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Glutamate preconditioning prevents neuronal death induced by combined oxygen–glucose deprivation in cultured cortical neurons

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ABSTRACT

The study of ischemic tolerance is critical in the development of strategies for the treatment of ischemic stroke. We used the oxygen and glucose deprivation (OGD) paradigm in cultured cortical neurons as an in vitro approach to elucidate the mechanism of protection conferred by glutamate preconditioning. Pretreatment of neurons with *N*-methyl-D-aspartate (NMDA) receptor antagonists prevented OGD-induced cell death whereas α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor and voltage-dependent Ca^{++} channel (VDCC) blockers were without effect. Neurons preconditioned with glutamate exhibited resistant to damage induced by OGD. The ischemic tolerance depended on the duration of preconditioning exposure and the interval between preconditioning exposure and test challenge. Protective efficacy was blocked by the NMDA or AMPA receptor antagonists but not by the VDCC blocker. Furthermore, neuroprotective effect was not seen if extracellular Ca^{++} was omitted or removed with EGTA. Pretreatment with staurosporin and 2-[*N*-(2-hydroxyethyl)]-*N*-(4-methoxybenzenesulfonyl)] amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine (KN93) but not 2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one (LY294002) or 1,4-diamino-2,3-dicyano-1, 4-bis[2-aminophenylthio] butadiene (U0126) significantly reduced ischemic tolerance. Preconditioning increased phosphorylated levels of cAMP responsive element binding protein (CREB) and pretreatment with CRE-decoy oligonucleotide completely blocked preconditioning-induced increase in cell viability. Importantly, glutamate preconditioning increased Bcl-2 expression that was blocked by KN93, staurosporin and CRE-decoy oligonucleotide. These results suggest that preconditioning with glutamate conferred neuroprotection against subsequent OGD by inducing p-CREB-mediated Bcl-2 expression.

1. Introduction

Learning from preceding noxious stimuli, most living organisms operate a self-protective mechanism to adapt or lessen upcoming lethal damage in order to survive adverse circumstances. This is exemplified by a phenomenon termed ischemic preconditioning or tolerance which was first demonstrated in the heart and later found in other organ systems including the brain. The brain is particularly vulnerable to anoxia or ischemia. However, if exposed to a brief period of sublethal ischemia, the brain acquires resistance to a subsequent severe ischemic insult (Dahl and Balfour, 1964; Kitagawa et al., 1991; Liu et al., 1992). Ischemic tolerance also occurs in humans such that patients with prior ipsilateral transient attacks exhibit attenuated stroke severity (Weih et al., 1999; Moncayo et al., 2000). Several ways of preconditioning have been applied to study the mechanisms of ischemic tolerance which is critical to develop the strategies for the treatment of ischemic stroke in humans (Kirino, 2002; Dirnagl et al., 2003; Gidday, 2006). A number of mediators have been reported to be responsible for ischemic resistance. These included NMDA receptors (Kato et al., 1992; Grabb and Choi, 1999), heat shock proteins (Kirino et al., 1991), adenosine and A1 receptors, ATP-dependent potassium channels (Heurteaux et al., 1995), hypoxia-inducible factor (Bernaudin et al., 2002), superoxide dismutase (Toyoda et al., 1997), metallothioneins (Trendelenburg et al., 2002), erythropoietin (Ruscher et al., 2002) and caspase-3 (McLaughlin et al., 2003). However, the mechanistic basis of ischemic tolerance has not been fully elucidated.

Bcl-2 belongs to a family of anti-apoptotic proteins that control cell death by regulating the intrinsic pathway of apoptosis (Newmeyer and Ferguson-Miller, 2003). Previous studies showed that neurotrophins such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) regulated cell survival by inducing Bcl-2 expression. For example, BDNF up-regulated Bcl-2 for survival in SH-SY5Y cells during neuronal differentiation (Wang et al., 2006). In sympathetic neurons, NGF promoted survival by inducing Bcl-2 expression through CREB-dependent transcriptional mechanisms (Riccio et al., 1999). A role for Bcl-2 expression in preconditioning-induced ischemic tolerance has been postulated. Bcl-2 expression increased in neurons that were resistant to focal ischemia (Chen et al., 1995) whereas Bcl-2 antisense oligonucleotide treatment that suppressed endogenous Bcl-2

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expression exacerbated neuronal loss (Chen et al., 2000). On the other hand, phosphorylation of CREB and the interaction of CREB and CREB-binding protein with the *Bcl-2* CRE were increased after ischemic preconditioning (Meller et al., 2005).

Glutamate is the major excitatory neurotransmitter in the brain. Glutamate concentration increases rapidly during brain ischemia because glutamate transporters operate in the reversed direction extruding glutamate to the extracellular space (Camacho et al., 2006). Transient exposure to glutamate mimicked the increase of the extracellular glutamate concentration during and after transient global ischemia. Only a few instances, however, has preconditioning with glutamate been studied to investigate the development of resistance to subsequent ischemic challenge (Grabb and Choi, 1999; Mabuchi et al., 2001). Here we use the oxygen and glucose deprivation paradigm in cultured cortical neurons as an in vitro approach to elucidate the mechanism of protection conferred by glutamate preconditioning.

2. Materials and methods

2.1. Primary cortical cultures

Primary neuronal cultures of cerebral cortex were obtained from embryos (E17–18) of Sprague–Dawley rats. Cerebral cortex was dissected, meninges were removed and cells were dissociated by mild trypsination and trituration as described by Culmsee et al. (2002). Cortical cells were then seeded onto poly-D-Lysine-coated 96-well plate culture dishes at a density of 5×10^4 (for survival analysis) or 60-mm culture dishes at a density of 3×10^6 (for immunoblot analysis). The neurons were cultured in neurobasal medium supplement with 2% B27, 0.5 mM l-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Invitrogen, San Diego, CA). All experimental treatments were performed on 9-day-old cultures, at which time they contained less than 5% astrocytes as determined by GFAP-immunocytochemistry. The neurons in these cultures express both *N*-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors, and were vulnerable to glutamate-induced apoptosis.

2.2. Oxygen–glucose deprivation (OGD)

Cortical neuronal cultures were washed three times with glucose-free balanced salt solution (BSS). BSS contained 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.01 mM glycine, and 10 mg/l phenol red lacking glucose, and was bubbled with N_2/CO_2 (95%/5%) to remove residual oxygen. Neuronal cultures were incubated in the solution (OGD solution) and were then put into an airtight chamber (~12 l in volume) equilibrated for 10 min with a continuous flux of gas (95% N_2/5% CO_2). The chamber was then sealed and placed in an incubator at 37 °C for 1, 3 and 5 h in the time course experiment and for 3 h in all other experiments. Control cell cultures not deprived of oxygen and glucose were placed in BSS containing 10 mM glucose, and not bubbled with anaerobic gas during the experiment. To terminate the oxygen–glucose deprivation, chamber was opened and the cells were returned to their normal culture conditions for 24 h before they were used for cell viability analysis.

2.3. Glutamate preconditioning

The cultured neurons were rinsed twice with balanced salt solution and were incubated in BSS containing 120 μM glutamate for 40 min. Our pilot study showed that this concentration of glutamate caused minor damage to neurons but conferred robust protection to subsequent OGD. To investigate the effects of various inhibitors on preconditioning, MK-801, APV or protein kinase inhibitors (LY294002, U0126, KN 93, staurosporin) were added to the medium 30 min before glutamate treatment.

2.4. Characterization and quantification of neuronal injury

OGD-induced neuronal death (apoptotic or necrotic) was characterized by use of Hoechst 33342 and propidium iodide (PI; Sigma-Aldrich) double staining. Twenty-four hours after OGD, cells were stained with 10 μg/ml Hoechst 33342 and 10 μg/ml PI for 30 min at 37 °C. After being washed with PBS twice, cells were fixed with 4% PFA in PBS for 30 min at 25 °C. Cells were mounted with Aqua Poly/Mount (Polysciences Inc., Warrington, PA), and imaged on a digidata camera attached to a fluorescence microscope. PI-positive cells and condensed nuclei are expressed as a percentage of the total number of the cells in the view (Hoechst positive). The average of four random views of a coverslip was assessed and counted, and experiments were repeated on six independent sets of cultures (control and OGD treatment).

2.5. TUNEL and PI double staining

Twenty-four hours after OGD, cells were stained with 10 μg/ml PI for 30 min at 37 °C. After being washed with PBS twice, cells were fixed with 4% PFA in PBS for 30 min at 25 °C, then were washed with PBS, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After washing with PBS, neurons were labeled (60 min, 37 °C) with a fluorescein TUNEL reagent mixture according to the manufacturer's suggested protocol (Roche Diagnostics, Indianapolis, IN). Neuronal cultures were mounted on slides and examined by fluorescence microscopy.

2.6. MTT (3-(4,5-dimethylthianol-2-yl)-2,5 diphenyl tetrazolium bromide) assay

The colorimetric MTT reduction assay that quantified cell viability was carried out as described previously (Mosmann et al., 1983). This method assessed mitochondrial activity by measuring the ability of cultured cells to convert yellow MTT to the purple formazan dye. Cortical neurons in 96-well plate were incubated with MTT (125 μg/ml) in growth medium (without phenol red) for 4 h at 37 °C. The precipitated formazan was solubilized with SDS (25 mg/ml) and quantified spectrophotometrically at a wavelength of 570 nm. Data were expressed as the percentage of viable cell in OGD-exposed plates compared with control normoxic plates.

2.7. Western blot assay

Cells were lysed in a lysis buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 1 mM phenyl methyl sulfonyl fluoride, and 100 lg/ml leupeptin. Lysates were centrifuged at 19,720 3 g for 10 min. Supernatants were collected, subjected to electrophoresis on 8.5% or 14% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. The blot was incubated in 5% nonfat dry milk for 60 min, reacted with primary antibodies overnight at 4 °C, and then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Immunoreactivity was detected by using the Western blot chemiluminescence reagent system (Perkin-Elmer, Boston, MA). Films were exposed at different time points to ensure the optimum density, but not saturated.

2.8. CRE-decoy oligonucleotide administration

To evaluate the importance of CREB in glutamate preconditioning, CRE-decoy and control oligoneucleotides were delivered into cortical cell 7.3 h before glutamate treatment. CRE-decoy oligonucleotide and control oligonucleotide were labeled by FITC. For immunohistochemical analysis of CRE-decoy, cells were washed twice with PBS after 8 h incubation and the distribution of FITC-labeled oligonucleotides was analyzed under a fluorescence microscope. The sequences of oligonucleotides are as follows: 24-mer CRE palindrome, 5′-TGACGTCATGACGTCA TGACGTCA-3′