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CONTRIBUTION OF A MITOCHONDRIAL PATHWAY TO EXCITOTOXIC NEURONAL NECROSIS

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Abstract

It is traditionally thought that excitotoxic necrosis is a passive mechanism that does not require the activation of a cell death program. In this study, we examined the contribution of the cytochrome c-dependent mitochondrial death pathway to excitotoxic neuronal necrosis, induced by exposing cultured cortical neurons to 1 mM glutamate for 6 hrs and blocked by the NMDA antagonist, dizocilpine. Glutamate treatment induced early cytochrome c release, followed by activation of caspase-9 and caspase-3. Preincubation with the caspase-9 inhibitor z-LEHD-fmk, the caspase-3 inhibitor z-DEVD-fmk or the specific pan-caspase inhibitor Q-VD-opf decreased the percentage of propidium iodide-positive neurons ($52.5 \pm 3.1\%$, $39.4 \pm 3.5\%$, $44.6 \pm 3\%$ respectively vs $65 \pm 3\%$ in glutamate + vehicle). EM studies showed mitochondrial release of cytochrome c in neurons in the early stages of necrosis, and cleaved caspase-3 immunoreactivity in morphologically necrotic neurons. These results suggest that an active mechanism contributes to the demise of a subpopulation of excitotoxic necrotic neurons.

Keywords

Glutamate; mitochondrial swelling; cytochrome c; caspase-3; primary cortical culture

INTRODUCTION

Necrosis may be the most common mode of neuronal death in many diseases including stroke, trauma and seizures, but it has received far less investigative attention than apoptosis (Choi et al., 1987; Gwag et al, 1995, 1997; Kato et al, 1997; Colbourne et al., 1999ab; Fujikawa et al., 2005; Niquet et al., 2003, 2007). In primary cultures or isolated preparations, mild and/or brief exposure to an excitotoxic insult may induce neuronal death that has features of apoptosis (Tenneti et al. 1998; Ientile et al., 2001; Yu et al., 2003). However, these experimental parameters do not mimic pathological conditions associated with severe and prolonged excitotoxic insults. High extracellular glutamate concentration (250 μ M–1mM) has been reported following ischemic insults (Hossmann, 1994). Neuronal firing causes transient but repeated release of millimolar concentration of glutamate in the

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synaptic cleft (Clements, 1996). Even though this is not universally recognized (Pollard et al., 1994), several ultrastructural studies have shown that these severe excitotoxic insults induce a form of neuronal death which is predominantly necrotic (Colbourne et al., 1999ab; Fujikawa et al., 2005; Niquet et al., 2007). These insults have also been shown to activate enzymes found in apoptotic pathways (Chen et al., 1998; Liu et al., 1999; Ouyang et al., 1999; Niwa et al., 2001), but this might indicate a direct role for those enzymes in excitotoxic necrosis rather than the presence of apoptotic or intermediate forms of cell death. We reported that severe hypoxia induces a form of death that is morphologically necrotic and may require the mitochondrial release of cytochrome c, which activates the caspase-9-caspase-3 cascade (Niquet et al., 2003).

In this study, we examined the possibility that glutamate excitotoxicity-induced necrosis might involve similar active mechanisms. The energy depletion induced by excitotoxicity (Greenwood et al., 2007ab) and seizures (Folbegrava et al., 1981) is less severe than that caused by severe hypoxia (Ekholm et al., 1992; Siesjo, 1992; Niquet et al., 2006), therefore the mitochondrial response to both conditions cannot be assumed to be identical. Exposure of primary cortical cultures to high concentration of glutamate (1 mM) has been reported to induce cytochrome c release and caspase-3-dependent neuronal death (Zhang and Bhavnani, 2005, 2006), but in those studies the mode of death (apoptotic or necrotic) and the cellular localization of caspase activation were not investigated in detail.

MATERIALS AND METHODS

Primary embryonic cortical cultures

Neocortices from Wistar rat embryos were dissected at gestational day 17 (E17), finely chopped with scissors, and incubated at 37 °C for 10 min with 0.25 % trypsin diluted in Neurobasal/B27 (2%; v/v). Mechanical dissociation was gently performed with a fire-polished Pasteur pipette in Neurobasal/Fetal bovine serum (10% v/v). After centrifugation (130 × g), dissociated cells (from the pellet) were resuspended in Neurobasal/FBS/B27 (5%/5%; v/v), and plated in poly-D-lysine (10 mg/ml molecular weight 300,000)-coated 8-well chamber slides, or in 12-, 24-well plates at density of 250,000 cells/ml. The culture medium consisted of Neurobasal, 2.5% fetal bovine serum, 1% B27 supplement, 1.25% (v/v) penicillin-streptomycin and 1% fungizone. The growth medium was refreshed at day in vitro (DIV) 5, 8, and 11. Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. At DIV 10, 42±4.4 % of the cells were neurons (identified by immunoreactivity against NeuN, a neuronal marker).

Glutamate and staurosporine treatment

All treatments were performed at DIV 10–13. In dose-response studies, cultures were exposed to 0.1–1 mM glutamate for 6 hrs, and then immediately fixed for neuronal death detection. In time-course studies, cultures were exposed to 1 mM glutamate for different durations (between 30 min and 24 hrs), and immediately fixed for neuronal death detection or ultrastructural studies. In MK-801 studies, cultures were preincubated with MK-801 (10 µM) 30 min before exposure to 0.1 M glutamate for 6 or 24 hrs, and then immediately fixed for neuronal death detection. In caspase inhibitor studies, cultures were preincubated with the caspase-9 inhibitor z-LEHD-fmk (Sigma, St Louis, Mo), the caspase-3 inhibitor z-DEVD-fmk (Sigma) or the general caspase inhibitor Q-VD-opf (R&D systems, Minneapolis) 45 min before exposure to 0.1 M glutamate for 6 or 24 hrs, and then immediately fixed for neuronal death detection. Control cultures were treated with vehicle (DMSO). When we wanted to induce apoptosis, cultures were treated with 300 nM staurosporine for 6 hrs.

Neuronal death detection

Cell death was detected with the death marker propidium iodide (10 mg/ml), added to the cultures 30 min prior to the end of glutamate treatment. Cultures were fixed with 4% paraformaldehyde in 0.1M phosphate buffer for 30 min at room temperature (RT), and incubated overnight with the neuronal marker NeuN (Chemicon; 1/100). Staining was revealed with anti-mouse monoclonal antibodies conjugated with Alexa Fluor 488, and cultures were counterstained with the nuclear stain Hoechst 33342. Neuronal death was determined by counting the percentage of propidium iodide-positive neurons in at least 3 wells per group (time point or treatment). Counting was performed in 5–6 fields per well ($\times$ 2000 cells). Each experiment was repeated at least four times to make sure of their reproducibility. Cell counting was performed by an observer who was blinded with respect to treatment group.

Ultrastructural studies

Cultures were fixed with 2.5 % glutaraldehyde and 1.6% paraformaldehyde in 0.1 M cacodylate buffer for 1 hour. After washing, the cultures were postfixed with 1% osmium tetroxide in cacodylate buffer for 45 min, rinsed with water and en bloc stained with 1% uranyl acetate for 1 hr. The cultures were then 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.

Light immunocytochemical studies

For cytochrome c and active caspase-3 (caspase-3_a) staining, cultures were fixed with a methanol/acetone mixture (1:1, v/v) for 10 min at -20°C . For active caspase-9 (caspase-9_a) staining, cultures were fixed with methanol for 10 min at -20°C . After washes, cultures were pre-incubated with 0.1M phosphate buffer containing 5% donkey serum for 60 min at RT, and incubated for 90 min at RT with primary antibody to cytochrome c (1:100, BD Biosciences) or caspase-3_a (1:300, Sigma), or 48 hrs at 4°C with primary antibody to caspase-9_a (1:100, Calbiochem). After washes, cultures were incubated for 90 min with antibodies conjugated to Alexa fluor and then counterstained with Hoechst 33342. The percentage of immunoreactive cells was determined in 5–6 fields per well by an observer unaware of the experimental conditions. Control experiments including incubation without primary antibody or non-immune IgG did not show any staining.

Electron microscopic immunocytochemistry

Preembedding immunogold labeling was used to localize caspase-3_a and cytochrome c immunoreactivity as described previously (Niquet et al., 2003, 2006). After paraformaldehyde fixation and washing, cultures were incubated with PBS containing 0.1% cold fish gelatin (CFG) and 1% normal goat serum for 15 min, then incubated overnight at 4°C with anti-rabbit polyclonal or anti-mouse monoclonal antibody conjugated to 1 nm gold particles (1/80; Ted Pella, Inc., CA), which was diluted in PBS containing 0.1% CFG. The cultures were washed with CFG containing 2.5M NaCl for 20min, and then several times with distilled water. Gold staining was intensified by silver enhancement (Ted Pella, Inc.) for 10–12 min., then cultures were washed with distilled water and postfixed with 0.1% osmium. After dehydration with alcohol, cultures were embedded in Spurr Epon and thin sectioned. Sections were stained with uranyl acetate and lead citrate and viewed on a Philips 201C TEM. Control experiments including incubation without primary antibody or non-immune IgG did not show any staining.

Statistical analysis

In figure 1A–B, figure 3A and figure 5A, data passed normality and equal variance tests and were presented as means $\pm$ SEM. Significance was determined by one-way ANOVA followed by Bonferroni's method for comparison of all groups vs control group (0 mM in 1A; 0 hr in 1B; Glu + vehicle in 5A). In figure 1C, 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 (6 hrs) or Kruskal-Wallis (24 hrs) methods for all pairwise multiple comparison.

RESULTS

Glutamate-induced neuronal death is excitotoxic

Dose-response studies showed that a majority of neurons ($64.0 \pm 5\%$) were propidium-positive when exposed to 1 mM glutamate for 6 hrs (Fig. 1A). We applied 1 mM glutamate in all subsequent experiments. Neuronal death was delayed to 4–6 hrs following glutamate application (Fig. 1B). Pre-incubation of the cultures with the NMDA receptor blocker MK-801 (10 μ M) fully prevented neuronal death induced by 6 hrs glutamate exposure. When glutamate exposure was prolonged to 24 hrs, MK-801 still provided high neuroprotection, showing that glutamate is excitotoxic (Fig. 1C). In cultures exposed to glutamate for 6 hrs, a large majority of neurons had pyknotic nuclei and were PI-positive, features suggestive of necrosis (Fig. 1D). By contrast, cortical cells exposed to 300 nM staurosporine (STS) for 6 hrs had fragmented nuclei and were PI-negative, features suggestive of apoptosis (Fig. 1D).

Ultrastructural evolution of glutamate-induced neuronal death

In control cultures, neurons had intact nuclei and healthy mitochondria, that were cigar-shaped or small and rounded (Fig 2A). After 30 min of glutamate treatment, a large majority of neurons (90 out of 100) displayed morphological features of early necrosis: many organelles were swollen, while the nuclei had an intact nuclear membrane and showed no sign of fragmentation (Fig. 2 B–C). After 6 hrs of glutamate treatment, neurons showed an advanced stage of necrosis with total loss of the plasma membrane and severe cytoplasmic swelling. Their nuclei displayed tigroid fragmentation of the chromatin, but their nuclear membrane was still visible (Fig 2D). By contrast, in cultures exposed to staurosporine for 6 hrs, apoptosis was characterized by the formation of large, round chromatin clumps and complete loss of nuclear membrane (Fig. 2 E).

Glutamate treatment induces release of cytochrome c, and caspase-9-dependent caspase-3 activation

Mitochondrial swelling may induce release of mitochondrial factors, such as cytochrome c, triggering cell death pathways. Cytochrome c release may activate caspase-3 through caspase-9 activation (Li et al., 1997). We first investigated by light immunocytochemistry the release of cytochrome c to the cytosol. Release was minimal in untreated cultures ($0.36 \pm 0.5\%$ of cells), increased 14-fold after 2 hrs glutamate treatment ($5.1 \pm 1.8\%$; $p < 0.05$ vs control by one way ANOVA) and was still observable at 6 hrs ($14.6 \pm 0.3\%$ $p < 0.05$ vs control by one way ANOVA). Glutamate treatment for 6 hrs increased caspase-9_a and caspase-3_a immunoreactivity compared to untreated controls (Fig. 3A). Caspase-3 activation was confirmed by enzymatic assays showing a significant increase of enzymatic activity in glutamate-treated cultures compared to controls (7.4 ± 0.22 nmol pNA/min/L in the glutamate group, vs 2.2 ± 0.15 in controls, $p < 0.01$ by t-test). All caspase-3_a-IR neurons were cytochrome c-positive, indicating its mitochondrial release (Figure 3 B–F), and pretreatment

with caspase-9 inhibitor Z-LEHD-fmk (100 μ M) significantly decreased (47%; $P < 0.01$ by t-test) the number of caspase-3_a-IR neurons.

Caspase-3-immunoreactive cells are morphologically necrotic

A large majority (90%) of caspase-3_a-IR or caspase-9_a-IR neurons displayed shrunken nuclei and were propidium iodide positive, suggestive of a necrotic process (Fig. 3 G–N). By EM immunocytochemistry, cytochrome c release was seen in neurons which were clearly necrotic, with swollen mitochondria, many of which had a ruptured outer membrane, swelling of endoplasmic reticulum (ER) and cytoplasm, and a relatively intact nucleus (Fig. 4A–B). After 6 hrs exposure to glutamate, 41 out of 50 caspase-3_a-immunoreactive neurons displayed features of late stage of necrosis with markedly swollen mitochondria and loss of plasma membrane (Fig. 4C), and 9 out of 50 had morphological features of apoptosis (large chromatin clumps with smooth edges), proving that glutamate-induced caspase-3 activation leads to necrotic death in the majority of neurons in this model.

Caspase inhibitors delay necrotic neuronal death

We pre-incubated the cultures with the specific pan-caspase inhibitor Q-VD-OPH (50 μ M), the caspase-9 inhibitor Z-LEHD-fmk (10 μ M), or the caspase-3 inhibitor Z-DEVD-fmk (10 μ M). In cultures exposed to glutamate for 6 hrs, propidium iodide uptake was partially prevented by pre-incubation of the cultures with any of those three caspase inhibitors (Fig. 5A). However, even at higher concentration (100 μ M), Z-DEVD-fmk failed to block nuclear condensation (Fig. 5B), or propidium iodide uptake when glutamate exposure was prolonged to 24 hrs (data not shown). Altogether, these results suggest that caspase activation promotes plasma membrane rupture seen in the early stages of necrosis.

DISCUSSION

In this study, we found that glutamate excitotoxicity can trigger a mitochondrial pathway, initiated by cytochrome c release and followed by caspase-9-dependent caspase-3 activation, that may contribute to the demise of a subpopulation of necrotic neurons. This “active” form of excitotoxic necrosis may be found in vivo, since we reported caspase-3 activation in morphologically necrotic cells following lithium-pilocarpine induced-seizures (Niquet et al, 2007). This mechanism may not be universal, since cell death with apoptotic features has been reported in a kainate model of seizures (Pollard et al, 1994), and caspase inhibition exacerbates glutamate-induced oxidative neuronal damage in cell line culture (Elphick et al., 2008).

It is traditionally thought that necrosis is a passive process involving intracellular ion influx leading to cell explosion (Choi et al, 1987). Our results show that necrosis can be an “active” process, but do not rule out the contribution of ionic influx. Actually, calcium may be a key element in “active” necrosis. In glutamate-induced excitotoxicity, massive calcium influx arising from overstimulation of the NMDA subtype of glutamate receptors may trigger the release of cytochrome c (Choi et al., 1987; Budd et al., 2000; Moltz et al., 2008). High concentrations of calcium are known to induce cytochrome c release in isolated brain mitochondria (Andreyev and Fiskum, 1999; Brustovetsky et al., 2002; Niquet et al., 2003). Brain mitochondria take up calcium whenever free $[Ca^{++}]$ in their environment exceeds 0.5 μ M, and have a large but not unlimited buffering capacity (200 nM/mg protein). Excessive cytosolic calcium concentration may overwhelm the calcium loading capacity of mitochondria, leading to mitochondrial swelling and possibly to rupture of the outer membrane, and release of mitochondrial factors such as cytochrome c (Griffiths et al., 1983; Brustovetsky et al., 2002; Niquet et al., 2003). This hypothesis is supported by EM images showing evidence of early cytochrome c release in neurons in which mitochondria are

swollen (Fig. 4B). In hypoxia, total energy failure with ATP levels at 0–5% of controls (Niquet et al., 2006) results in a rapid collapse of the mitochondrial membrane potential (Niquet et al., 2003). In excitotoxicity, ATP levels are often at 50% of controls (or at 80% in seizures, Folbetrova et al., 1981) and mitochondrial depolarization is partial (Greenwood et al., 2007a), but the large Ca^{++} influx through NMDA receptors may overload the mitochondrial matrix ($>1500 \text{ nM } \text{Ca}^{++}/\text{mg}$ protein, Chalmers and Nicholls, 2003) and trigger permeability pore transition and lethal calcium release. These results do not rule out a contribution of caspase-independent mechanisms, such as the apoptosis-Inducing factor pathway, cathepsins or calpains, indeed this study does not reveal the mechanism of injury in the many necrotic neurons that did not show caspase activation.

The participation of a mitochondrial death pathway in necrotic neuronal death raises the possibility that neurons could be rescued after an excitotoxic insult before “executioner” caspases cause irreparable damage. The ability of caspase inhibitors to reduce excitotoxic cell death supports that possibility. However, any therapeutic intervention would have to be rapid in order to be effective, since outer membrane rupture and cytochrome c release are already present after 2 hrs of glutamate exposure. Furthermore, it might only rescue a fraction of injured neurons. In the present study, the neuroprotective effect of caspase inhibitors was partial, and caspase activation was only detected in a minority of necrotic cells, raising the possibility that several cell death mechanisms may coexist and that any monotherapy could only correct a fraction of the damage.

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Figure 1.

The graphs A, B and C display glutamate neurotoxicity determined by the loss of membrane competency after staining with propidium iodide. **(A)** Dose-response curve of glutamate neurotoxicity after 6 hrs exposure. $**P < 0.05$ vs control (0 mM glutamate). **(B)** Time course of neurotoxicity induced by 1 mM glutamate. $**P < 0.05$ vs 0 hr. **(C)** The NMDA antagonist MK-801 (10 μ M) blocked neuronal death induced by 6 hrs exposure to 1 mM glutamate treatment ($*P < 0.05$ vs. control; $**P < 0.05$ vs glutamate). Protection decreased slightly when exposure was prolonged to 24 hrs. ($*P < 0.05$ vs. control; $**P < 0.05$ vs glutamate; $\#P < 0.05$ vs control). **(D)** Triple staining of primary cortical cultures with the nuclear marker Hoechst 33342, the neuronal marker NeuN, and propidium iodide in untreated controls (CONT), after 6 hrs exposure to 1 mM glutamate (GLU), or after 6 hrs exposure to 300 nM

staurosporine (STS). Arrows show a neuron with a PI-positive pyknotic nucleus. Arrowheads show a neuron with a PI-negative fragmented nucleus. Bars = 20 μ M.

Figure 2. Ultrastructure of primary cortical cultures treated with glutamate or staurosporine. **(A)** control neuron. **(B–C)** Early necrosis after 30 min of glutamate treatment. Image C is a higher magnification of the insert seen in image B, and shows intact nuclear membrane (arrow), swollen mitochondria (arrowheads), and dilated endoplasmic reticulum. **(D)** Advanced stage of necrosis after 6 hrs of glutamate treatment. Arrowheads show the nuclear membrane. **(E)** Apoptotic morphology after 6 hrs of staurosporine treatment. Bars = 3 μ M.

Figure 3.

(A) The graph displays the percentage of neurons that release cytochrome c, and/or express caspase-9_a or caspase-3_a after 6 hrs exposure to 1 mM glutamate. *P < 0.05 vs control. (B–F) Triple staining of a neuron (arrowhead) with cytochrome c (B) and caspase-9_a (C) immunoreactivity and the nuclear marker Hoechst 33342 (D), after 6 hrs exposure to 1 mM glutamate. Images E and F are overlays of images B–C and B–D respectively. Bars = 25 μM. (G–N) Triple staining of primary cortical cultures with caspase-3_a (G and I, green), caspase-9_a (K and M, green), propidium iodide (H–I, L–M; red) and the nuclear marker Hoechst 33342 (J and N; blue) after 6 hrs exposure to 1 mM glutamate. Arrows and arrowheads show caspase-3_a or caspase-9_a-IR neurons respectively. Bars = 25 μM.

Figure 4.

Ultrastructure of neurons releasing cytochrome c (B) or expressing caspase-3_a (C). (A–B) By EM, cytochrome c IR, revealed by gold particles (arrowheads), was located in the mitochondrial intermembrane space in a control neuron (A). B shows a neuron after 2 hrs of glutamate exposure, with a relatively intact nucleus, swollen mitochondria with ruptured outermembranes and cytochrome c release into the cytosol. (C) Example of a caspase-3_a-immunoreactive neuron with necrotic morphology after 6 hrs of glutamate exposure. Gold particles reveal caspase-3_a localization (arrows). Bars = 3 μM.

Figure 5. Effect of caspase inhibitors on glutamate neurotoxicity. **(A)** The graph shows a partial neuroprotection of caspase inhibitors against 6 hrs exposure to 1 mM glutamate. * $P < 0.05$ vs “Glu + vehicle” group. **(B)** Triple staining showed that neurons exposed to 1 mM glutamate for 6 hrs took up propidium iodide and had pyknotic nuclei (arrows). After 100 μ M Z-DEVD-fmk treatment, neurons excluded propidium iodide but still displayed nuclear condensation (arrowheads). Bars = 20 μ M.