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Programmed Neuronal Necrosis and Status Epilepticus

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Summary: We examined the mechanism of neuronal necrosis induced by hypoxia in dentate gyrus cultures or by status epilepticus (SE) in adult mice. Our observations showed that hypoxic necrosis can be an active process starting with early mitochondrial swelling and loss of the mitochondrial membrane potential, followed by cytochrome c release and caspase-9–dependent activation of caspase-3. This sequence of events (or program) was independent of protein synthesis and may be induced by energy failure and/or calcium overloading of mitochondria. We called this form of necrosis “programmed necrosis.” After SE in adult mice, CA1 and CA3 pyramidal neurons displayed a necrotic morphology, associated with caspase-3 immunoreactivity and with double-stranded DNA breaks, suggesting that “programmed necrosis” may be involved in SE-induced neuronal loss. Key Words: Status epilepticus—Programmed necrosis—Neuronal loss.

MATERIALS AND METHODS

DG cultures

Cultures were prepared as described by Niquet et al. (16). In brief, DG were dissected from postnatal day 3 (P3) Wistar rat pups, dissociated enzymatically and mechanically, and plated in Neurobasal/fetal bovine serum (FBS). Cultures were incubated at 37°C in a 5% CO2/95% O2 incubator, and culture medium was removed and replaced by Neurobasal/B-27 at day in vitro 1 (DIV 1) and DIV 5. Hypoxia was induced at 7 DIV by 45-min exposure to 10 mM NaCN. Some cultures were treated with 300 nM staurosporine. The research protocol was approved by the Institutional Animal Care and Use Committee at the performance site.

Ultrastructural studies

Cultures were fixed with 2.5% glutaraldehyde and 1.6% paraformaldehyde in 0.1 M cacodylate buffer for 1 h, and processed as described by Niquet et al. (16).

Light-microscopic immunocytochemistry

After paraformaldehyde 4% (wt/vol) fixation, cultures were incubated with an antibody to the p20 active fragment of caspase-3 (caspase-3a) (R&D system; AF835), and/or an antibody to cytochrome c (Pharmingen; 556432). Immunocytochemical staining was revealed as described by Niquet et al. (16). Some cultures were preincubated with 200 nM Mitotracker Orange (Molecular Probes; M-7510) before paraformaldehyde fixation.
Electron-microscopic immunocytochemistry

Preembedding immunogold labelling was used to localize caspase-3 or for cytochrome c immunoreactivity as reported (16).

Caspase inhibitors

Cultures were preincubated with caspase inhibitors (Enzyme Systems Products) 45 min before NaCN exposure, and this was continued during and after hypoxic treatment. Control and untreated hypoxic wells were incubated with vehicle [0.2% dimethylsulfoxide (DMSO)]. Neuronal survival was determined 24 h after hypoxia by trypan blue assay, as described elsewhere (16).

Isolated brain mitochondria

Mitochondria were isolated from adult rat brain and incubated with increasing concentrations of calcium, as described elsewhere (16).

KA model of SE

Adult mice were perfused with 4% paraformaldehyde 3 days after the induction of SE with kainic acid (KA; 35 mg/kg). Coronal frozen sections (30 mm thick) through the hippocampi were incubated with rabbit anti–caspase-3 antiserum (Santa Cruz Biotechnology) or stained by in situ end-labeling of nuclear DNA fragments by using terminal deoxynucleotidyl transferase and biotinylated dUTP (TUNEL), as described elsewhere (7).

RESULTS

In neuronal death induced in hippocampal DG neurons by a transient exposure (45 min) to a high concentration (10 mM) of the mitochondrial poison sodium cyanide, several electron-microscopic (EM) criteria showed that degenerative neurons had a necrotic morphology: mitochondrial swelling was the first alteration induced by cyanide application, at a time when nuclear morphology was intact (Fig. 1B); it was followed by severe mitochondrial swelling with frequent breaks of the outer membrane, marked cytoplasmic swelling, and plasma membrane rupture, whereas irregular ("tigroid") chromatin fragmentation was a late event (Fig. 1C). By contrast, in the same cultures treated with staurosporine, an apoptotic morphology could be easily recognized by the presence of round chromatin clumps with regular contour (Fig. 1D). Several observations showed that caspase-3, the executioner of the cell, contributes to sodium cyanide–induced neuronal necrosis. First, we showed by light microscopy (LM) an increase of caspase-3a immunoreactivity (Fig. 2E), confirmed by Western blots (not shown). We confirmed by EM that caspase-3a immunoreactive cells had a necrotic morphology (Fig. 3A and B). Second, the caspase-3 inhibitor Z-DEVD-fmk improved neuronal survival (Fig. 3C).

In classic apoptosis, cytochrome c release from the mitochondria to the cytoplasm and subsequent caspase-9 activation are two key events leading to caspase-3 activation in the so-called intrinsic pathway. We wondered whether cytochrome c and caspase-9 were also involved in caspase-3 activation in necrotic cell death. We confirmed by immunocytochemistry the release of cytochrome c, starting within minutes of the sodium cyanide application, and preceding caspase-3 activation (Fig. 2E). Seventy-five percent of caspase-3a immunoreactivity was colocalized with cytochrome c, 5 h after hypoxic exposure (Fig. 4A–F). Caspase-9 activation seemed to be a key event in hypoxic necrosis, because the caspase-9 inhibitor Z-LEHD-fmk improved neuronal survival (Fig. 3C) and inhibited caspase-3 activation (16), suggesting that caspase-9 activation is an event upstream of caspase-3 activation. These results showed that hypoxic necrosis depends on a program starting with cytochrome c release, inducing caspase-9–dependent caspase-3 activation. In this experimental paradigm, necrosis is not just a passive process with swelling and ionic lysis of neurons, but an active

FIG. 1. Hypoxia induces early swelling of mitochondria and necrotic neuronal death. Ultrastructure of control (A), hypoxic (1 and 24 h after hypoxia; B and C) and staurosporine-treated neurons (24 h after staurosporine treatment; D) was studied by electron microscopy. One hour after hypoxia, most cytosolic and nuclear morphology is intact, and mitochondrial swelling (arrows in B) is the first sign of cell damage. It evolves toward necrosis with severe cytoplasmic swelling, including mitochondrial swelling, membrane rupture, and tigroid-type chromatin fragmentation (24 h after hypoxia; C). By contrast, the staurosporine-treated neuron displays a characteristic apoptotic morphology with large masses of chromatin (D). Bars, 1 µM.
Control neurons (no triton, peroxidase technique) are not cytochrome c-immunoreactive (A) and have a high mitochondrial membrane potential indicated by orange mitotracker dye (C). Hypoxic neurons 3 min after NaCN application are cytochrome c-immunoreactive (arrows in B) and show a severe loss of mitochondrial membrane potential (D). E: Cytochrome c release preceded caspase-3 activation. Sister cultures were stained with anti-cytochrome c and anti-caspase-3a antibodies. Data are expressed as mean \( \pm \) SEM of four independent determinations in each group, analyzed by one-factor ANOVA with post hoc t tests using the pooled standard deviation and \( \alpha = 0.05 \), \( * < 0.05 \) or \( ** < 0.001 \) vs. 0 min for cytochrome c. \#< 0.05 or \#\# < 0.001 vs. 0 min for caspase-3a. F–H: Electron photomicrographs of mitochondria stained with anti-cytochrome c antibodies in control (F) and after 45 min of NaCN exposure (G, H). In control cultures, mitochondria are undamaged and cigar-shaped. Cytochrome c staining, revealed by gold particles, is located in the intermembrane space. In hypoxic cultures, mitochondria are swollen, and outer membranes are sometimes ruptured (arrow). Cytochrome c immunoreactivity is located in the cytosol. Bars, 0.5 \( \mu \)M.

Because “programmed necrosis” and classic apoptosis share a final common pathway, we wondered whether they required the same mechanism upstream of cytochrome c release. Initial events of apoptosis include immediately early gene upregulation, causing increased expression of death-signaling proteins such as Bax, which is translocated from the cytoplasm to the mitochondrial outer membrane and causes cytochrome c release (17). We tested the dependency of hypoxic “programmed necrosis” on protein synthesis by preincubating the cultures with cycloheximide (CHX; 1 \( \mu \)g/ml). This treatment was effective in blocking cytochrome c release and caspase activation in staurosporine-induced apoptosis of DG neurons, but was totally ineffective in hypoxic necrosis of the same cells (Fig. 6). This is not surprising because gene expression or synthesis of new proteins requires large amounts of energy and is an improbable events in conditions of strong...
FIG. 3. A, B: Five hours after NaCN treatment, a neuron with relatively intact nucleus, ruptured plasma membrane, and grossly swollen organelles is clearly necrotic, but activated caspase-3 (caspase-3α) immunoreactivity (gold particles, arrows) is present throughout the cytoplasm (A). In another necrotic neuron at 24 h (B), caspase-3α has diffused into the nucleus as well. Bars, 1 µM. (C) Inhibitors of caspase-3 (DEVD-fmk), caspase-9 (LEHD-fmk), and the general caspase inhibitor ZVAD-fmk reduce hypoxic neuronal death, whereas caspase-1 inhibitor (YVAD-fmk) has no effect. Cell survival was determined 24 h after NaCN by using trypan blue. All these inhibitors had no effect on control cultures. Data are expressed as mean ± SEM of seven independent determinations in each group, analyzed by one-factor ANOVA with post hoc t tests by using the pooled standard deviation and α = 0.05 (*<0.05; **<0.01; ***<0.001).

energy depletion such as chemical hypoxia. Protein synthesis is not required for “programmed necrosis” because the caspase-3 proenzyme is constitutively expressed in control neurons and does not need to be synthesized. A proteolytic cleavage, requiring little energy, is sufficient to activate caspase-3. Similarly, no synthesis would be necessary for the constitutively expressed death effector Bax, the translocation of which may contribute to cytochrome c.

FIG. 4. In the control neuron (A–C, triton-treated), the nucleus (stained with Hoechst 33342, A) is large, cytochrome c staining is punctate, reflecting its mitochondrial localization (B), and no caspase-3α immunoreactivity is visible (C). Five hours after hypoxia, a hypoxic neuron has a shrunken nucleus (D), diffuse cytochrome c staining throughout the cytoplasm (E), and is immunoreactive for caspase-3α (F).

FIG. 5. Status epilepticus induces caspase-3 expression and double-stranded DNA breaks in the hippocampus of adult mice. A–D: Immunocytochemical analysis of caspase-3 immunoreactivity. In the saline-injected mice, a small amount of caspase-3-ir was detected in hippocampus (A, C). Three days after injection of kainate (35 mg/kg), a dramatic increase was seen in caspase-3-ir in neurons through the entire pyramidal layer (B, D). E–H: TUNEL staining. None was apparent in saline-treated controls (E, G). Large numbers of TUNEL-positive neurons were dispersed throughout the CA1 layer (F), and significant numbers were scattered in the CA3 subfield (H) of adult mice 3 days after kainate injection (Scale bar, 125 mm).
release (17), and its role in hypoxic “programmed necrosis” is currently investigated in our laboratory. Interestingly, LM observations showed Bax translocation to the mitochondria in SE-induced necrotic neurons (7). Furthermore, in less severe energetic depletion, as observed during SE, protein synthesis is severely inhibited, but we cannot exclude death-effector protein upregulation as a contributor to some forms of necrotic cell death.

Calcium overloading of mitochondria, a well-established result of energy failure and/or excitotoxic injury (18), may be sufficient to induce cytochrome c release in “programmed necrosis.” We showed that high concentration of calcium induced cytochrome c release from isolated brain mitochondria (16). In hypoxic cultures, two observations suggest a contribution from mitochondrial calcium influx, known to cause mitochondrial depolarization, opening of the mitochondrial transition pore (MTP), and/or mitochondrial swelling when mitochondrial buffering capacity is exceeded (18). First, a progressive loss of the mitochondrial membrane potential, which started within minutes of the hypoxic treatment, was synchronous with cytochrome c release (Fig. 2A–D). We speculate that this mitochondrial depolarization is induced by calcium overloading and may trigger cytochrome c release through MTP opening. Second, we observed cytochrome c release by EM in hypoxic neurons with swollen mitochondria and broken outer mitochondrial membrane, suggesting an alternate mechanism for its release from calcium-overloaded mitochondria (Fig. 2G–H). More work is needed to distinguish the contribution of these two mechanisms in hypoxic “programmed necrosis.”

Our results suggest a speculative mechanism for hypoxic “programmed necrosis”: when the calcium-buffering capacity of mitochondria is exceeded, they may depolarize and/or rupture, and release cytochrome c, which activates constitutively expressed pro-caspase-9, and caspase-9 in turn may activate caspase-3, causing proteolytic damage around mitochondria, and later diffusing to the nucleus, where it destroys the nuclear membrane and activates DNAases (Fig. 7). Because SE has been shown to deplete cerebral energy reserves and to cause mitochondrial calcium influx that can exceed their buffering capacity (5), neuronal “programmed necrosis” might be expected to occur in SE. These results suggest that SE-induced impairment of mitochondrial function may have rapidly fatal consequences for vulnerable central neurons, by activating the caspase cascade in a manner that short-circuits the apoptotic process and does not require expression of new genes or cell-death proteins.

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REFERENCES