 75–90 min of otherwise lethal OGD 24 h later (Fig. 1B). Coapplication of 200 \(\mu\)M MnTBAP during NMDA preconditioning significantly suppressed OGD tolerance (Fig. 1B). Preconditioning by exposing cultures to 15–25 \(\mu\)M NMDA applied for 15–25 min rendered neurons significantly resistant to 30–40 \(\mu\)M NMDA applied for 15–25 min 24 h later (Fig. 1C). Application of 200 \(\mu\)M MnTBAP during NMDA preconditioning suppressed tolerance to otherwise lethal NMDA (Fig. 1C). None of the drugs investigated in this study was neurotoxic on their own (data not shown).

Other antioxidants. Having identified reactive oxygen species as important to preconditioning, the effect of other antioxidants on NMDA preconditioning was investigated. Application of \(100\ \mu\)M Zn(II)/TBAP, but not a less active analog, 160 \(\mu\)M Mn(III)/TPPS, during NMDA preconditioning resulted in significant suppression of OGD tolerance (Fig. 2). Scavenging abilities by MnTBAP and Zn(II)/TBAP include superoxide, H\(_2\)O\(_2\), peroxides, peroxynitrite, lipid peroxidation products, and carbonic radicals (13, 42, 62). Application of a much more potent superoxide dismutase and catalase mimetic, 50 \(\mu\)M Mn(III)/TE-PyP(2), for 1 h before, during, and 24 h post-NMDA preconditioning did not significantly suppress OGD tolerance (Fig. 2).
Role of Mitochondria Ca\(^{2+}\) in Preconditioning

**Mitochondrial transport of superoxide.** Tolerance to otherwise lethal OGD was significantly suppressed by inclusion of 0.5 mM DIDS during OGD or NMDA preconditioning (Fig. 3). Application of 0.5 mM DIDS during NMDA preconditioning also suppressed tolerance to otherwise lethal NMDA; however, in the non-preconditioning control experiment, transient exposure to DIDS alone rendered cultures significantly more susceptible to NMDA applied 24 h later (Fig. 3).

**Mitochondrial Ca\(^{2+}\) loading.** We considered whether a source of the MnTBAP/Zn(II)/TBAP- and DIDS-sensitive component to OGD and NMDA preconditioning was nonlethal reactive oxygen species generated downstream of mitochondrial Ca\(^{2+}\) loading. Reactive oxygen species may be generated within cortical neuronal mitochondria as a result of mitochondrial Ca\(^{2+}\) loading consequent to an NMDA-initiated increase in [Ca\(^{2+}\)]\(_i\) (14, 50). MnTBAP can access neuronal cortical mitochondria (33).

We determined whether NMDA preconditioning caused mitochondrial Ca\(^{2+}\) loading by using a micropenetration protocol. Uptake of mitochondrial Ca\(^{2+}\) after transient NMDA exposure was measured as the Ca\(^{2+}\) detected by cytosolic fluorescence indicators after mitochondrial depolarization induced by the protonophore FCCP (8). Single-neuron fluorescence was monitored on cultures loaded with the 2+ -sensitive dyes, Fluo-3 and fura red, and micropenetrated with glucose-containing buffer for 5 min, 25 μM NMDA for 5 min, buffer for 5 min, Ca\(^{2+}\)-free buffer for 30 s, and finally 2 μM FCCP in Ca\(^{2+}\)-free buffer to release Ca\(^{2+}\) present within mitochondria into the cytosol. NMDA typically induced a reversible rise in Fluo-3/fura red fluorescence.

**Fig. 1.** Effect of a catalytic antioxidant, Mn(III)tetra(4-carboxyphenyl)porphyrin (MnTBAP), on preconditioning-induced tolerance. A: cultures were exposed to 60–70 min of oxygen-glucose deprivation (OGD) preconditioning or nonpreconditioning followed by exposure to 75–90 min of lethal OGD 24 h later. B: cultures were exposed to 15–25 min of 15–25 μM N-methylaspartate (NMDA) preconditioning or nonpreconditioning wash ± 200 μM MnTBAP and then subjected to 75–90 min of lethal OGD 24 h later. C: cultures were exposed to 15–25 min of 15–25 μM NMDA preconditioning or nonpreconditioning wash ± 200 μM MnTBAP, followed by exposure to 15–25 min of 30–40 μM lethal NMDA applied 24 h later. Values represent the percentage of the maximal fluorescence intensity measured 24 h later in propidium iodide (PI)-exposed cultures. Each bar represents means ± SE from 3–4 platings comprising 4–6 cultures per condition. *Significant differences between preconditioned cultures ± MnTBAP; †significant differences between preconditioned and nonpreconditioned cultures were shown using an ANOVA.

**Fig. 2.** Effect of catalytic antioxidants on NMDA preconditioning-induced tolerance to OGD. Cultures were exposed to 15–25 min of 15–25 μM NMDA preconditioning or nonpreconditioning wash ± 100 μM Zn(II)/tetra(4-carboxyphenyl)porphyrin [Zn(II)/TBAP] or 160 μM Mn(III)/tetra(4-sulfonatophenyl)porphyrin [Mn(III)/TPPS] and then subjected to 75–90 min of lethal OGD 24 h later. Alternatively, 50 μM Mn(III)/tetra(N-ethyl-2-pyridyl)porphyrin chloride [Mn(III)/TE-PyP(2)] was applied for 1 h before, during, and after post-NMDA preconditioning or nonpreconditioning wash. Values represent the percentage of the maximal fluorescence intensity measured 24 h later in PI-exposed cultures. Each bar represents means ± SE from 3–4 experiments comprising 3–4 platings per condition. * Significant difference between preconditioned cultures ± Zn(II)/TBAP; †significant differences between preconditioned and nonpreconditioned cultures were shown using an ANOVA.
nescence ratio, followed by a substantial increase in the neuronal fluorescense ratio with FCCP treatment (Fig. 4). In this protocol, FCCP did not increase the fluorescense ratio in cultures that were not preexposed to NMDA (data not shown).

**Mitochondrial uncouplers.** Mitochondrial generation of reactive oxygen species can be indirectly prevented by using the mitochondrial protonophore FCCP or the mitochondrial uncoupler DNP to depolarize the mitochondrial membrane and prevent mitochondrial Ca\(^{2+}\) loading (7, 50, 69). Coapplication of 0.75 μM FCCP, but not 200 μM DNP, during 25 μM NMDA preconditioning significantly suppressed tolerance to OGD 24 h later (Fig. 5). The loss of protection with FCCP was incomplete, though, because cultures were still significantly protected from otherwise lethal OGD.

**Fig. 4.** NMDA preconditioning induces mitochondrial Ca\(^{2+}\) uptake. Confocal microscopic images of neurons dual loaded with the Ca\(^{2+}\) indicators Fluo-3 and fura red. Time-lapse series of images (top) and the corresponding mean Fluo-3/fura red intensity ratios (bottom) in response to microperfusion of wash, 25 μM NMDA, washout (in 0 mM extracellular Ca\(^{2+}\)), and 2 μM FCCP (in 0 mM extracellular Ca\(^{2+}\)).

**Fig. 5.** Effect of mitochondrial uncoupling on NMDA preconditioning-induced tolerance to OGD. Cultures were exposed to 15–25 min of 15–25 μM NMDA preconditioning or nonpreconditioning wash followed by exposure to 75–90 min of lethal OGD 24 h later. Values represent the percentage of the maximal fluorescence intensity measured 24 h later in PI-exposed cultures. Each bar represents means ± SE from 3–4 platings comprising 3–4 cultures per condition. *Significant differences between preconditioned or nonpreconditioned cultures were shown using an ANOVA.
The effect of each of these inhibitors on cytosolic \([\text{Ca}^{2+}]\) was also examined. Application of 0.75 \(\mu\text{M}\) FCCP or 200 \(\mu\text{M}\) DNP during 15 min of NMDA preconditioning resulted in increases in \([\text{Ca}^{2+}]\) that were 115 \(\pm\) 0 and 109 \(\pm\) 3% that of a 25 \(\mu\text{M}\) NMDA induced increase, respectively. This confirmed that an NMDA preconditioning-induced increase in cytosolic \([\text{Ca}^{2+}]\) was not significantly suppressed by FCCP or DNP (60). A 15-min application of 0.75 \(\mu\text{M}\) FCCP or 200 \(\mu\text{M}\) DNP alone increased \([\text{Ca}^{2+}]\) by 68 \(\pm\) 15 and 3 \(\pm\) 3%, respectively, measured relative to a 25 \(\mu\text{M}\) NMDA preconditioning-induced increase. In control experiments, subjecting cultures to 15 min buffer alone caused no significant increase in \([\text{Ca}^{2+}]\).

**Nitric oxide-linked pathways in OGD preconditioning.** Inclusion of nitric oxide synthase (NOS) inhibitors, 100 \(\mu\text{M}\) 7-NI or 100 \(\mu\text{M}\) l-NNA, for 30 min before, during, and 24 h post-OGD preconditioning did not eliminate OGD tolerance (Fig. 6A). We addressed in further detail the basis for the negligible role for nitric oxide in our cultures, given a report of Gonzalez-Zulueta et al. (18) of the central role for this neurotransmitter in a similar model of OGD preconditioning. An intracellular dye, DAF-2, allows detection of NMDA receptor-mediated production of nitric oxide (31) and has been used to confirm nitric oxide production during hypoxic preconditioning in cardiomyocytes (32). As a positive control, cortical cultures preloaded with 10 \(\mu\text{M}\) DAF-2 were exposed to a nitric oxide donor, DEA/NONOate, which rapidly produces nitric oxide (half-life of 16 min at 25°C) (16). DEA/NONOate caused an immediate and sustained increase in neuronal fluorescence (Fig. 7, A and B). Plotting neuronal fluorescence intensity vs. [DEA/NONOate] resulted in a slope of 1 (correlation coefficient = 0.99) (Fig. 7C). In contrast, a 10-min application of 100 \(\mu\text{M}\) NMDA did not significantly increase neuronal fluorescence (data not shown) and correlated with negligible generation of nitric oxide (Fig. 7C). NADPH-diaphorase staining, a marker of neuronal NOS-containing neurons (11), was also negligible (data not shown). Hence, the current cultures likely do not generate enough nitric oxide to participate in a lethal or preconditioning stress due to an insufficient number of neurons containing the neuronal isoform of NOS.

Mitogen-activated protein (MAP) kinases are reported to be activated downstream of OGD preconditioning-induced nitric oxide production (18). However, inclusion of a MAP kinase inhibitor employed in that study, 50 \(\mu\text{M}\) PD-98059, or another inhibitor, 5 \(\mu\text{M}\) U0126, for 30 min before, during, and 24 h post-OGD preconditioning did not eliminate OGD tolerance (Fig. 6A).

Nitric oxide produced by NOS can activate poly-(ADP-ribose) polymerase (PARP) to neurotoxic levels (61), but moderate stimulation of PARP may suppress cerebral ischemia (39) or cardiac ischemic preconditioning (34). However, inclusion of the PARP inhibitor, 10 \(\mu\text{M}\) IQL, for 30 min before, during, and 24 h post-OGD preconditioning did not eliminate OGD tolerance (Fig. 6A).

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**Fig. 6.** A: tolerance to otherwise lethal OGD 24 h post-OGD preconditioning or control wash \(\pm\) inhibitors of nitric oxide synthase, MAP kinase, or poly(ADP-ribose) polymerase (PARP). Cultures were exposed to the nitric oxide synthase inhibitors 100 \(\mu\text{M}\) 7-nitroindazole (7-NI) or 100 \(\mu\text{M}\) \(L\)-nitro-l-arginine (l-NNA) or the MAP kinase inhibitors 50 \(\mu\text{M}\) PD-98059 or 5 \(\mu\text{M}\) U1026 and to PARP inhibitor 10 \(\mu\text{M}\) 1,5-isoquinolinediol (IQL) for 30 min before, during, and 24 h post-OGD preconditioning or control wash, followed by exposure to otherwise lethal 75–90 min of lethal OGD. Values represent the percentage of the maximal fluorescence intensity measured 24 h later in PI-exposed cultures. Each bar represents means \(\pm\) SE from 3–4 platings comprising 4–6 cultures per condition. *Significant differences between preconditioned and control were inferred using an ANOVA.
A1 agonist, 100 μM R-PIA, for 30 min before, during, and 24 h post-OGD preconditioning did not eliminate OGD tolerance (Fig. 6B).

Role of CaMKII in OGD and NMDA preconditioning. CaMKII is a kinase the function of which is integrally coupled with NMDA receptor activation (15). After 75–90 min of otherwise lethal OGD, the %PI uptake was significantly higher between cultures preconditioned 24 h earlier in the absence and presence of a CaMKII inhibitor, 5 μM KN-62, applied for 180 min before and during 60–70 min of OGD preconditioning (Fig. 8A). This loss of protection was not complete, though, because cultures preconditioned in the presence of KN-62 were still significantly protected from otherwise lethal OGD. Similar results were obtained when examining NMDA preconditioning-induced tolerance to otherwise lethal NMDA (Fig. 8B). In contrast, inclusion of KN-62 during NMDA preconditioning did not significantly increase the %PI uptake in cultures subsequently exposed to otherwise lethal NMDA 24 h later (Fig. 8C). However, in the control experiment, treatment of cultures with KN-62 24 h before exposure to otherwise lethal NMDA also significantly decreased the %PI uptake, so a role for CaMKII cannot be ruled out in this pathway. KN-62 has been reported to inhibit voltage-dependent Ca2+ channels (58); however, acute application of KN-62 did not significantly inhibit an NMDA-induced rise in [Ca2+]i (data not shown).

Comparison of cellular signaling activated by OGD/NMDA preconditioning or lethal OGD/NMDA. To compare cellular signaling activated by lethal vs. preconditioning stress, for each agent or class of agents the %suppression of OGD toxicity was plotted against the %suppression of OGD preconditioning-induced tolerance to OGD (preconditioning data were normalized to 0% for the drug-free condition) (Fig. 9A). A slope of unity (dashed lines) represents a perfect correlation between the degree of suppression of lethal and preconditioning stress for that particular agent or class of agents. First, as for OGD, agents that suppress NMDA preconditioning fall into two categories. The NOS inhibitors, MAP kinase inhibitors, PARP inhibitor, and adenosine A1 antagonists and agonists did not protect neurons against 75–90 min of lethal OGD, whereas MnTBAP, DIDS, and KN-62 were significantly protective (data not shown). These latter three compounds suppress OGD preconditioning (Figs. 1, 2, and 8, respectively) and, therefore, plot near the (0,0) coordinate. Hence, the degree of suppression of OGD preconditioning correlates with the degree of suppression of OGD toxicity for all compounds investigated.

Similarly, the %suppression of NMDA-toxicity was plotted against the %suppression of NMDA preconditioning-induced tolerance to OGD (Fig. 9B). Inhibitors that suppress NMDA preconditioning fall into two categories. First, as for OGD, agents that suppress NMDA preconditioning [MnTBAP, Zn(II)TBAP, and KN-62 in Figs. 1, 2, and 8, respectively] also significantly protect

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**fig. 7.** Response of neurons loaded with the nitric oxide-sensitive dye DAF-2 to the nitric oxide donor DEA/NONOate. Representative same-field phase-contrast (A) and fluorescence images (B) of cultures loaded with 10 μM DAF-2 acquired 15 min after application of 60 μM DEA/NONOate, which produced a slope of 1 having a correlation coefficient of 0.99. In sister cultures, 100 μM NMDA produced 30 fluorescence units, equivalent to <1 μM nitric oxide.

Reactive oxygen species induce release of adenosine in the hippocampus (2), which may in turn stimulate nitric oxide production (4), possibly during ischemic or hypoxic preconditioning (9, 44). However, inclusion of the adenosine A1 receptor antagonists, 10 μM DPCPX, 10 μM 8-CPT, or 150 μM 8-SPT, for 30 min before, during, and 24 h post-OGD preconditioning did not significantly suppress tolerance to otherwise lethal OGD (Fig. 6B).

A1 receptor agonists can be beneficial in various models of cerebral ischemia, possibly through suppression of glutamate release through hyperpolarization of presynaptic neuronal terminals (53). Because glutamate is a key mediator in OGD preconditioning (20), the alternative possibility of activating the adenosine A1 receptor during OGD preconditioning was considered. However, application of 1 mM adenosine or the...
against lethal NMDA (data not shown), whereas those compounds that do not suppress NMDA preconditioning [Mn(III)TPPS and Mn(III)TE-PyP(2) in Fig. 2] do not protect either (data not shown). Second, the mitochondrial uncouplers FCCP and DNP suppress lethal NMDA toxicity (data not shown) more effectively than NMDA preconditioning (Fig. 5), so the values for FCCP and DNP plot well below the unity slope (Fig. 9B).

Effect of preconditioning on extracellular glutamate levels during otherwise lethal OGD. Lethal OGD results in excessive levels of extracellular glutamate ([\text{glutamate}]_{\text{ex}}). A reduction in [\text{glutamate}]_{\text{ex}} observed during otherwise lethal ischemia may represent a common end effector of divergent models and signaling pathways initiated by preconditioning (20, 28, 56). Cultures were subjected to NMDA preconditioning or non-preconditioning wash, and 24 h later buffer was collected at the termination of 75–90 min of lethal OGD. Preconditioning resulted in a significant reduction in [\text{glutamate}]_{\text{ex}} (Fig. 10). PI analysis performed 24 h later confirmed significant protection (data not shown).

DISCUSSION

The primary purpose of this study was to identify early events in OGD preconditioning of cortical neurons responsible for inducing subsequent tolerance to...
otherwise lethal OGD. A secondary purpose was to determine the degree of overlap in signaling with the NMDA receptor-mediated pathway previously identified in these cultures (64), as well as with other ischemic/OGD preconditioning models in brain and other tissue. Taken together, the data show that an NMDA-mediated increase in cytosolic [Ca\(^{2+}\)], and not in mitochondrial Ca\(^{2+}\) uptake, appears to underlie the sensitivity of OGD or NMDA preconditioning or cortical neuron cultures to the antioxidants MnTBAP and Zn(II)/TBAP, DIDS, and the CaMKII inhibitor KN-62. Key differences in signaling between other laboratories are also identified, even compared with ostensibly similar models of OGD preconditioning in neuronal cortical culture. To account for this, OGD and NMDA tolerance may be induced only through abbreviated neurotoxic signaling. Hence, early signaling activated by ischemic preconditioning may be largely tissue and model specific, overlapping with other models only if a pathway has the potential to be universally lethal.

**MnTBAP- and Zn(II)/TBAP-Dependent Preconditioning**

The suppression of tolerance by including MnTBAP and the structurally related Zn(II)/TBAP during OGD or NMDA preconditioning of cortical neurons implicates reactive oxygen species participation (Figs. 1 and 2). The fact that all three tolerance pathways investigated are suppressed by MnTBAP (Fig. 1, A–C) is consistent with our working hypothesis that NMDA preconditioning-induced tolerance to NMDA is a crucial subpathway of OGD preconditioning-induced tolerance to OGD and that reactive oxygen species are involved in this process (64). MnTBAP has been extensively investigated in neurons. Neurotoxic NMDA decreases aconitase activity in cultured cortical neurons, indicative of overproduction of intracellular reactive oxygen species, whereas MnTBAP protects by preserving aconitase activity (33). MnTBAP protects against the classic apoptotic agent staurosporine (41). MnTBAP does not block NMDA receptor-mediated whole cell currents, acute or delayed rises in [Ca\(^{2+}\)], or mitochondrial depolarization (43, 69). Collectively, this evidence suggests specificity of MnTBAP against reactive oxygen species generated downstream of an NMDA receptor-mediated increase in [Ca\(^{2+}\)]\(_i\) (42). It is difficult to identify which reactive oxygen species are involved: possibilities include superoxide, H\(_2\)O\(_2\), lipid peroxyl radicals, peroxynitrite, and carbonate anion radicals (12, 13, 47, 62, 75). Several possibilities could account for the different effects of MnTBAP and Mn(III)/TE-PyP(2) in preconditioning. MnTBAP achieves higher cytosolic levels than Mn(III)/TE-PyP(2) in cortical neurons (33). Intracellular localization or other properties may be affected by differences in overall net charge between MnTBAP (−3 charge) and Mn(III)/TE-PyP(2) (+5 charge). Structure/activity relationships determined in cell-free assays may not directly translate to cells. The scavenging properties of each antioxidant can be differentially altered by endogenous reductants, reaction rates, intermediate species generated during reactions, intracellular compartmentalization/binding, or degradation of the antioxidant. N-alkylpyridinium-based porphyrins may be inactivated through binding to intracellular proteins or degraded by reaction with H\(_2\)O\(_2\) (51). Whatever the basis for these differences, the ineffectiveness of Mn(III)/TE-PyP(2) is internally consistent, failing to suppress a lethal NMDA stress as well (Fig. 8B and Ref. 33).

**Mitochondria-Independent Preconditioning**

Despite mitochondrial Ca\(^{2+}\) loading during NMDA preconditioning (Fig. 4), the uncoupler-based data do not support a major role for generation of reactive oxygen species within the mitochondria. The inability of DNP to mimic FCCP by at least partially suppressing NMDA preconditioning-induced tolerance against OGD (Fig. 5) cannot be attributed to ineffective uncoupling of the mitochondria because both uncouplers completely protect neurons against lethal NMDA (60) (Fig. 8B). Hence, FCCP may partially suppress preconditioning through an alternative mechanism, perhaps related to the ability of FCCP to increase [Ca\(^{2+}\)]\(_i\) on its own, possibly through plasma membrane depolarization (72). Consequently, MnTBAP likely suppresses preconditioning by mitochondrial-independent scavenging of oxygen radicals. In studies reporting inhibition of preconditioning by MnTBAP, a potential link with mitochondrial production of oxidants has not been investigated (30, 37, 74).

The minimal involvement of mitochondrial reactive oxygen species in cortical neurons (Fig. 5) suggests that DIDS suppresses preconditioning (Fig. 3) by a mitochondria-independent mechanism. In contrast,
suppression of hypoxic preconditioning by DIDS in myocytes has been attributed elsewhere to blocking the transport of superoxide through a mitochondrial anion channel, recently identified as voltage-dependent anion channels (22), to the cytosol (68). Complex III in the mitochondrial respiratory chain is believed to be the source of superoxide (68); however, a key distinction with the present study is the 10-min interval between preconditioning and the lethal insult. Alternative possibilities include a weak NMDA receptor block (65) or targeting downstream signaling common to excitotoxic and apoptotic stimuli (59).

**Nitric Oxide-Independent Preconditioning**

No evidence supporting production of nitric oxide or associated cellular signaling was found (Figs. 6 and 7), in contrast to a report that nitric oxide production is not only required during OGD preconditioning but can induce OGD tolerance on its own in cortical neurons (18). A wide range of concentrations of nitric oxide appear effective because identical concentrations of the two compounds used in that study to precondition would produce very different amounts of nitric oxide, i.e., NOR-3 and diethylenetriamine (DETA) NONOate have half-lives of 40 and 3,400 min, respectively (16). In vivo models of cerebral ischemic preconditioning also do not support NMDA receptor-mediated production of nitric oxide (17, 46). Possible reasons cited for negligible neuronal NOS expression in cortical culture (54), such as plating neurons on an established glial bed, cannot account for this discrepancy in the present cultures.

Agents that prevent preconditioning or toxicity in the current study can act independently of nitric oxide. For instance, hypoxic preconditioning-induced generation of reactive oxygen species, as well as subsequent hypoxic tolerance, is suppressed by DIDS but not by NOS inhibitors in cardiac myocytes (68). In neuron cultures for which NOS inhibitors are ineffective, FCCP, DNP, and MnTBAP suppress NMDA-mediated generation of reactive oxygen species and toxicity (14, 60, 67). Taken together, these findings show that OGD preconditioning can induce dramatically different cellular signaling to achieve OGD-resistant phenotypes in cortical neurons. Specifically, NMDA receptor-mediated Ca\(^{2+}\) influx and subsequent generation of MnTBAP/Zn(II)/TBAP-sensitive reactive oxygen species represents a tolerance-inducing pathway that parallels a nitric oxide-based one in another laboratory (18).

The lack of effect of 50 μM PD-98059, which inhibits both MAPK/extracellular signal-regulated kinase 1 (MEK1) and MEK2 (1), as well as a more potent MEK1 and MEK2 inhibitor U0126, extends earlier work of ours, demonstrating a lack of effect of 20 μM PD-98059 (which inhibits MEK1) on OGD preconditioning-induced tolerance to otherwise lethal OGD (63). In contrast, 50 μM PD-98059 inhibited OGD preconditioning (18) in the same laboratory that demonstrated a nitric oxide-dependent component (11). These results add a further level of complexity to an already diverse role for MAP kinases in in vivo models of cerebral ischemic preconditioning, which generally report a necessary role for this kinase (26). PARP was investigated because this enzyme appears to be activated downstream of nitric oxide in cortical cultures during OGD or NMDA receptor activation (18), and pharmacological blockade of PARP is neuroprotective (61). As well, PARP may be activated during nonlethal transient cerebral global ischemia (39) and myocardial ischemic preconditioning (34). The inability of MAPK and PARP inhibitors to inhibit OGD preconditioning in the current study (Fig. 6A) might reflect a negligible role for nitric oxide.

The ineffectiveness of adenosine A1 receptor antagonists (Fig. 6B) contrasts with findings reported for in vivo models of delayed ischemic preconditioning in brain (23, 25) and in heart (45). A1 receptor activation can result in production of nitric oxide (44) or protein kinase C (5), events linked with ischemic preconditioning models (5, 44) other than ours (63). Similarly, the failure of agonists to abolish OGD preconditioning further highlights differences with intact brain because we have confirmed that adenosine A1 receptor agonists or antagonists may not protect against OGD in cortical neuron culture (35), despite benefits in various models of cerebral ischemia (53).

**CaMKII-Dependent Preconditioning**

A neurotoxic role for CaMKII has previously been identified in cerebral ischemia. KN-62 protects cortical neurons against lethal OGD or NMDA (21) and degrada-

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independent setting in the present study. Thus the suppression of preconditioning by KN-62 (Fig. 8) may involve disrupted CaMKII function and protein targeting of CaMKII as opposed to restricting transit to subcellular locales. Taken together, these findings imply a dual role for CaMKII in cerebral ischemic preconditioning: first, CaMKII activation by preconditioning is required to induce tolerance (Fig. 8), although suppression of CaMKII activity may be required after the otherwise lethal event.

The mechanism by which CaMKII contributes to OGD tolerance may be pre- and postsynaptic in origin. CaMKII is prominent in glutamatergic synapses and phosphorylates multiple synaptic proteins and is involved in neurotransmitter synthesis and release (15). The lower [glutamate]ex observed during OGD in preconditioned cultures (Fig. 10) may result from CaMKII being involved in minimizing neuronal presynaptic glutamate release. CaMKII phosphorylates a number of proteins in the postsynaptic density, with NR2B functioning as a major acceptor site (15). Possibly, this could contribute to the tolerance observed against otherwise lethal NMDA. It is not known whether production of reactive oxygen species or CaMKII activation by preconditioning is coincident or represents separate paths toward inducing tolerance. It is noted, though, that kinases have been identified as targets for oxidants produced during cardiac preconditioning (10).

Preconditioning Results in Reduced Extracellular Glutamate Levels During Otherwise Lethal OGD

We chose to measure [glutamate]ex to determine whether the response or end effector of preconditioned cortical neuronal cultures to OGD can also differ between laboratories. The marked reduction in [glutamate]ex (Fig. 10) accounts for our previous finding that a lethal OGD-induced rise in [Ca\(^{2+}\)]i is suppressed in NMDA-preconditioned cultures (64). In view of our finding of postsynaptic NMDA tolerance (64), the reduction in [glutamate]ex may consequently represent suppressed presynaptic glutamate release from neurons. A different laboratory reports that reduced glutamate release may be an important mediator in similar models of preconditioning in cortical neurons (20). Reduced [glutamate]ex during otherwise lethal hypoxia is also noted in acute models of hypoxic preconditioning (28, 56). Taken together, these findings indicate that reduced [glutamate]ex may represent a common end effector between different models of preconditioning.

Tolerance Induction Through Abbreviated Neurotoxic Cellular Signaling

Only cellular signaling that has the potential to be neurotoxic may be involved in preconditioning. Conversely, signaling that does not have the potential to be neurotoxic is not activated by preconditioning (Fig. 9). Investigation into the relationship between preconditioning and lethal stress was prompted by the realization that the laboratory reporting nitric oxide-dependent OGD preconditioning (18) previously reported profound nitric oxide-dependent OGD or NMDA toxicity (11). Hence, nitric oxide and other signalers may be involved in preconditioning only if the potential exists to be involved in a lethal stress. Reduction of a preconditioning stress may account for a recent finding that ischemic preconditioning is eliminated in neuronal NOS-knockout mice (3). Numerous laboratories have also reported NMDA induces nitric oxide-independent, but nonetheless reactive oxygen species-dependent, toxicity in cortical neuron cultures (for instance, see Ref. 24). OGD or NMDA preconditioning may also induce nitric oxide-independent signaling in those systems.

If tolerance- and toxicity-inducing stimuli are also strongly linked in other ischemia models, then the variability in preconditioning-induced signaling observed between models and tissues may largely reflect variability in toxic signaling. For instance, blockade of the NMDA receptor, which can be neuroprotective in ischemia models, almost universally inhibits ischemic/hypoxic/OGD preconditioning (for example see Ref. 19). Examples of this dual role in tolerance induction are becoming increasingly appreciated in ischemic preconditioning (38), as well as in “chemical” preconditioning, i.e., preconditioning could be triggered by molecules that in larger doses would be toxic.

The tolerance- and toxicity-inducing pathways diverge downstream of cytosolic Ca\(^{2+}\) at the mitochondrial Ca\(^{2+}\) level. Indeed, complete depolarization of the mitochondrial membrane by the uncouplers during NMDA preconditioning largely leaves the tolerance-induction machinery intact (Fig. 5). Hence, the beneficial effects provided by preconditioning may not preclude potential concomitant therapy based on neuronal mitochondrial uncoupling.

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