HYPOXIA IN PRESENCE OF BLOCKERS OF EXCITOTOXICITY INDUCES A CASPASE-DEPENDENT NEURONAL NECROSIS

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Abstract—When excitotoxic mechanisms are blocked, severe or prolonged hypoxia and hypoxia–ischemia can still kill neurons, by a mechanism which is poorly understood. We studied this “non-excitotoxic hypoxic death” in primary cultures of rat dentate gyrus neurons. Many neurons subjected to hypoxia in the presence of blockers of ionotropic glutamate receptors developed the electron microscopic features of necrosis. They showed early mitochondrial swelling, loss of mitochondrial membrane potential and cytoplasmic release of cytochrome c, followed by activation of caspase-9, and by caspase-9-dependent activation of caspase-3. Caspase inhibitors were neuroprotective. These results suggest that “non-excitotoxic hypoxic neuronal death” requires the activation in many neurons of a cell death program originating in mitochondria and leading to necrosis. Published by Elsevier Ltd on behalf of IBRO.

Key words: apoptosis, cytochrome c, MK-801, NBQX, ATP, mitochondrial membrane potential.

Neurons die from hypoxia or hypoxia–ischemia much faster than other cells, and that is probably due to the fact that they express a biochemical machinery involved in excitotoxicity (Simon et al., 1984; Zipfel et al., 2000; Arundine and Tymianski, 2004). Excitotoxic cell death is induced by an excessive calcium influx following the activation of ionotropic (NMDA and KA/AMPA) glutamate receptors (Arundine and Tymianski, 2003). However, hypoxia–ischemia-induced neuronal death may also have a non-excitotoxic component, since potent blockers of excitotoxicity such as MK-801 are not fully neuroprotective in a variety of models of hypoxic-ischemic death in vivo or in vitro (Goldberg et al., 1987; Hattori et al., 1989; Gwag et al., 1995; Endres et al., 1998; Ma et al., 1998; Schulz et al., 1998; Hicks et al., 1999; Olsson et al., 2003). Caspase inhibitors can reduce hypoxia or hypoxia–ischemia-induced neuronal death in the presence of glutamate receptor blockers, but the mechanism of caspase activation or the type of death (apoptotic or necrotic) that results is not clear (Gottron et al., 1997; Ma et al., 1998; Schulz et al., 1998). This problem is of some practical importance to our neuroprotective strategies in stroke and hypoxic–ischemic neuronal injury, and will become critical as we develop better therapeutic agents. As we become able to block the rapid, excitotoxic form of neuronal death, our ability to stop the slower neuronal death which is independent of glutamate receptors will become a key to therapeutic success. Indeed, anoxia–ischemia kills cells which do not express glutamate receptors, albeit slowly. In this study, we examined the mechanisms of neuronal cell death in presence of blockers of ionotropic glutamate receptors in an in vitro model of chemical hypoxia. NMDA receptor blockers are not fully neuroprotective when the hypoxic insult is severe or prolonged. We found that severe hypoxia in presence of blockers of excitotoxicity induces early mitochondrial swelling, cytochrome c release and activation of the caspase-9, caspase-3 cascade, and that, at early time points, this caspase-dependent neuronal death is predominantly necrotic.

EXPERIMENTAL PROCEDURES

Primary cultures of dentate gyrus (DG)

Cultures were prepared as previously described elsewhere (Niquet et al., 2003). Briefly, DG were dissected from 3 days-old Wistar rat pups (Simonsen Laboratories, Gilroy, CA, USA), incubated at 37 °C for 15 min with trypsin (Sigma, St. Louis, MO, USA) diluted in Neurobasal/B27 (Invitrogen, Carlsbad, CA, USA) and mechanically dissociated with Pasteur pipettes in Neurobasal/fetal bovine serum (FBS) (10%; v/v). Tissue was then centrifuged, and dissociated cells (from the pellet) were resuspended in Neurobasal/FBS and plated in eight well chamber slides or 96 well plates. More than 90% of cell population is NeuN-immunoreactive after 7 days in vitro and calbindin-immunoreactive after 14 days in vitro.

Model of chemical hypoxia

Seven days in vitro DG cultures were exposed to 3 or 10 mM sodium cyanide for 45 min, washed, and incubated again with Neurobasal/B27. Some cultures were exposed to 3 mM sodium cyanide for 90 or 180 min, washed, and incubated again with Neurobasal/B27. Some cultures were preincubated with blockers of excitotoxicity (10 μM MK-801, and/or 10 μM NBQX, both from Sigma) 30 min before sodium cyanide (Sigma) treatment and incubated again with the same blockers during the hypoxic period.
Some cultures were exposed to general caspase inhibitor Z-VAD-fmk (100 μM; Sigma), or caspase-9 inhibitor Z-LEHD-fmk (100 μM; Sigma) or caspase-3 inhibitor Z-DEVD-fmk (100 μM; Sigma) 45 min before, during and after hypoxia. Control cultures were treated with the vehicle (DMSO 0.2%). Some cultures were treated with caspase inhibitor M867 (10 μM) 45 min before, during and after hypoxia. M867 was a generous gift of Merck Frosst (Methot et al., 2004). Some cultures were exposed to the protein synthesis inhibitor cycloheximide (CHX; 1 μg/ml) 1 h before, during and after the hypoxic treatment. Cytotoxicity assay Cell death was assayed by measuring the enzymatic activity of lactate dehydrogenase (LDH) released by degenerative cells to the medium using LDH cytotoxicity detection kit (Takara, MK401, Otsu, Japan). Basal enzymatic activity from Neurobasal/B27 was deduced in each value. In Fig. 1A, toxicity values obtained in experimental cultures are expressed as % of the untreated control cultures (100%). In Fig. 1B, percentage of neuroprotection is a mean value of four independent determinations calculated as followed: % neuroprotection=[1−(A/B)]×100. A=“NaCN+MK801+NBQX” group minus mean untreated control group; B=mean “NaCN” group minus mean untreated control group. In Fig. 5B and C, toxicity values obtained in inhibitor-treated groups are expressed as % of the untreated hypoxic cultures (100%). Survival assay Percentage of cell survival was calculated after counting alive cells (excluding Trypan Blue dye) in five to six fields per well. ATP measurement Total amount of ATP in each well was determined by a bioluminescent kit using firefly luciferase (Sigma FD-ASC). Immunocytochemical studies Cultures were fixed with paraformaldehyde (4%;w/v) in PB 0.1 M for 30 min. After three washes with PB 0.1 M, cultures were saturated with PB 0.1 M containing 0.2% gelatin (PB-G) for 1 h, and incubated for 90 min at room temperature with primary antibodies against cytochrome c (1/100; Pharmingen), or active caspase-3 (1/1500; Research and Development), or the cleavage

Fig. 1. Blockers of excitotoxicity fail to protect DG neurons from severe hypoxia. (A) MK-801 (10 μM) and 10 μM NBQX fully protect cultures from 3 mM NaCN treatment, but only partially protect cultures treated with 10 mM NaCN. LDH activity was measured 7 h after hypoxic treatment. MK-801 and NBQX had no effect on control cultures. Data are mean±S.E.M. of four independent determinations in each group, analyzed by one way ANOVA. * P<0.05 vs. 3 mM NaCN; ** P<0.05 vs. 10 mM NaCN; # P<0.05 vs. control. (B) Protection provided by 10 μM MK-801 and 10 μM NBQX decreases when exposure to 3 mM NaCN is prolonged to 180 min. Data are mean±S.E.M. of four independent determinations in each group, analyzed by one way ANOVA (* P<0.05 vs. 45 min). (C) Higher concentrations of MK-801 have no effect on control cultures and do not improve neuroprotection in NaCN-treated cultures. Cell survival was determined by Trypan Blue assay 24 h after hypoxia. Data are median mean of nine independent determinations in each group, analyzed by one way ANOVA (* P<0.05 vs. NaCN). (D) Pretreatment with 10 μM NBQX has no protective effect on hypoxic cell death induced by 10 mM NaCN. Cell survival was determined by Trypan Blue assay 24 h after hypoxia. Data are median values of four independent determinations in each group, analyzed by one way ANOVA (* P<0.05 vs. NaCN).
Fig. 2. NEH induces early energy failure, mitochondrial membrane potential loss and mitochondrial swelling. (A–C) Neurons (arrows) have a high mitochondrial membrane potential, detected by Mitotracker Orange, in control cultures. Complete loss of the potential was observed at the end of the NEH period (B) and substantial recovery of the potential was seen 4 h after the NEH period (C). (D) In the absence or in the presence of blockers of excitotoxicity, NaCN treatment triggers severe ATP depletion at the end of the hypoxic period. Only a very partial recovery was observed during the posthypoxic period. This graph displays the level of ATP, measured with a bioluminescent assay kit. Data are mean ± S.E.M. of three to four independent determinations in each group, analyzed by one way ANOVA (* P<0.05 vs. NaCN). (E, F) Example of microphotographs of a control neuron (E) and a hypoxic neuron (F) at the end of the NEH treatment. Note that NEH treatment induces early mitochondrial swelling (arrows in F).
product of actin by caspase-3 (fractin; 1/3000; Pharmingen). After washes, some cultures were incubated for 90 min with antibodies conjugated to peroxidase (1/100; Amersham) diluted in PB-G and staining was revealed with diaminobenzidine (DAB; Sigma). The protocol for the antibodies against active caspase-9 (1/100; Cell Signaling Technology) was identical, except for the fixation time (10 min) and incubation conditions (overnight at 4 °C). Cultures were counterstained with the nuclear dye Hoechst 33342 and percentage of caspase-3 or cytochrome c-immunoreactive cells was determined in five to six fields per well. Some cultures were preincubated with nuclear dye propidium iodide (10 μg/ml) for 30 min before paraformaldehyde fixation to assess cytoplasmic membrane competency, and immunostaining was revealed with secondary antibodies to Alexa Fluor 588 (1/100; Molecular Probes).

**Fig. 3.** NEH treatment induces cytochrome c release. (A, B) NEH treatment induces cytochrome c release. Since cultures were not permeabilized with Triton X-100, brown staining (product of peroxidase–DAB reaction) revealed cytochrome c release to the cytosol (objective ×40). A few cells were cytochrome c immunoreactive in control cultures (A), while many of them were immunoreactive at the end of the NEH period (B). (C) Blockers of excitotoxicity (MK-801 and NBQX) partially inhibit NaCN-induced cytochrome c release. The graph displays the percentage of cells immunoreactive for cytochrome c. Cultures were fixed after 45 min of NaCN exposure. Data are mean ± S.E.M. of six to nine independent determinations in each group, analyzed by one way ANOVA (* P < 0.05 vs. control; # P < 0.05 vs. NaCN). (D) Microphotographs of mitochondria stained with anti-cytochrome c antibodies at the end of the NEH period. Mitochondria are swollen and outer membranes are sometimes broken (arrow). Some gold particles, revealing cytochrome c staining, are located in the cytosol. In the inset, a mitochondrion from a control untreated neuron has a normal cigar shape. Cytochrome c immunoreactivity is located in the intermembrane space. (Scale bar = 1 μM.)
Ultrastructural studies

Early morphologic changes were observed by routine electron microscopy (EM) procedures. Briefly, at the end of the hypoxic treatment, cultures were fixed with 2.5% glutaraldehyde and 1.6% paraformaldehyde in 0.1 M cacodylate buffer (CB); washed with CB and postfixed with 1% osmium tetroxide in CB. Cultures were then rinsed with distilled water and en bloc stained with 1% uranyl acetate, dehydrated with graded ethanols and embedded in Spurr Epon. The thin sections were stained with uranyl acetate and lead citrate and viewed using a Philips 201C TEM.

Electron microscopic immunocytochemical studies

Caspase-3 and cytochrome c immunoreactivity was revealed by a preembedding immunogold method. Fixation and incubation with primary antibodies were performed as described above for light microscopic immunocytochemistry. Cultures were washed, saturated with PBS containing 0.07% cold fish gelatin (P-CFG) and 1% normal goat serum (NGS) for 15 min and incubated overnight at 4 °C with P-CFG containing anti-rabbit polyclonal or anti-mouse monoclonal antibodies conjugated to 1 nm gold particles (1/80; Ted Pella, Inc.). The cultures were washed with P-CFG containing 2.5 M NaCl for 20 min, and then several times with distilled water. Gold staining was intensified by silver enhancement (Ted Pella, Inc.) for 10 –12 min. Cultures were washed with distilled water and postfixed with 0.1% osmium. After dehydration with ethanol, cultures were embedded with Spurr Epon and thin sectioned. Sections were stained with uranyl acetate and lead citrate and viewed on a Philips 201C TEM. The mode of death (necrotic or apoptotic) of 50 randomly chosen caspase-3-immunoreactive cells was determined in cultures 12 and 24 h after NEH. For these counts, we defined necrosis as severe cytoplasmic swelling (often with plasma membrane rupture) and a tigroid nucleus. Apoptosis was defined by fragmentation of the nucleus into large, round clumps of chromatin.

Statistical analysis

In Figs. 1A–B, 2D, 3C, 5A (caspase-9; fractin), 5B, 5D–F, and 6E, data passed normality and equal variance tests and were presented as means ± S.E.M. (except Fig. 5A). Significance was determined by one-way ANOVA followed by Student-Newman-Keuls method for all pairwise multiple comparison procedures. In Figs. 1C–D, 5A (caspase-3) and 5C, normality or equal variance tests failed, and data were presented as median values. Significance was determined by one-way ANOVA followed by Student-Newman-Keuls method (Figs. 1C–D and 5A) or by Dunn’s method for comparison of all groups vs control group (Fig. 5C; 7 h time point).

RESULTS

The contribution of ionotropic glutamate receptors to hypoxic neuronal death depends on the severity of the insult

We first examined the contribution of ionotropic glutamate receptors to hypoxic neuronal death. For this purpose, DG cultures were incubated with the NMDA receptor blocker MK-801 and the non-NMDA receptor blocker NBQX, before, during and after 45 min exposure to NaCN. As shown in Fig. 1A, preincubation of cultures with 10 μM MK-801 and 10 μM NBQX fully protected cultures from 45 min exposure to 3 mM NaCN. This neuroprotection was reduced when exposure was extended to 180 min (Fig. 1B) or when NaCN concentration was increased to 10 mM (Fig. 1C). Neuroprotection was not improved when cultures were pre-incubated with higher concentrations of MK-801 (or 100 μM AP-5; data not shown) suggesting that nearly complete blockage of NMDA receptors did not fully
Fig. 5. Caspase-9 and caspase-3 contribute to neuronal death. (A) Hypoxia triggers the caspase cascade even in presence of MK-801 and NBQX. The graph displays the percentage of cells immunoreactive for caspase-9, caspase-3 or fractin. Cultures were fixed 12 h after NaCN exposure. Data are median values of six independent determinations in each group, analyzed by one way ANOVA (\(*P<0.05\) vs. control). (B) Inhibitors of caspase-9 (Z-LEHD-fmk), caspase-3 (Z-DEVD-fmk) and the general caspase inhibitor Z-VAD-fmk reduce neuronal death induced by hypoxia. LDH release was measured 7 h after treatment. Data are mean ± S.E.M. of four to 11 independent determinations in each group, analyzed by one way ANOVA (\(*P<0.05\) vs. NaCN). (C) Caspase inhibitors inhibit early LDH release induced by NEH. Data are median values of four to 11 independent determinations in each group, analyzed by one way ANOVA (\(*P<0.05\) vs. NaCN). Analyzed by t-test, the Z-LEHD-fmk group (7 h time point) is statistically different from the “NaCN + MK-801/NBQX” group. (D) Z-DEVDFmk (100 \(\mu\)M) improves neuronal survival determined by Trypan Blue assay 7, 12 or 24 h after hypoxia. Data are mean ± S.E.M. of six to 11 independent determinations in each group, analyzed by one way ANOVA (\(*P<0.05\) vs. control).
protect cultures from severe hypoxia (Fig. 1C). AMPA/kainate receptors were not critical to hypoxic cell death, since the addition of 10 μM NBQX to MK-801 did not improve neuroprotection (Fig. 1D). Altogether, these results suggested that increasing the duration or the severity of hypoxia revealed a second, non-excitotoxic form of cell death which, in the absence of treatment, was masked by the more rapid excitotoxic death.

Non-excitotoxic hypoxia (NEH) induces early loss of mitochondrial integrity and cytochrome c release

Treatment of DG cultures with 10 mM NaCN, a potent inhibitor of the complex IV of the respiratory chain, is expected to deeply affect neuronal mitochondrial metabolism. Using Mitotracker Orange dye, a severe loss of the mitochondrial membrane potential was observed in the whole neuronal population at the end of the hypoxic treatment in absence (data not shown) or presence of blockers of excitotoxicity (Fig. 2B). This loss was substantially reversed in surviving cells 4 h after NEH treatment (Fig. 2C). Conversely, measurement of ATP level at the end of NaCN treatment showed a severe energy failure, which was identical in absence or presence of the blockers (Fig. 2D). A partial ATP level recovery was observed at later time points. EM studies showed that mitochondrial swelling was an early sign of cell damage, since it was already prominent by the end of the NEH treatment, when nuclei were still relatively intact (Fig. 2E–F). Altogether, these results showed that severe hypoxia primarily induces loss of mitochondrial integrity, which is partially prevented by blockers of excitotoxicity.

Mitochondrial membrane potential loss and/or mitochondrial swelling may induce release of mitochondrial factors triggering cell death pathways. For example, release of cytochrome c from mitochondrial intermembrane space to the cytosol can induce cell death by initiating the caspase cascade. At the end of the NEH period, cytochrome c release was already detectable by immunocytochemistry (Fig. 3A–C), and mitochondrial swelling and disruption of their outer membrane were visible by EM immunocytochemistry in neurons undergoing cytochrome c release (Fig. 3D). When we studied mitochondria in these neurons, 34 of 50 showed a ruptured outer membrane.

Caspase-9 and caspase-3 contribute to NEH-induced neuronal death

Cytochrome c release may activate caspase-9, triggering activation of caspase-3, the executioner of the cell. We examined their contribution to NEH death by immunocytochemical studies. Caspase-9 and -3 activation were already detectable 12 h after NEH treatment and were still visible 24 h after treatment (Fig. 4 and 5A). Caspase-3 was functional, since fractin immunoreactivity, revealing actin cleavage by caspase-3, was increased (Figs. 4 and 5A). We pre-incubated DG cultures with the general caspase inhibitor Z-VAD-fmk, the caspase-9 inhibitor Z-LEHD-fmk and the caspase-3 inhibitor Z-DEVD-fmk before, during and after 45 min exposure to NaCN. As measured by LDH release, these caspase inhibitors reduced hypoxic cell death monitored 7 h after NaCN treatment, in absence (Fig. 5B) or presence of blockers of excitotoxicity (Fig. 5C). No significant neuroprotection was detectable by the LDH release assay 12 and 24 h after hypoxia (Fig. 5C), perhaps because the peak of necrotic death was over, and most apoptotic neurons still had a competent membrane by 24 h (Fig. 6G), and thus were unlikely to have released much LDH by that time. Even though low concentrations of Z-DEVD-fmk were not neuroprotective (Fig. 5E), preincubation of DG cultures with the caspase inhibitor M867 (10 μM) diminished cell death, as monitored by Trypan Blue exclusion 12 h after NEH treatment (15.3 ± 1.5% cell death vs 22.5 ± 1.9%; P < 0.05; t-test). This confirms that caspase activation contributes to NEH-induced injury. We also showed that Z-LEHD-fmk inhibited caspase-3 activation, suggesting that caspase-9 activation was upstream of caspase-3 (Fig. 5F). Altogether, these results suggest that the caspase-9/caspase-3 cascade contributes to NEH-induced neuronal death. Note that these results do not rule out a participation of calpain or cathepsin in hypoxic cell death.

At early time points, caspase-3-immunoreactive cells have a necrotic morphology

We identified by EM the type of death (necrotic or apoptotic) of 50 caspase-3-immunoreactive neurons 12 and 24 h after NEH treatment (Fig. 6A–D). Forty-seven out of 50 immunoreactive neurons had a necrotic morphology 12 h after NEH treatment (Fig. 6A and D) while 27 of 50 immunoreactive neurons were necrotic 24 h after treatment (Fig. 6B and D). The expression of apoptotic cell death usually depends on synthesis of death effector proteins. By contrast, necrotic cell death does not require protein synthesis. The protein synthesis blocker CHX was unable to block NEH death 12 h after treatment, when death was overwhelmingly necrotic, but partially increased cell survival 24 h after NEH treatment, when nearly half the deaths were apoptotic (Fig. 6E).

Altogether, our results show that caspase inhibitors partially block NEH death, and caspase-3-immunoreactive neurons are mainly necrotic 12 h after treatment, suggesting that caspase-3 contributes to NEH-induced necrosis. To ensure that death reduction is attributable to necrosis

NaCN = MK-801/NBQX in each group. (E) Dose-response analysis of neuroprotective effect of Z-DEVD-fmk. Cell survival was determined by Trypan Blue assay 7 h after NEH treatment. Data are mean ± S.E.M. of nine independent determinations in each group, analyzed by one way ANOVA. * P < 0.05 vs. NaCN = MK-801/NBQX. (F) The increase of caspase-3 IR cells is blocked when hypoxic cultures are preincubated with the caspase-9 inhibitor Z-LEHD-fmk, the caspase-3 inhibitor Z-DEVD-fmk before, during and after 45 min exposure to NaCN. As measured by LDH release, these caspase inhibitors reduced hypoxic cell death monitored 7 h after NaCN treatment, in absence (Fig. 5B) or presence of blockers of excitotoxicity (Fig. 5C). No significant neuroprotection was detectable by the LDH release assay 12 and 24 h after hypoxia (Fig. 5C), perhaps because the peak of necrotic death was over, and most apoptotic neurons still had a competent membrane by 24 h (Fig. 6G), and thus were unlikely to have released much LDH by that time. Even though low concentrations of Z-DEVD-fmk were not neuroprotective (Fig. 5E), preincubation of DG cultures with the caspase inhibitor M867 (10 μM) diminished cell death, as monitored by Trypan Blue exclusion 12 h after NEH treatment (15.3 ± 1.5% cell death vs 22.5 ± 1.9%; P < 0.05; t-test). This confirms that caspase activation contributes to NEH-induced injury. We also showed that Z-LEHD-fmk inhibited caspase-3 activation, suggesting that caspase-9 activation was upstream of caspase-3 (Fig. 5F). Altogether, these results suggest that the caspase-9/caspase-3 cascade contributes to NEH-induced neuronal death. Note that these results do not rule out a participation of calpain or cathepsin in hypoxic cell death.

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At early time points, many caspase-3-immunoreactive cells have a necrotic morphology, and death does not depend on protein synthesis. (A–C) Microphotographs showing caspase-3-immunoreactive cells 12 h (A), or 24 h (B, C) after NEH. Gold particles, revealing caspase-3 localization (arrows), are visible in cells morphologically necrotic (A, B) as well as in cells displaying an apoptotic morphology (C). Scale bars = 1 μM. (D) Graph showing the number of caspase-3-immunoreactive cells displaying an apoptotic or necrotic morphology. The mode of death of 50 immunoreactive cells was identified 12 and 24 h after the NEH treatment. (E) The protein synthesis inhibitor CHX (1 μg/ml) improves cell survival 24 h after NEH treatment. CHX is not neuroprotective 12 h after NEH. Cultures were exposed to CHX 1 h before, during and after the NEH treatment. Cell survival was assayed by Trypan Blue exclusion. Data are mean ± S.E.M. of six independent determinations in each group, analyzed by one way ANOVA (* p < 0.05 vs. NaCN + MK-801/NBQX). (F) Examples of cells triple stained with caspase-3 antibodies (green), nuclear marker Hoechst 33342 (blue) and propidium iodide (PI; red). Upper images show an immunoreactive cell with a pyknotic nucleus stained by PI, suggestive of a necrotic morphology. Lower images show a cell with a fragmented nucleus and PI exclusion, suggesting an apoptotic process. (G) Graph representing the number of caspase-3-immunoreactive cells with a pyknotic or a fragmented nucleus, and with or without PI exclusion. Most immunoreactive cells have pyknotic nuclei and are stained by PI 12 h after NEH treatment, while many cells have fragmented nuclei and exclude PI at 24 h.
reduction and not apoptosis inhibition, we studied by light microscopy the membrane competency and nuclear morphology of caspase-3-immunoreactive cells. Necrotic cells usually have a pyknotic nucleus and their cytoplasmic membrane is incompetent, while apoptotic cells have a nucleus showing large fragmented chromatin masses and a competent cytoplasmic membrane. Most caspase-3-immunoreactive cells had a pyknotic nucleus and an incompetent membrane 12 h after treatment (Fig. 6F and G). Caspase inhibitors may also block early stages of apoptosis, but this inhibition is probably minimal, since at the 12 h time point most caspase-3-immunoreactive cells with a fragmented nuclei excluded propidium iodide and, since they had a competent membrane, would not be expected to contribute to LDH release and Trypan Blue exclusion assays, which we used as measures of cell death.

**DISCUSSION**

Our results suggest that (1) if excitotoxicity is blocked, hypoxic neuronal death still takes place, providing hypoxic exposure is long or severe enough; (2) this non-excitotoxic component of severe hypoxia can be reduced by caspase inhibitors, confirming previous reports of a neuroprotective synergy between caspase-3 inhibitors and MK-801 in cerebral ischemia (Ma et al., 1998) and in chemical hypoxia (Schulz et al., 1998), and of unmasking caspase-dependent cell death by blockade of the excitotoxic component of ischemia in neuronal culture (Gottron et al., 1997). None of these studies had identified the type (necrotic or apoptotic) or mechanism of death. In this present report, electron microscopic immunocytochemical studies and cell survival assays showed two forms of NEH-induced, caspase-dependent cell death by blockade of the excitotoxic component of ischemia in neuronal culture (Gottron et al., 1997). The other form appears earlier, has morphological features of necrosis and does not require protein synthesis. This active necrosis is a fast process since caspase-3-dependent LDH release was seen early, suggesting early breakdown of the plasma membrane.

It has long been believed that necrotic neuronal death is a passive process of cytoplasmic swelling and lysis following overwhelming ionic influx. This present study shows that neuronal necrosis can be an active process requiring the activation of a mitochondrial death program, with early mitochondrial swelling followed by cytochrome c release and caspase activation. Our laboratory has recently shown a similar form of "active necrosis" in neuronal cultures exposed to hypoxia in the absence of blockers of excitotoxicity (Niquet et al., 2003). In vivo observations suggest that "active necrosis" also occurs in rodent CA1 neurons after transient cerebral ischemia. Activation of the mitochondrial pathway and a necrotic morphology have been described in models of transient cerebral ischemia (Chen et al., 1998; Colbourne et al., 1999a,b; Ouyang et al., 1999; Niwa et al., 2001).

In both excitotoxic and NEH, "active necrosis" of DG neurons seems to be initiated by mitochondrial dysfunction (swelling, rupture of the outer membrane, and/or opening of the transition pore) leading to cytochrome c release and to the activation of the caspase cascade. Our studies do not exclude the involvement of other mitochondrial factors (such as apoptosis inducing factor) or proteolytic enzymes (such as calpain, cathepsins) to "active necrosis." Initial energy failure may initiate several death pathways, and their inhibition may reduce or delay but not fully block cell demise.

Blockers of excitotoxicity decrease cytochrome c release, suggesting that calcium influx through glutamate ionotropic receptors may contribute to it, probably by provoking excessive mitochondrial calcium uploading (Andreyev et al., 1998). Additional experiments will be necessary to determine whether other sources of calcium or calcium-independent mechanisms contribute to NEH-induced cytochrome c release. The direct effect of energy failure on mitochondria may also be a contributor by inducing loss of the mitochondrial membrane potential and opening of the mitochondrial transition pore (Nicholls and Budd, 2000).

This type of death may be masked, in most experimental models of cerebral hypoxia–ischemia, by the more rapid excitotoxic death of neurons. However, as non-toxic blockers of excitotoxicity are developed, the slower NEH death may take on increasing importance. In the core of cerebral ischemic infarcts, where death is predominantly necrotic (Lipton, 1999; Snider et al., 1999), the severity of the insult may be sufficient to trigger NEH (or non-excitotoxic ischemic) death, as well as excitotoxic death, and both will need to be treated to improve neuronal viability. The therapeutic implications of these results are that ionotropic glutamate receptor blockers alone may be inadequate for rescuing neurons from severe injury, but that combined use of these agents with caspase inhibitors or with agents that reduce cytochrome c release (e.g. bcl-2) may be worth testing.

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