

The temperature dependence and involvement of mitochondria permeability transition and caspase activation in damage to organotypic hippocampal slices following *in vitro* ischemia

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Abstract

The aggravating effect of hyperglycemia on ischemic brain injury can be mimicked in a model of *in vitro* ischemia (IVI) using murine hippocampal slice cultures. Using this model, we found that the damage in the CA1 region following IVI in the absence or presence of 40 mM glucose (hyperglycemia) is highly temperature dependent. Decreasing the temperature from 35 to 31°C during IVI prevented cell death, whereas increasing the temperature by 2°C markedly aggravated damage. As blockade of the mitochondrial permeability transition (MPT) is equally effective as hypothermia in preventing ischemic cell death *in vivo*, we investigated whether inhibition of MPT or of caspases was protective following IVI. In the absence of glucose, the MPT blockers cyclosporin A and Melle⁴-CsA but not the immunosuppressive compound

FK506 diminished cell death. In contrast, following hyperglycemic IVI, MPT blockade was ineffective. Also, the pan-caspase inhibitor Boc-Asp(OMe)fluoromethyl ketone did not decrease cell death in the CA1 region following IVI or hyperglycemic IVI. We conclude that cell death in the CA1 region of organotypic murine hippocampal slices following IVI is highly temperature dependent and involves MPT. In contrast, cell death following hyperglycemic IVI, although completely prevented by hypothermia, is not mediated by mechanisms that involve MPT or caspase activation.

Keywords: apoptosis, hyperglycemia, mitochondria, neuroprotection, organotypic hippocampal slice culture, temperature.

J. Neurochem. (2005) **95**, 1108–1117.

We recently described a model of *in vitro* ischemia (IVI), using mouse hippocampal organotypic slice cultures, in which slices are subjected to oxygen and glucose deprivation in a medium with an ion composition similar to that of the extracellular fluid of the ischemic brain (Rytter *et al.* 2003). In this model, cell death is restricted to the CA1 region and is aggravated when glucose is present during IVI [hyperglycemic IVI (HG-IVI)] (Cronberg *et al.* 2004). This aggravating effect of glucose mimics the deleterious effect of hyperglycemia on ischemic neuronal injury seen in rodents (Rehncrona *et al.* 1981; Pulsinelli *et al.* 1982; Nedergaard 1987), primates (Myers and Yamaguchi 1977) and man (Pulsinelli *et al.* 1983). The mechanism whereby glucose aggravates ischemic cell death is still unknown. In the organotypic hippocampal slice culture, cell death following IVI is dependent on NMDA receptor activation and extracellular calcium ions while, in the presence of glucose, neither NMDA or α -amino-3-hydroxy-5-methylisoxazole-4-propionate receptor blockade nor removal

of calcium ions or treatment with free radical scavengers diminishes cell death (Rytter *et al.* 2003; Cronberg *et al.* 2005a,b).

It is well known that lowering cerebral temperature by 3–5°C during experimental global (Busto *et al.* 1987; Olsson

Received May 27, 2005; revised manuscript received June 23, 2005; accepted July 12, 2005.

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Abbreviations used: Ac-YVAD-cmk, Ac-Tyr-Val-Ala-Asp-chloromethylketone; BAF, Boc-Asp(OMe) fluoromethyl ketone; BAPTA, 1, 2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid; CsA, cyclosporin A; HG-IVI, hyperglycemic *in vitro* ischemia; IB, isolation buffer; IVI, *in vitro* ischemia; MFI, mean fluorescence intensity; MPT, mitochondrial permeability transition; NIM811, Melle⁴-CsA; NR, NMDA receptor; PI, propidium iodide.

et al. 2003) or focal (Chen *et al.* 1992), ischemia is highly protective. Neuroprotection by hypothermia has also been described in several *in vitro* models of hypoxic-ischemic cell damage, cerebellar and cortical cultures (Bruno *et al.* 1994), hippocampal organotypic cultures (McManus *et al.* 2004) and acute hippocampal slice (Okada *et al.* 1988). Multiple mechanisms are involved in brain protection by hypothermia, including glutamate release (Busto *et al.* 1989) and free radical mechanisms as well as mitochondrial-mediated processes (Bossenmeyer-Pourie *et al.* 2000). The latter include the activation of the mitochondrial permeability transition (MPT) that leads to a loss of mitochondrial membrane potential, mitochondrial swelling and eventually rupture of the outer membrane (Zoratti and Szabo 1995; Halestrap *et al.* 1997). Mitochondria harbor proteins, such as cytochrome *c*, that may induce apoptosis (Saelens *et al.* 2004) by activating caspases implicated in neuronal cell death following brain ischemia (Gillardon *et al.* 1997; Namura *et al.* 1998; Li *et al.* 2000). The MPT is blocked by cyclosporin A (CsA) which is also an inhibitor of calcineurin (Zoratti and Szabo 1995). CsA suppresses ischemic cell death (Uchino *et al.* 1995; Matsumoto *et al.* 1999). The CsA analogs MeVal⁴-CsA and MeIle⁴-CsA (NIM811) also block MPT (Khaspekov *et al.* 1999; Hansson *et al.* 2004b) but do not inhibit calcineurin (Waldmeier *et al.* 2002). FK506 on the other hand does not block MPT but is a potent calcineurin inhibitor and diminishes infarct size (Sharkey and Butcher 1994).

The aim of the present investigation was first to assess the temperature dependence of cell death in murine organotypic hippocampal cultures subjected to IVI in the absence or presence of glucose and second to assess the possible involvement of MPT and caspases in these processes.

Materials and methods

Hippocampal organotypic tissue cultures

All animal experiments were approved by the Malmö/Lund ethical committee on animal experiments (approval no. M108-01). In brief, hippocampal organotypic tissue cultures were prepared as previously described (Stoppini *et al.* 1991; Rytter *et al.* 2003). Hippocampi from 6-day-old Balb/c mice were dissected out in Hank's balanced salt solution with 20 mM HEPES, 100 units Penicillin-Streptomycin/mL and 3 mg/mL D-glucose and cut into 250- μ m thick transverse sections with a McIlwain Tissue Chopper. Sections were plated onto Millicell culture inserts, one per insert (0.4 μ m Millicell-CM, 12 mm in diameter, Millipore Corp., Bedford, MA, USA). Slices were cultured at 35°C and 90–95% humidity in a CO₂ incubator (Thermo Forma Scientific, Marietta, MA, USA) for 3 weeks before experiments. The culture medium consisted of 50% Eagle's with Earl's balanced salt solution, 25% horse serum, 18% Hank's balanced salt solution and was supplemented with 4 mM L-glutamine, 50 units Penicillin-Streptomycin/mL, 20 mM D-glucose and had a pH of 7.2, adjusted with NaHCO₃. During the first week of culture 2% B27 was included in the medium. The culture medium

was changed three times per week. All substances were obtained from Invitrogen (Carlsbad, CA, USA) with the exception of D-glucose (Sigma-Aldrich, St Louis, MO, USA).

Induction of *in vitro* ischemia

The ischemia experiments were performed as previously described (Rytter *et al.* 2003; Cronberg *et al.* 2004). In each experiment, the experimental groups contained slices from six different pups. Cultures were washed in glucose-free medium, transferred to the anaerobic incubator (Electrotek Ltd, Shipley, UK), which had an atmosphere of 10% H₂, 5% CO₂ and 85% N₂, and placed in anoxic IVI medium. The IVI medium, ischemic cerebrospinal fluid, contained (in mM): CaCl₂, 0.3; NaCl, 70; NaHCO₃, 5.25; KCl, 70; NaH₂PO₄, 1.25; MgSO₄, 2; sucrose, 40, pH 6.5. In the HG-IVI experiments slices were pre-incubated in culture medium with 40 mM glucose for 1 h prior to IVI. In the HG-IVI medium, sucrose was replaced by 40 mM glucose. The temperature was maintained at 35.0 \pm 0.2°C throughout the experiments. CsA (2 and 10 μ M), NIM811 (2 and 10 μ M) and FK506 (1 and 10 μ M) were added to the slices the day before the experiments and were present during both IVI and recovery. For inhibition of caspases, the pan-caspase inhibitor Boc-Asp(OMe) fluoromethyl ketone (BAF, 100 μ M) was added to the medium 1 h before IVI, to the IVI medium and to the culture medium during the 48-h recovery period. CsA, FK506 and BAF were purchased from Sigma-Aldrich. NIM811 was a kind gift from P. Waldmeier (Novartis Institute for Biomedical Research, Basel, Switzerland). Stock solutions of all drugs were prepared in dimethylsulfoxide. The temperature in the incubator and medium was kept at 31, 35 or 37 \pm 0.2°C. After 15 min of IVI cultures were returned to oxygenated culture medium and transferred back to the CO₂ incubators.

Quantification of cell damage

Quantification of cell death was performed as previously described (Rytter *et al.* 2003; Cronberg *et al.* 2004). The fluorescent cell death marker propidium iodide (PI) was present in the medium from 24 h prior to the experiments and throughout the recovery period. For image processing the commercial software Image-Pro Plus 4.0 (Media Cybernetics, MD, USA) was used. Fluorescence intensity was measured in standardized areas in the CA1 region and in the dentate gyrus. Background staining was measured in a small hexagon placed in an undamaged area outside the CA2/3 cell band (Fig. 1). Values of cell damage were obtained by subtracting the

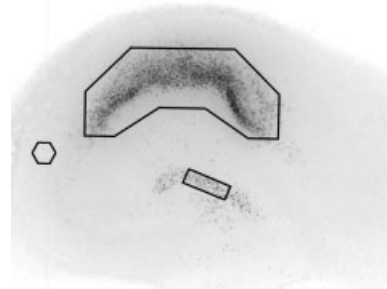


Fig. 1 An inverted fluorescent image showing the standardized areas for measuring propidium iodide intensity and mean fluorescence intensity (MFI) in the CA1 region and dentate gyrus. Background MFI is measured in a standardized hexagon outside the CA2–CA3 region.

mean fluorescence intensity (MFI) in the background area from the MFI levels in the standardized CA1 area. To obtain the maximal dynamic range of detection, the settings of the detector may vary among different studies and hence the MFI value may of different magnitude.

Immunohistochemistry

Unsectioned slices, fixed at 2, 4, 8, 12, 24 and 48 h after IVI, were immunostained for the active form of caspase 3. Slices were fixed with 4% paraformaldehyde in the culture medium for 4 h and, if not immediately processed, placed in 25% sucrose overnight and then stored in antifreeze medium (12.5% ethyleneglycol, 12.5% glycerol in 0.5 M phosphate-buffered saline) at -20°C until use. Before staining, slices were treated with 3% hydrogen peroxide in 10% methanol for 20 min to quench endogenous peroxidase activity. After rinsing, slices were pre-incubated with 5% normal serum (DAKO, Glostrup, Denmark) diluted in phosphate-buffered saline with 0.5% Triton X-100 for 1 h prior to overnight incubation with the primary antibody, monoclonal rabbit anti-caspase 3 (R and D Systems, Abingdon, UK), diluted in phosphate-buffered saline containing 5% normal serum and 0.5% Triton X-100. This antibody detects activated caspase 3 in brain tissue (Kristensen *et al.* 2003). After rinsing, slices were incubated with a biotinylated secondary antibody (swine anti-rabbit IgG antibody; DAKO) diluted in phosphate-buffered saline containing 0.5% Triton X-100 and 2% swine serum for 90 min. This was followed by incubation with the avidin-biotin-peroxidase complex (ABC, Vectastain elite, Vector Laboratories, Burlingame, CA, USA) for 1 h before reaction with the chromogen 3,3'-diaminobenzidine supplemented with 0.06% nickel (DAB-Safe; 0.5 $\mu\text{g}/\text{mL}$; Saveen-Werner AB, Malmö, Sweden).

Isolation of mitochondria

Hippocampal mitochondria were isolated from 21-day-old Balb/c mice using a discontinuous Percoll gradient [method B (Sims 1990) with slight modifications (Hansson *et al.* 2003)]. The mice were briefly sedated with halothane and decapitated. The brains were rapidly dissected and transferred to ice-cold isolation buffer (IB) containing 320 mM sucrose, 2 mM EGTA, 10 mM Trizma base, pH 7.4. For each experiment, hippocampi from three mice were homogenized in IB containing 12% (v/v) Percoll using a 2-mL Teflon Homogenizer, size 19 (Kontes, Vineland, NJ, USA). The homogenate was added to a Percoll gradient (40 and 26%, respectively) and centrifuged in an Optima Ultracentrifuge (100.3 rotor, Beckman, Palo Alto, CA, USA) at 30 700 *g* for 7 min, yielding a dense fraction between the two lower Percoll layers (fraction 3). The latter was collected and diluted 1 : 4 with IB, followed by a washing step at 16 700 *g* for 12 min. A last washing step in IB containing bovine serum albumin (1 mg/mL) was performed in a microcentrifuge (Eppendorf, Hamburg, Germany) at 7300 *g* for 6 min. This yielded a dense mitochondrial pellet that was resuspended and diluted in IB. The protein content was quantified according to the method of Bradford (1976) with bovine serum albumin as standard.

Flow cytometric analysis

The flow cytometric protocol for analysis of MPT in isolated brain mitochondria has previously been used to analyse CsA-mediated inhibition of MPT in de-energized brain mitochondria (Mattiasson

et al. 2003) as well as in energized mitochondria (Hansson *et al.* 2004a). Briefly, we used a FACSCalibur equipped with a 488-nm Argon laser (Becton and Dickinson, San Jose, CA, USA) and analysed data with the CellQuest software (Becton and Dickinson). Mitochondria were selectively stained with non-yl acridine orange (Mattiasson *et al.* 2003). The experiments were performed at room temperature (21°C) and 10 000 events, i.e. non-yl acridine orange-positive particles per sample, were collected. Hippocampal mitochondria were evaluated for properties of MPT under de-energized conditions. Experiments were performed in an isotonic KCl-based buffer containing the respiratory complex blockers rotenone and anti-mycin (both 0.5 μM) and the calcium ionophore A23187 (2 μM) to ensure free diffusion of Ca^{2+} over the mitochondrial membranes. Mitochondria (25 $\mu\text{g}/\text{mL}$) were incubated with non-yl acridine orange (100 nM) and either vehicle (dimethylsulfoxide), CsA (2 μM), NIM811 (2 μM) or FK506 (1 μM). MPT was detected as a decrease in side scattering. A baseline value for side scattering was detected after 3 min of incubation and, at 4 min of incubation, CaCl_2 corresponding to 200 μM of free calcium was added to induce MPT. Values for side scattering were registered every minute up to 6 min of incubation with calcium. The experiment was terminated by the addition of 7.5 $\mu\text{g}/\text{mL}$ alamethicin to induce maximal swelling. Background swelling was detected in vehicle-treated samples without added calcium and was subtracted from all samples before comparison. Duplicate samples in three independent mitochondrial preparations were analysed and the swelling responses (% of maximal swelling) were compared using the geometrical mean values (CellQuest).

Statistics

In the IVI experiments, compared groups were always run in parallel inside the anaerobic incubator and all statistical groups consist of data obtained from three separate experiments. Data are expressed as mean \pm SD. Differences between groups were analysed by two-way ANOVA with Scheffé's post-hoc test. Variability between experimental dates was compensated for by including the date of the experiment as a factor. The mitochondrial swelling response was evaluated using one-way ANOVA followed by Bonferroni post-hoc test. For statistical analyses the commercial software Statview 4.0 (Abacus Concepts Inc., Berkeley, CA, USA) was used. A *p*-value less than 0.05 was considered significant.

Results

Temporal development of damage following *in vitro* ischemia and hyperglycemic *in vitro* ischemia

To follow the temporal development of damage following 15 min of IVI or HG-IVI, images of PI-stained slices were captured after 2, 4, 8, 12, 24 and 48 h of recovery (Fig. 2a). IVI led to a homogenous uptake of PI, restricted to the CA1 region. The MFI increased at 4 h and continued to increase until 48 h of recovery (Fig. 2b top). After 48 h of recovery there was no further increase in MFI (data not shown). Cultures pre-incubated for 1 h in 40 mM glucose prior to IVI in ischemic cerebrospinal fluid, HG-IVI, displayed a delayed

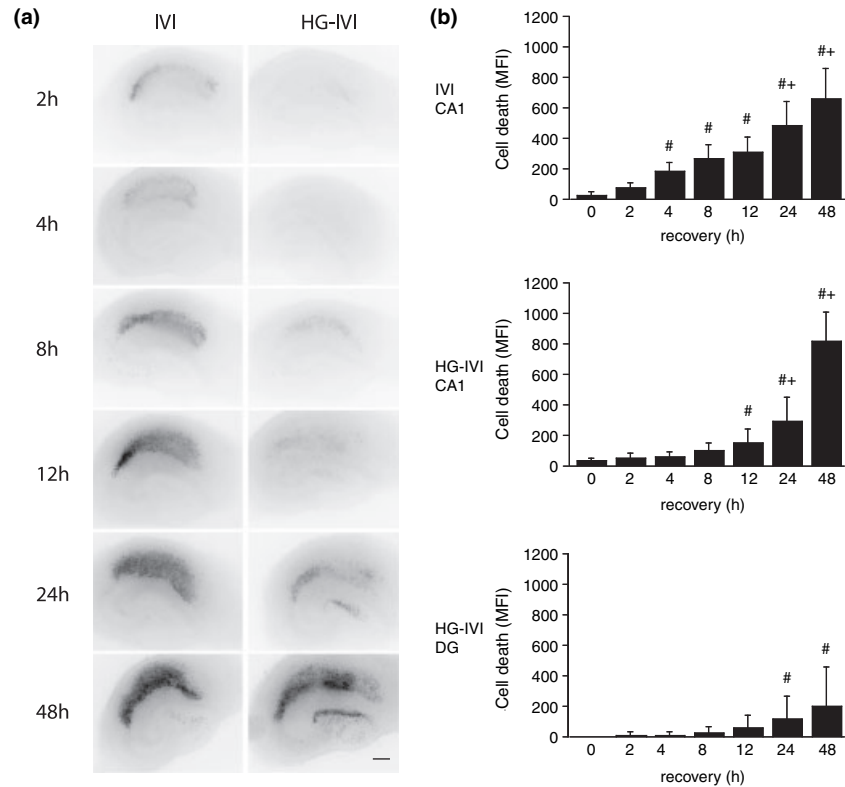


Fig. 2 (a) Inverted fluorescent images of propidium iodide-stained hippocampal slices after 2, 4, 8, 12, 24 and 48 h of recovery following *in vitro* ischemia (IVI) in the absence and presence of 40 mM glucose [hyperglycemic IVI (HG-IVI)]. Scale bar, 200 μ m. (b) Cell death [mean fluorescence intensity (MFI)] following IVI in the CA1 region (upper) and HG-IVI in the CA1 region (middle) and dentate gyrus (DG) (lower). #Significant ($p < 0.05$) differences compared with before the insult (0 time-point). *Significant differences in the same group between two successive time-points ($p < 0.05$), ANOVA with Scheffé's post-hoc test. Data are shown as means \pm SD with $n = 18$ obtained from three independent experiments.

and augmented cell death compared with glucose-free IVI slices (Fig. 2a). Damage was not restricted to the CA1 region but was also seen in the dentate gyrus (Fig. 2b). After 12 h of recovery, damage increased in the CA1 region (Fig. 2b) and continued to increase until 48 h of recovery. At 24 h of recovery cell death was significantly increased in the dentate gyrus (Fig. 2b).

Effect of temperature on cell death following glucose-free *in vitro* ischemia

The temperature dependence of cell death was studied after glucose-free IVI (Fig. 3). In a first series, slices were exposed to 15 min IVI at 31, 35 and 37°C, respectively and the slices were returned to 35°C (Fig. 3, top row). Cell death, mainly found in the CA1 region, was less in the 31°C group compared with groups subjected to IVI at 35 or 37°C (Fig. 3a). After 48 h of recovery cell death was more extensive in cultures exposed to 37°C during IVI. Hence, cell death following IVI increases with increasing temperature during IVI. Next, we investigated the effect of the same temperature during and following IVI. At a temperature of 31°C scattered cell death was found and this increased in the 35 and 37°C groups (Fig. 3b). Control cultures kept at the three temperatures, respectively, for 72 h did not display any distinct PI uptake. Subsequently, the influence of post-IVI temperature was assessed. When cultures were exposed to IVI at 35°C and then placed at 31, 35 or 37°C, respectively during recovery, the cell death was similar in all groups (Fig. 3c).

Effect of temperature on damage following hyperglycemic *in vitro* ischemia

Similar experiments were performed with HG-IVI (Fig. 4). Hippocampal cultures subjected to HG-IVI at 31°C displayed less cell death compared with those exposed to 35 and 37°C during IVI. There was also significantly more cell death after HG-IVI at 37 than at 35°C (Fig. 4a). If the same temperature was applied during and following IVI, no damage was seen in the group kept at 31°C (Fig. 4b). Cell damage was more extensive in the 37°C group compared with the 35°C group. Evidently, damage is less at 31°C during and after IVI compared with 31°C during IVI and recovery at 35°C, indicating an ongoing detrimental temperature-sensitive process during recovery. When slices were subjected to HG-IVI at 35°C and then placed at 31, 35 or 37°C, respectively, cell death increased (Fig. 4c) and there was no difference in cell death among the groups.

Permeability transition in isolated hippocampal mouse mitochondria

We next investigated the MPT in hippocampal mitochondria isolated from 21-day-old pups, i.e. the same age as the slices (De Simoni *et al.* 2003), and the swelling response (% of maximum; mean \pm SD) of isolated mitochondria over time following the addition of calcium in the presence of vehicle (control sample), 2 μ M CSA, 2 μ M NIM811 or 1 μ M FK506 was studied (Fig. 5). Mitochondrial swelling was detected as

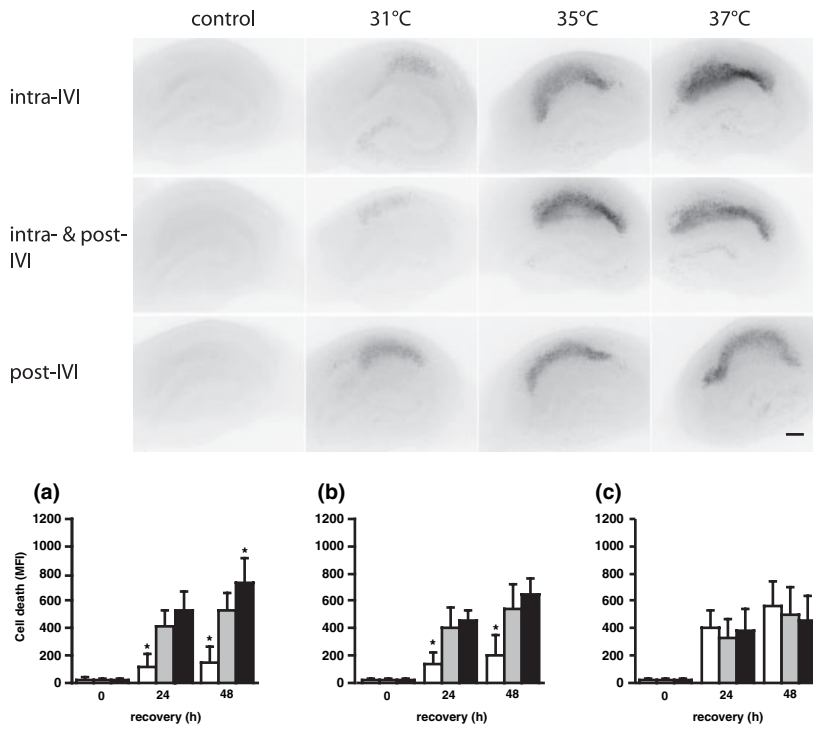


Fig. 3 Representative inverted fluorescent images of propidium iodide (PI)-stained hippocampal slices subjected to *in vitro* ischemia (IVI) and/or recovery at 31, 35 or 37°C. Scale bar, 200 μ m. Cell death [mean PI fluorescence intensity (MFI)] in the CA1 region of (a) cultures exposed to 15 min of IVI at 31°C (□), 35°C (▒) and 37°C (■); (b) cultures exposed to 31°C (□), 35°C (▒) and 37°C (■) during both IVI and recovery and (c) cultures subjected to IVI at 35°C and then placed at 31°C (□), 35°C (▒) and 37°C (■). *Significant ($p < 0.05$) difference compared with the group subjected to IVI and recovery at 35°C at the same time-point. ANOVA with Scheffé's post-hoc test. Data are shown as means \pm SD with $n = 9-30$, obtained from at least three independent experiments.

a distinct decrease in side scattering in the control samples and samples treated with FK506, whereas swelling was robustly inhibited in the samples treated with either CsA or NIM811. No differences in swelling response between

samples treated with CsA and NIM811 or between samples treated with vehicle and FK506 were detected. Thus, both CsA and NIM811 but not FK506 inhibited MPT in hippocampal mitochondria from 21-day-old mice.

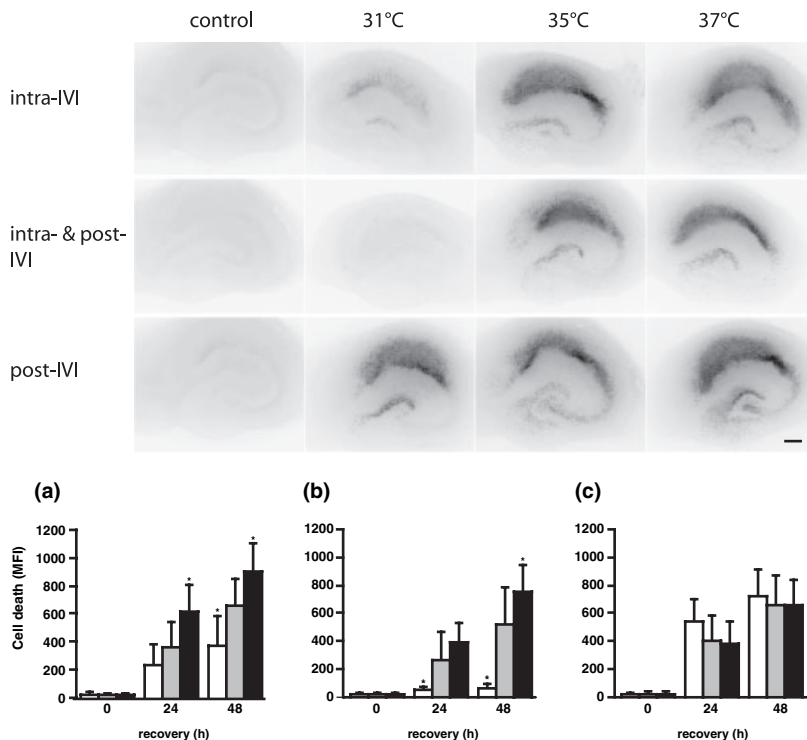


Fig. 4 Representative inverted fluorescent images of propidium iodide-stained hippocampal slices subjected to hyperglycemic *in vitro* ischemia (HG-IVI) and/or recovery at 31, 35 or 37°C. Scale bar, 200 μ m. Cell death [mean fluorescence intensity (MFI)] in the CA1 region of organotypic hippocampal slices (a) subjected to 15 min of HG-IVI at 31°C (□), 35°C (▒) and 37°C (■) and then placed at 35°C; (b) exposed to 31°C (□), 35°C (▒) and 37°C (■) during both HG-IVI and during recovery and (c) subjected to 15 min of HG-IVI at 35°C and then placed in incubators at 31°C (□), 35°C (▒) or 37°C (■). * $p < 0.05$ differences compared with the control group at each time-point (ANOVA with Scheffé's post-hoc test). Results are from at least three different experiments.

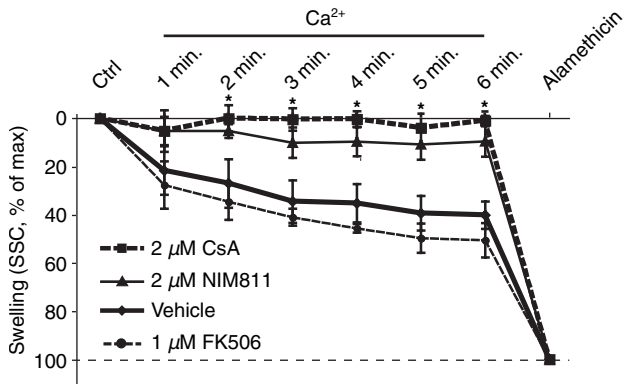


Fig. 5 Flow cytometric detection of mitochondrial permeability transition in isolated hippocampal mitochondria. Mitochondrial swelling detected by side scattering (SSC), % of the maximal, following exposure to calcium under de-energized conditions. The experiments were terminated by addition of alamethicin to induce maximal swelling (100%). *Significant difference in swelling response compared with vehicle. Values are presented as means \pm SD. Data were obtained from three separate experiments. CsA, cyclosporin A; NIM811, Melle⁴-CsA.

Effect of blocking mitochondrial permeability transition on cell death following *in vitro* ischemia and hyperglycemic *in vitro* ischemia

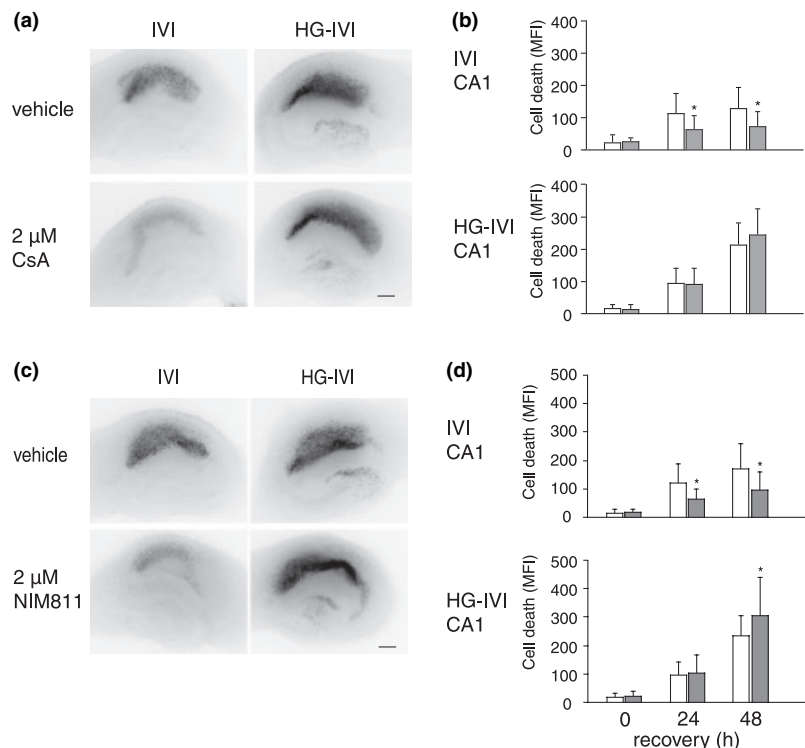
To investigate the involvement of MPT in IVI, CsA (2 μ M) was included in the medium from the day before IVI to the end of the experiment (Fig. 6). CsA significantly diminished

cell death at 24 and 48 h of recovery (Figs 6a and b). In contrast, slices subjected to HG-IVI with and without CsA did not show any difference in cell death. No cell death was seen in control cultures incubated with CsA for 72 h in the CO₂ incubator. Likewise, 2 μ M NIM811 administered to the slices prior to, during and following IVI (Figs 6c and d) reduced cell death but did not affect cell death after HG-IVI. FK506 (1 μ M) slightly aggravated damage following IVI (Fig. 7) but not following HG-IVI. Control slices placed in medium containing FK506, but not subjected to IVI, did not display any damage.

Caspase 3 activation following *in vitro* ischemia and hyperglycemic *in vitro* ischemia

Caspase 3 activation following IVI and HG-IVI was studied using an antibody directed to the active form of caspase 3 (Fig. 8). In control cultures, not exposed to IVI, there was a weak and diffuse background immunostaining (Fig. 9). Cultures stained at 2, 4, 8, 12, 24 and 48 h of recovery after IVI showed no distinct immunostaining in the CA1 region and only a few cells in the dentate gyrus showed clear staining (Fig. 8). In slices subjected to HG-IVI, diffuse staining in the CA1 region was seen after 8 h of recovery that increased at 24 h. In the dentate gyrus there was a distinct staining of cells after 2 h of recovery that increased with time and was maximal at 24 h of recovery. Damage was mostly seen in the dorsal blade of the dentate gyrus. In the dentate gyrus both cell bodies and dendrites were immunostained whereas only cell bodies were stained in the CA1 region

Fig. 6 (a) Inverted fluorescent images of propidium iodide (PI)-stained slices, showing the effect of 2 μ M cyclosporin A (CsA) in slices exposed to *in vitro* ischemia (IVI) and hyperglycemic IVI (HG-IVI) and 48 h of recovery. Scale bar, 200 μ m. (b) Damage in the CA1 region following IVI and HG-IVI. CsA (2 μ M, ■) or the vehicle (dimethylsulfoxide, □) was present in the media from 1 day prior to, during IVI and in the recovery period. (c) Slices subjected to IVI and HG-IVI and 48 h of recovery in 2 μ M Melle⁴-CsA (NIM811). (d) Cell death in the CA1 region following IVI (top graph) and HG-IVI (lower graph). Vehicle (□), 2 μ M NIM811 (■). Data are expressed as means \pm SD, * p < 0.05 compared with the vehicle group at the same time-point, two-way ANOVA with Scheffé's post-hoc test. Data are obtained from three separate experiments, n = 12–16. MFI, mean fluorescence intensity.



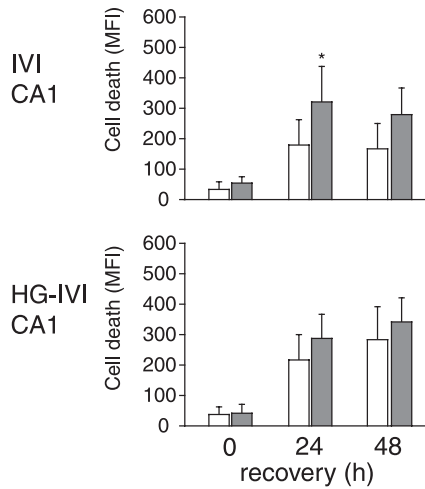


Fig. 7 The effect of FK506 following *in vitro* ischemia (IVI) and hyperglycemic IVI (HG-IVI). Quantification of cell death [mean fluorescence intensity (MFI)] in the CA1 region following IVI and HG-IVI. FK506 or the vehicle (dimethylsulfoxide) was present in the media from 1 day prior to experiment, during IVI and in the recovery period. Vehicle (□), 1 μ M FK506 (■). Data are expressed as means \pm SD, * p < 0.05 compared with the vehicle group at the same time-point, two-way ANOVA with Scheffé's post-hoc test.

(Fig. 9). No specific staining was detected when the primary antibody was excluded.

Effect of caspase inhibition on cell death following *in vitro* ischemia and hyperglycemic *in vitro* ischemia

The caspase inhibitor BAF (100 μ M) was added 1 h before IVI and was present during the recovery phase (Fig. 10). There was no decrease in cell death in the CA1 region in the BAF-treated group compared with the vehicle-treated group. In slices subjected to HG-IVI there was a delay in the progression of cell death. PI uptake did not increase 24 h after HG-IVI but after 48 h cell death was similar to that in the untreated group. In the dentate gyrus of slices exposed to HG-IVI, BAF significantly inhibited damage at both 24 and 48 h of recovery. Control cultures incubated in culture medium containing 100 μ M BAF for 48 h displayed no damage.

Discussion

In this study we show that damage in the CA1 region of organotypic hippocampal slice cultures following both glucose-free IVI and HG-IVI is highly temperature dependent, that cell death following glucose-free IVI but not HG-IVI is markedly decreased by MPT blockade and that inhibition of caspases does not prevent cell death in the CA1 region following glucose-free IVI or HG-IVI.

Following glucose-free IVI, cell death appears by 4 h of recovery, indicating that cell degeneration occurs relatively

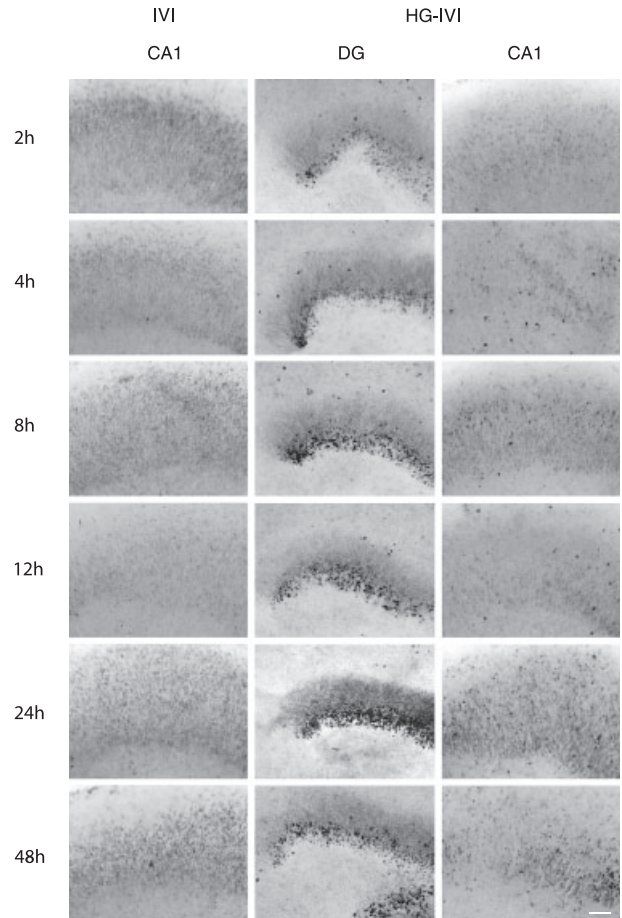


Fig. 8 Photomicrographs of immunoreactivity of the active form caspase 3 at 2, 4, 8, 12, 24 and 48 h of recovery following *in vitro* ischemia (IVI) and hyperglycemic IVI (HG-IVI). The first column shows representative images of the CA1 region following IVI and the next two columns show the CA1 region and dentate gyrus (DG) of slices subjected to HG-IVI. Scale bar, 50 μ m.

fast. The rate of cell death in the CA1 region was clearly delayed when glucose was present during IVI and appeared first after 12 h of recovery. The delayed progression and particular distribution of damage are similar to those found *in vivo* in the mouse hippocampus following global cerebral ischemia (Olsson *et al.* 2003). The results also mimic findings described *in vivo* that hyperglycemia aggravates ischemic damage (Myers and Yamaguchi 1977; Siemkiewicz and Hansen 1978; Pulsinelli *et al.* 1982, 1983). However, *in vivo*, damage develops faster following hyperglycemic ischemia than after normoglycemic ischemia (Smith *et al.* 1988), presumably due to post-ischemic seizures driven by subcortical structures (Inamura *et al.* 1987) not present in isolated hippocampal slices.

Damage following glucose-free IVI in organotypic slice cultures is NMDA receptor and calcium dependent (Rytter *et al.* 2003; Cronberg *et al.* 2005a,b). Calcium overload

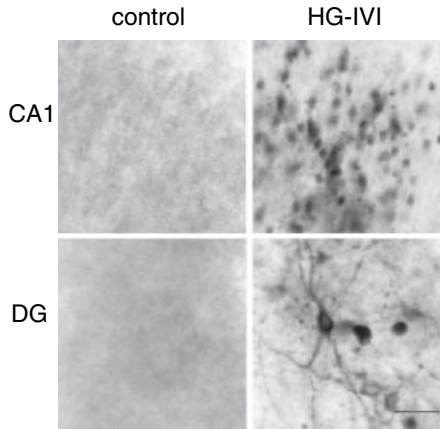


Fig. 9 Photomicrographs of immunoreactivity of the active form of caspase 3 of the CA1 region and dentate gyrus (DG) in cultures subjected to control conditions or hyperglycemic *in vitro* ischemia (HG-IVI). Scale bar, 50 μm .

causes mitochondrial calcium uptake and a subsequent activation of MPT (Halestrap 2005). This clearly occurs in the CA1 cells following IVI as the MPT blocker CsA and NIM118 decrease cell death. These results are in line with observations *in vivo* showing marked protection by the non-immunosuppressive CsA analog MeVal⁴-CsA following focal ischemia (Matsumoto *et al.* 1999). The blockade of calcineurin by FK506 does not provide any protection following IVI, indicating that calcineurin does not contribute to cell death in this model. On the contrary, there is an aggravation of damage by the FK506 treatment, suggesting that calcineurin is required for cell survival following IVI. This is in contrast to findings *in vivo* where FK506 protects against brain damage after middle cerebral artery occlusion in the rat (Sharkey and Butcher 1994). This difference could be explained by the systemic immunosuppressive actions of FK506 preventing the inflammatory response following middle cerebral artery occlusion.

From the available data it appears that the cellular calcium overload that follows NMDA receptor activation during glucose-free IVI activates MPT that disrupts ATP formation and terminates the synthesis of cellular constituents, causing increased cellular degradation and eventually cell death that can be prevented by hypothermia (Fig. 11). The lack of protection by both glutamate receptor blockade and hypothermia when instigated after IVI suggests that the detrimental events taking place during the IVI insult may be harmful to such an extent that although cell death is not seen until 4 h after the insult it is essentially irreversible by the end of IVI.

Following HG-IVI, MPT does not contribute to the cell death process in the CA1 neurons. This is in line with the observation that neither NMDA receptor blockade nor removal of calcium prevents cell death following HG-IVI (Cronberg *et al.* 2005b). In contrast, we show here that

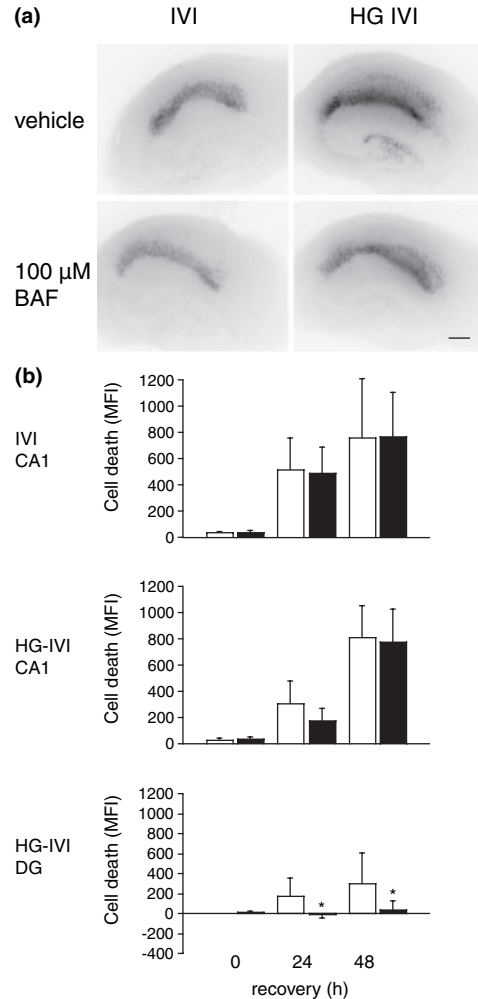


Fig. 10 (a) Representative inverted fluorescence images showing the effect of the pan-caspase inhibitor Boc-Asp(OMe) fluoromethyl ketone (BAF) on cultures subjected to control conditions, *in vitro* ischemia (IVI) or hyperglycemic IVI (HG-IVI) and 48 h of recovery. Scale bar, 200 μm . (b) Cell death [mean fluorescence intensity (MFI)] in the CA1 region of slices subjected to IVI (top) or HG-IVI (middle) and in the dentate gyrus (DG) of hyperglycemic slices (bottom). Slices were treated with BAF (100 μM), present in the medium from 1 h prior to and during IVI and during recovery (■) or subjected to the same treatment with vehicle (□). *Significant differences ($p < 0.05$) between groups at the same time-point. Data are presented as means \pm SD, $n = 18$ or 24 derived from three or four experiments.

changes in temperature have dramatic effects on cell death development. In addition to the neuroprotective effect of decreased temperature during HG-IVI, we found that if the low temperature (31°C) continues during recovery damage is further decreased. This demonstrates that post-IVI processes do indeed contribute to cell death in this experimental paradigm. Such mechanisms could include apoptosis. The rupture of the outer mitochondrial membrane during MPT would release apoptogenic factors and activate the apoptotic cascade including caspase 3. We did not detect an increase in

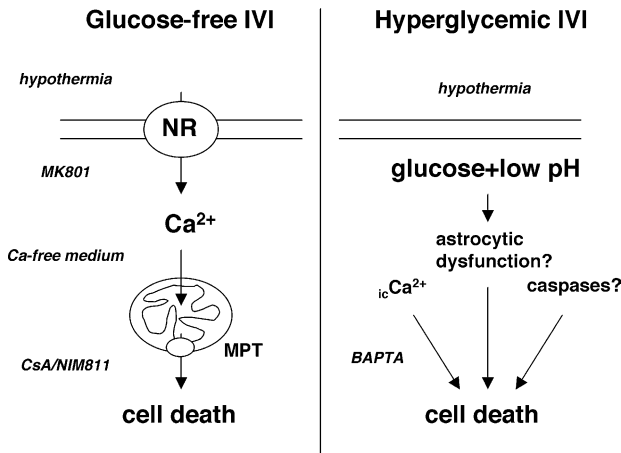


Fig. 11 Tentative cell death mechanisms activated during glucose-free *in vitro* ischemia (IVI) and hyperglycemic IVI, respectively. Words in italics are measures that protect against damage. BAPTA, 1, 2-bis(o-aminophenoxy) ethane-N,N,N¹,N¹-tetraacetic acid; CsA, cyclosporin A; MPT, mitochondrial permeability transition; NIM811, Melle⁴-CsA; NR, NMDA receptor.

activated caspase 3 in any cell types following IVI and the pan-caspase inhibitor BAF was also not protective. Again, this could be due to a rapid loss of ATP production during IVI which precludes the formation of the apoptosome. In a model of oxygen and glucose deprivation using rat hippocampal slices, Ray *et al.* (2000) showed protection by the caspase inhibitor Ac-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-cmk) but the inhibitor had no effect when the oxygen and glucose deprivation period was extended. It is therefore possible that our insult to the CA1 region is too severe to allow apoptosis and caspase 3 activation to occur. During HG-IVI, the presence of glucose will allow some ATP formation and hence the insult is less severe, the apoptosome could be formed and caspase 3 activated. Indeed, after 2 h of recovery there is a clear increase in immunoreactivity for active caspase 3 in some granule cells in the dentate gyrus, which increases until 24 h of recovery. Also, BAF completely abolishes cell death in the dentate gyrus, demonstrating that BAF indeed inhibits caspases in the slice preparation. Interestingly, active caspase 3 immunoreactivity increases at a time-point when cell death defined by the loss of plasma membrane integrity (PI uptake) is not evident until after 24 h of recovery. Similar findings have been reported in the dentate gyrus when hippocampal slices were exposed to colchicine (Kristensen *et al.* 2003). The activation of caspase 3 in the CA1 region following HG-IVI is less obvious.

In conclusion, the present data complement our earlier data suggesting that two distinct cell death mechanisms are induced during glucose-free and HG-IVI, respectively (Fig. 11). NMDA receptor activation, cellular calcium overload (Choi 1992) and activation of the MPT (Crompton

1999) contribute to the cell death process following glucose-free IVI. Damage following HG-IVI is dependent on glucose and acidosis, possibly involving astrocytic dysfunction. It does not involve glutamate toxicity or MPT activation but may have an apoptotic component. Further elucidation of the mechanisms of cell death following HG-IVI may provide novel neuroprotective therapies against ischemic brain damage.

Acknowledgements

The authors thank Blanka Boberg for excellent technical assistance. This work was supported by the Swedish Medical Research Council (grant 8644), Bergendahl Foundation, Royal Physiographic Society, Thorsten and Elsa Segerfalk Foundation, Laerdal Foundation and The Swedish Brain Foundation. CMPC was sponsored by a FEBS Fellowship.

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