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Neurobiology of Disease

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Neurobiology of Disease 20 (2005) 27 – 37

Contribution of caspase-mediated apoptosis to the cell death caused by oxygen–glucose deprivation in cortical cell cultures

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Received 13 September 2004; revised 7 January 2005; accepted 31 January 2005
Available online 17 March 2005

Several evidences suggest that cell death after cerebral ischemia involves both necrosis and apoptosis. However, it is still unknown which is the relative contribution of both types of cell death. Exposing rat cortical cultures to oxygen–glucose deprivation (OGD), we show the simultaneous presence of necrotic and apoptotic cells. The relative contribution of necrosis and apoptosis was dependent on the duration of the OGD. OGD-mediated apoptotic cell death is caspase-dependent because the addition of a pan-caspase inhibitor specifically blocked the apoptotic component of the OGD-mediated cell death. Moreover, we observed the activation of caspase-3, -7, and -9 after OGD in neurons and microglial cells. No activation of these caspases was observed in GFAP positive cells. Our results also show that calpain is related to OGD-mediated proteolysis of caspase-3 and -9 but not of caspase-7. These data suggest that different pathways could be involved in OGD-mediated caspase activation.

Keywords: Apoptosis; Caspase; Calpain; Neuron; Microglia; Ischemia; Cell culture

Introduction

Ischemic neuronal death was considered for a long time to be necrotic, mainly produced by a persistent stimulation of divalent cations (Ca^{2+} or Zn^{2+})-permeable membrane channels, such as the *N*-methyl-D-aspartate (NMDA) or α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) glutamate receptor subtypes (Choi, 1988; Rothman and Olney, 1986; Simon et al., 1984; Yatsugi et al., 1996). However, over the last few years, the notion that ischemic neuronal death occurs strictly by necrosis has been challenged. Mounting evidences indicate that the loss of neurons via apoptosis might be important in cerebral ischemia (Banasiak et al., 2000; Lee et al., 1999).

Apoptosis is a form of programmed cell death which is characterized by several ultrastructural and biochemical hallmarks (Hengartner, 2000; Strasser et al., 2001). Strong support for the existence of apoptotic cell death in ischemia came from the observation of active caspases in ischemic brain tissue. Activation of caspase-3 has been reported several hours after transient focal ischemia in cerebral cortex, hippocampus, and striatum (Chen et al., 1998; Hara et al., 1997; Namura et al., 1998; Ni et al., 1998; Velier et al., 1999). Other reports have also suggested an eventual activation of caspase-1, -8, and -11 in permanent focal ischemia (Kang et al., 2000; Velier et al., 1999). Accordingly, the administration of some caspase inhibitors has been shown to reduce the infarct size and cell death in transient cerebral ischemia (Bose et al., 1998; Han et al., 2002; Hara et al., 1997). Data obtained in vitro also support the existence of apoptotic cell death in ischemic insults. Cultured cortical neurons, transiently deprived of oxygen and glucose, have been reported to die by apoptosis when AMPA and NMDA receptors are pharmacologically blocked (Gottron et al., 1997; Gwag et al., 1995). This apoptotic death was attenuated with the caspase inhibitor z-VAD-fmk (Gottron et al., 1997).

Although it seems reasonable to predict that eventual future therapeutic approaches should target both necrotic and apoptotic cell death to be successful (Lee et al., 1999), at present, it is not clear yet which is the exact contribution of necrosis and apoptosis to ischemia-mediated cell death. Moreover, the contribution of apoptosis to the ischemic brain damage could be underestimated mainly due to the phagocytosis of apoptotic cells in vivo (Savill and Fadok, 2000).

Here, we use the oxygen and glucose deprivation (OGD) paradigm in mixed cortical cell cultures (Goldberg and Choi, 1993) as an in vitro approach to evaluate the relative importance of necrotic and apoptotic death in ischemic damage. We have also studied the possible implication of caspase-3, -7, and -9 in the observed apoptotic cell death under OGD. Our results clearly show the simultaneous presence of necrotic and apoptotic cells in the cultures. Moreover, although the percentage of necrotic death increases with the duration of OGD, apoptotic death represents, at least, 50% of total cell death. The observed apoptosis parallels the activation of caspase-3, -7, and -9 in neurons and microglial cells.

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Available online on ScienceDirect (www.sciencedirect.com).

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doi:10.1016/j.nbd.2005.01.028

Materials and methods

Chemicals

Eagle's Basal Medium (BME), horse serum (HS), and fetal calf serum (FCS) were obtained from Invitrogen. Cultures dishes were obtained from TPP or Falcon. Hoechst 33258 was obtained from Molecular Probes. All other reagents were from Sigma-Aldrich or Calbiochem.

Cell cultures

The cerebral cortices obtained from fetal rats at 17 days of gestation were enzymatically and mechanically dissociated after removal of the meninges. Dissociated cells were resuspended at a density of 3×10^5 cells/cm^2 in BME supplemented with 5% FCS, 5% HS, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, and 10 mM glucose, and plated onto poly-L-lysine precoated wells. Cultures were kept at 37°C, 100% humidity and in 95% air/5% CO_2 atmosphere until 7 days in vitro (DIV) when the plating medium was replaced by BME supplemented with 10% horse serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, 10 mM glucose, and 10 μM cytosine arabinoside to arrest non-neuronal cell proliferation. All the experiments were performed with mature cultures (13–14 DIV).

Oxygen–glucose deprivation

Cortical cells cultures were oxygen and glucose deprived (OGD) accordingly to the procedures described by Goldberg and Choi (1993) with minor modifications. The culture medium was replaced (and kept for its use after the OGD period) by thorough exchange with a glucose-free Earle's balanced salt solution (BBS; composition, in mM: NaCl 116 mM, KCl 5.4 mM, $MgSO_4{\cdot}7H_2O$ 0,8 mM, $NaH_2PO_4{\cdot}2H_2O$ 1 mM, $NaHCO_3$ 26.2 mM, Glycine 0.01 mM, $CaCl_2{\cdot}2H_2O$ 1.8 mM, pH 7.4) which had previously been saturated with 95% N_2/5% CO_2 at 37°C. Cultures were then placed in an airtight incubation chamber (CBS Scientific) equipped with inlet and outlet valves, and were equilibrated for 15 min with a continuous flux of gas (5% CO_2/95% N_2). The chamber was then sealed and placed into an humidified incubator at 37°C for 15–60 min. OGD was terminated by removing the cultures from the airtight chamber, exchanging the deoxygenated and glucose-free BBS with the pre-OGD culture medium and returning the cells to the normoxic conditions. Control sister culture plates were exposed to oxygenated BSS containing 5.5 mM glucose in normoxic conditions during the same period of time as OGD cultures. When used, the antagonists of ionotropic glutamate receptors and the inhibitors of caspases were present in the culture medium 1 h before, during, and after the OGD insult.

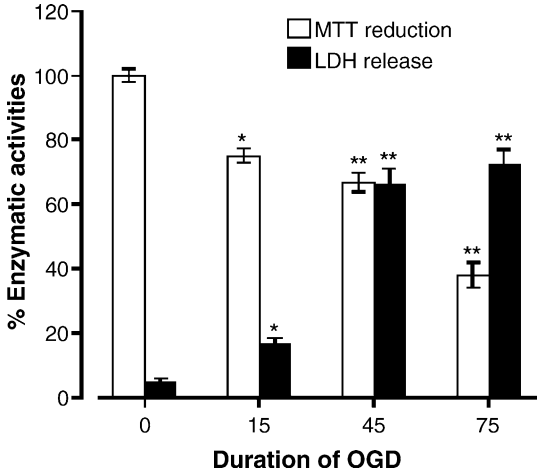

Fig. 1. Effect of OGD duration on cell viability. Cortical cultures were exposed to OGD (30, 45, and 75 min), and MTT reduction (open column) or LDH activity (solid column), released in the culture medium, was determined 24 h later. Data are expressed as percentage of MTT reduction in normoxic cultures or as percentage of LDH released by treatment with 1 mM glutamate for 24 h. Values represent the mean ± SEM of 4 independent experiments performed in triplicate. Significant differences (*$P < 0.05$ and **$P < 0.01$) from control were determined by ANOVA followed by Tukey's test.

Table 1
Blockade of OGD-mediated cell death by antagonists of ionotropic glutamate receptors

Treatment	MTT reduction	
	Control culture	75-min OGD
Non-treated	100 ± 4.5	40 ± 3.9
+ MK-801	98 ± 2.0	92 ± 3.5$^{\#}$
+ CNQX	102 ± 3.2	66 ± 4.1$^{*,\#}$

Control or oxygen–glucose-deprived (OGD; 75 min) cortical cultures were treated with 10 μM of the NMDA receptor antagonist MK-801 or the AMPA receptor antagonist CNQX. Both antagonists were present in the incubation medium during the OGD insult and the subsequent 24-h recovery period (see Materials and methods). Cell viability was determined by MTT reduction 24 h after the beginning of the OGD insult. Values are mean ± SEM of four independent experiments. Significant difference (*$P < 0.05$ vs. non-treated control culture; $^{\#}P < 0.05$ vs. non-treated OGD cultures) was determined by ANOVA followed by Tukey's test.

Cell viability

Cell viability was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay as described (Mossmann, 1983). Briefly, MTT (0.3 mg/ml) was added to the 24-well plates and incubated for 45 min at 37°C. The reaction media were then gently aspirated and DMSO was added to solubilize the blue formazan product. Formazan–DMSO mixtures were then transferred to 96-well plates and quantified using a Multiskan plate reader at 570 nm (Labsystems Multiskan RC). Results were expressed as the percentage of viable cells.

Determination of necrosis

The presence of necrotic cells in the cultures was assessed with the fluorescent dye propidium iodide (PI), a highly polar compound which could only enter the cells when the plasma membrane is damaged, a typical feature of necrosis. PI (10 μM) was added to culture medium after OGD and fluorescence was viewed with a Leica DMRB microscope. In some experiments, overall necrosis in the cultures was determined by measuring the lactate dehydrogenase (LDH) released from damaged cells