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Research report

Chelation of intracellular calcium reduces cell death after hyperglycemic in vitro ischemia in murine hippocampal slice cultures

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Abstract

The aggravating effect of high glucose levels during cerebral ischemia has been extensively documented in clinical studies and in vivo models of global and focal ischemia. Detailed mechanistic studies of hyperglycemic ischemia have so far been hampered by the lack of in vitro models since glucose during anoxia in vitro is highly protective. We have previously reported glucose toxicity in murine hippocampal organotypic slice cultures exposed to anoxia in an acidotic medium containing high potassium and low calcium. In the present study, we compared the importance of calcium, nitric oxide and free radicals during in vitro ischemia (IVI) and hyperglycemic (40 mM) IVI. Extracellular calcium was a ubiquitous factor for cell death after IVI, but its removal from the medium had no effect on cell death after hyperglycemic IVI. When intracellular calcium was chelated by the 1,2-Bis(2-amino-5-fluorophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethyl) ester (BAPTA-AM) cell death appeared earlier but was mitigated in hyperglycemic IVI, while it was increased in glucose-free IVI. Addition of the nitric oxide synthase (NOS) inhibitor N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) or the free radical scavengers *N-tert*-butyl-α-phenylnitrone (PBN), deferoxamine and *N*-acetyl-L-cysteine (NAC) did not affect cell damage in either paradigm. We conclude that the aggravating effect of hyperglycemia during in vitro ischemia is partially mediated by calcium ions released from intracellular stores.

Theme: Disorders of the nervous system
Topic: Ischemia

Keywords: Brain ischemia; Hyperglycemia; Cell culture; Calcium; Free radicals; Nitric oxide; Anoxia

1. Introduction

Stroke and cardiac arrest are common clinical conditions where focal and global cerebral ischemia leads to cell death in the brain and death or disability of the patient.

The knowledge of the basic pathogenic mechanisms of cerebral ischemia has increased tremendously (for review, see [11,47]), and a large number of putative neuroprotective agents have been identified. Even if many of these have reached clinical trials [21], no truly neuroprotective drug has yet come into clinical use in stroke or cardiac arrest.

The in vivo models of global or focal cerebral ischemia offer integrated systems where the role of the cerebral circulation and blood borne factors can be appreciated and the extent of damage is estimated both in morphological and functional terms. Although less complex in nature, the in vitro systems have the advantages of a high degree of control and accessibility for electrophysiological and functional studies, drug delivery and manipulation of the external environment.

We have developed an in vitro model of cerebral ischemia in organotypic tissue cultures from the mouse

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hippocampus. In this model, we have found that an adaptation of the extracellular ionic composition during a brief oxygen and glucose deprivation (OGD) to levels measured in the ischemic brain [17] leads to selective and delayed neuronal death in the CA1 region [40] similar to in vivo global ischemia. Particularly interesting was that the change of ischemic paradigm from conventional OGD revealed a dose-dependent toxicity of glucose [10]. An aggravating effect of high glucose levels during cerebral ischemia has been extensively documented in experimental studies in vivo [30,36] and in clinical studies [33,37] but not previously shown in vitro. In our in vitro ischemia (IVI) model, hyperglycemia further delayed and aggravated cell death in a manner that was dependent on concomitant acidosis but independent of lactate production [10]. Cell death following hyperglycemic IVI was also independent of ionotropic glutamate receptor activation [10].

In the present investigation, we have studied the role of extra- and intracellular calcium ions, nitric oxide and free radicals in the two new models of in vitro ischemia (IVI) and hyperglycemic IVI.

2. Materials and methods

2.1. Hippocampal organotypic tissue cultures

The Malmoe/Lund ethical committee approved all animal experiments (approval nr. M108-01). The method for preparation of murine hippocampal organotypic slice cultures, originally modified from Stoppini et al. [44], has been described previously in [40], with modifications in [10]. Briefly, hippocampi from 6-day-old Balb/c mice were dissected out in ice-cold HBSS, cut into 250 μm thick slices on a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd, Goose Green, UK) and plated onto Millicell culture inserts (0.4 μm Millicell-CM, 12 mm in diameter, Millipore Corp., Bedford, MA) one slice per insert. Cultures were maintained in humidified atmosphere at 35 °C in a CO_2 incubator (Thermo-Forma Scientific, Marietta, MA) for 3 weeks before experiments. The culture medium, with osmolarity 330 mosM, consisted of 50% MEM (Eagles with Earl's balanced salt solution), 25% heat inactivated horse serum, 18% HBSS and 2% B27 and was supplemented with 4 mM L-glutamine and 50 units of penicillin–streptomycin/ml. D-glucose was added to a final concentration of 20 mM. B27 was omitted after the first week of culture. All substances were from Invitrogen, Carlsbad, CA, with the exception of D-glucose, which was from Sigma, St. Louis, MO.

2.2. In vitro ischemia (IVI)

All cultures received a 1-h pre-incubation in new medium with or without drug and with 20 (IVI) or 40 mM glucose (hyperglycemic IVI, Fig. 1) before ischemia.

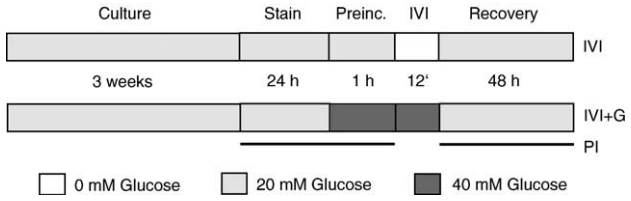

Fig. 1. Experimental paradigms for in vitro ischemia (IVI) and hyperglycemic IVI (IVI + G). Propidium iodide (PI) was added to the culture medium 24 h before the insult and throughout recovery. Cultures were made hyperglycemic by an increase in glucose concentration of the culture medium 1 h before insult and addition of 40 mM glucose to the ischemic medium during the insult.

The nitrocellulose membrane of the 3-week-old cultures was then washed twice from beneath by sequentially placing them in wells with pre-warmed, glucose-free medium. While in 24-well plates, cultures were transferred to the anaerobic incubator (Elektrotek ltd., England) and moved to wells with anoxic IVI medium. Temperature was kept at 35 ± 0.3 °C throughout experiments. After 12 min of IVI, slices were returned to culture medium and placed in the CO_2 incubator. The IVI medium, ischemic cerebrospinal fluid (iCSF), contained (in mM): 0.3 $CaCl_2$, 70 NaCl, 5.25 $NaHCO_3$, 70 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 40 sucrose, pH 6.8 and osmolarity 353 mosM. In experiments on calcium-free environment, $CaCl_2$ was omitted from the medium without correction for osmolarity. In hyperglycemic IVI, sucrose was replaced by 40 mM glucose. The pH of anaerobic iCSF with different drugs was routinely measured in a standard micro-sampler blood–gas monitor (ABL 50 Radiometer Copenhagen, Copenhagen, Denmark).

1,2-Bis(2-amino-5-fluorophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethyl) ester (BAPTA-AM) was dissolved in dimethyl sulfoxide. Stock solutions from *N-tert*-butyl-α-phenylnitrone (PBN) were made in culture medium and IVI medium. Deferoxamine mesylate salt, *N*-acetyl-L-cysteine (NAC) and N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) were all dissolved in sterile water. BAPTA-AM, DMSO (>95%), deferoxamine mesylate salt and NAC were purchased from SIGMA. L-NAME was from Tocris Cookson, St. Louis, MO.

2.3. Measurement of cell death

The fluorescent cell death marker propidium iodide (PI) was added to the culture medium from 24 h before experiment and throughout the recovery period (Fig. 1). Approximately 10% of cultures were discarded before the start of experiments due to bad morphology or localized PI uptake. Cell death was assessed by measuring mean fluorescence intensity (MFI) in a standardized area in the CA1 region and in a hexagon placed outside the CA2/CA3 cell band which was used as background (Fig. 2) Cell death was calculated by subtracting the MFI in the background area from the MFI measured in CA1.