Pergamon

PII: S0306-4522(99)00292-4

Neuroscience Vol. 93, No. 4, pp. 1219–1222, 1999

0306-4522/99 $20.00+0.00

Letter to Neuroscience

INHIBITION OF CASPASES PREVENTS CELL DEATH OF HIPPOCAMPAL CA1 NEURONS, BUT NOT IMPAIRMENT OF HIPPOCAMPAL LONG-TERM POTENTIATION FOLLOWING GLOBAL ISCHEMIA

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Key words: apoptosis, cerebral ischemia, Z-DEVD-FMK, LTP.

An essential role for caspases in programmed neuronal cell death has been demonstrated in various *in vitro* studies, and synthetic caspase inhibitors have recently been shown to prevent neuronal cell loss in animal models of focal cerebral ischemia and traumatic brain injury, respectively. The therapeutic utility of caspase inhibitors, however, will depend on preservation of both structural and functional integrity of neurons under stressful conditions. The present study demonstrates that expression and proteolytic activity of caspase-3 is up-regulated in the rat hippocampus after transient forebrain ischemia. Continuous i.c.v. infusion of the caspase inhibitor *N*-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethyl ketone significantly attenuated caspase-3-like enzymatic activity, and blocked delayed cell loss of hippocampal CA1 neurons after ischemia. Administration of *N*-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethyl ketone, however, did not prevent impairment of induction of long-term potentiation in post-ischemic CA1 cells, suggesting that caspase inhibition alone does not preserve neuronal functional plasticity. © 1999 IBRO. Published by Elsevier Science Ltd.

An involvement of caspases in neuronal cell death following growth factor deprivation or glutamate exposure *in vitro* has been demonstrated using specific inhibitors like cowpox virus crmA protein or synthetic peptide methyl ketones.[1,5,15] More importantly, synthetic caspase inhibitors recently showed neuroprotective effectiveness in animal models of focal cerebral ischemia and traumatic brain injury, indicating a potential therapeutic utility of caspase inhibitors in the treatment of neurodegenerative diseases.[8,17] After transient global ischemia in adult rats, we have demonstrated an increase in caspase-3 mRNA expression in vulnerable hippocampal CA1 neurons and a concomitant increase in caspase-3-like proteolytic activity in hippocampal protein extracts, suggesting that caspases may also be involved in delayed CA1 cell death after cerebral ischemia.[7] Using histochemical and electrophysiological analysis, we have therefore investigated whether continuous i.c.v. infusion of the synthetic caspase inhibitor *N*-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethyl ketone (Z-DEVD-FMK) can prevent neuronal cell loss and functional disturbances in the post-ischemic hippocampus.[6,9,10] To assess whether different expression levels of endogenous caspase inhibitors might contribute to selective neuronal vulnerability, we analysed mRNA expression of the X-linked inhibitor of apoptosis (XIAP), which inhibits cell death *in vitro* by specific binding to active caspase-3, thereby blocking proteolytic activity.[2,4,16] Our findings indicate that ischemia-sensitive CA1 neurons can be rescued by synthetic caspase inhibitors, but do not regain fully intact synaptic function.

Male Wistar rats (300–350 g; from own breeding colony) were subjected to 15 min of four-vessel occlusion with hypotension, as described.[6] This treatment reproducibly causes 80–100% hippocampal CA1 cell loss within one week. Only animals showing isoelectric electroencephalogram during four-vessel occlusion were included in the study. Immediately following re-circulation, a cannula device connected to a subcutaneously implanted osmotic minipump (Alzet model 1007D) was inserted in the lateral ventricle. Z-DEVD-FMK (Calbiochem), which shows some selectivity for inhibition of caspase-3-like proteases,[15] was delivered i.c.v. at a rate of 50 pmol/h for seven days. Control groups included animals receiving i.c.v. infusions of vehicle (artificial cerebrospinal fluid, ACSF) following ischemia, as well as animals receiving i.c.v. infusions of either Z-DEVD-FMK or *N*-benzyloxycarbonyl-Phe-Ala-fluoromethyl ketone (Calbiochem) after sham operation. Animals were anesthetized (1% halothane in nitrous oxide/oxygen) throughout surgical treatment and core temperature was maintained at 37°C up to 30 min after ischemia. Caspase-3-like proteolytic activity was assayed in extracts from microdissected hippocampal tissue using the ApoAlert CPP32 Fluorescent Assay Kit (Clontech), as described.[7]

Densitometric analysis of *in situ* hybridization

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Abbreviations: ACSF, artificial cerebrospinal fluid; fEPSP, field excitatory postsynaptic potential; LTP, long-term potentiation; TUNEL, terminal deoxynucleotidyl transferase-mediated biotin–dUTP nick end labeling; XIAP, X-linked inhibitor of apoptosis; Z-DEVD-FMK, *N*-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethyl ketone.

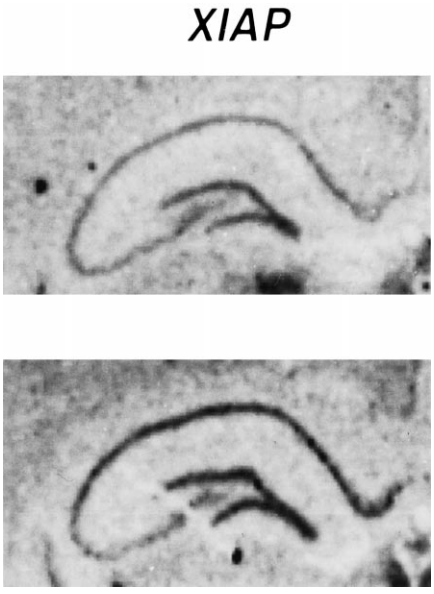


Fig. 1. Representative *in situ* hybridization autoradiograms exhibiting *XIAP* mRNA expression in the rat hippocampus 24 h after either sham treatment (upper panel) or transient forebrain ischemia (lower panel). *In situ* hybridization was performed in brain cryosections (16 μm) using 3′-[^{35}S]dATP-labeled oligonucleotide probes corresponding to the mouse *XIAP* gene (bases 1112–1166), as described in detail elsewhere.[7,16]

autoradiograms revealed a significant ($P < 0.05$, Mann–Whitney *U*-test) increase in caspase-3 mRNA levels by 185 ± 12% selectively in the hippocampal CA1 cell layer of the hippocampus at 24 h after transient four-vessel occlusion, similar to our findings in cardiac arrest and resuscitation.[7] *XIAP* mRNA was constitutively expressed at low levels in the granule cell layer of the dentate gyrus and the pyramidal cell layer of the hippocampus. Expression of *XIAP* mRNA in granule cells and CA1 neurons tended towards an increase at 24 h following transient forebrain ischemia which, however, did not reach statistical significance ($P > 0.05$, Mann–Whitney *U*-test; Fig. 1). Caspase-3-like immunoreactivity was not detectable in sections from control brains. Twenty-four hours after transient ischemia, however, distinct caspase-3 immunolabeling became apparent in the cytoplasm of CA1 hippocampal neurons. Caspase-3-like immunoreactive material displayed a granular distribution in neuronal cell bodies and proximal axonal processes (Fig. 2). Twenty-four hours after ischemia and subsequent i.c.v. infusion of ACSF, caspase-3-like proteolytic activity in hippocampal extracts increased to 206 ± 10% (mean ± S.D., $n = 5$) of non-ischemic control tissue. Infusion of Z-DEVD-FMK significantly ($P < 0.05$, Mann–Whitney *U*-test) reduced ischemia-induced caspase-3 activation in the ipsilateral hippocampus to 133 ± 21% (mean ± S.D., $n = 5$) of control values.

Seven days after transient cerebral ischemia, numerous hippocampal neurons exhibiting nuclear DNA fragmentation were detected in the CA1 region of vehicle-treated animals (all of five animals; Fig. 3). Continuous infusion of the caspase inhibitor Z-DEVD-FMK completely prevented DNA cleavage in CA1 cells of the ipsilateral hippocampus (all of five animals), whereas numerous terminal deoxynucleotidyl transferase-mediated biotin–16-dUTP nick end labeling (TUNEL)-positive CA1 neurons were still detectable on the contralateral side (Fig. 3). Cell counts in Cresyl Violet-stained sections revealed 87 ± 15% CA1 cell loss in vehicle-treated hippocampi versus 12 ± 5% in Z-DEVD-FMK-treated hippocampi (mean ± S.D., $n = 5$ each).

To assess whether CA1 neurons, which appear morphologically intact, are also functionally preserved, synaptic responses (field excitatory postsynaptic potentials, fEPSPs) were recorded in the CA1 sector of the ipsilateral hippocampus 14 days after ischemia. In sham-operated, ACSF-treated animals, brief bursts of high-frequency stimulation elicited a sustained enhancement of synaptic responses, indicating an intact capacity for long-term potentiation (LTP) (Fig. 4). By contrast, in ischemic, ACSF-treated animals, tetanic stimulation failed to evoke hippocampal LTP. Importantly, administration of Z-DEVD-FMK after ischemia resulted in a tendency to increase fEPSP slopes after tetanic stimulation, but without recovering LTP. Induction of LTP was not affected by i.c.v. infusion of Z-DEVD-FMK in sham-operated rats (Fig. 4), suggesting that caspase inhibition *per se* does not impair synaptic function. Additionally, administration of *N*-benzyloxycarbonyl-Phe-Ala-fluoromethyl ketone, a cathepsin B inhibitor, which has been used as a control compound in similar studies, had no effect on LTP, thus excluding toxic side-effects of the fluoromethyl ketone moiety (data not shown).[8]

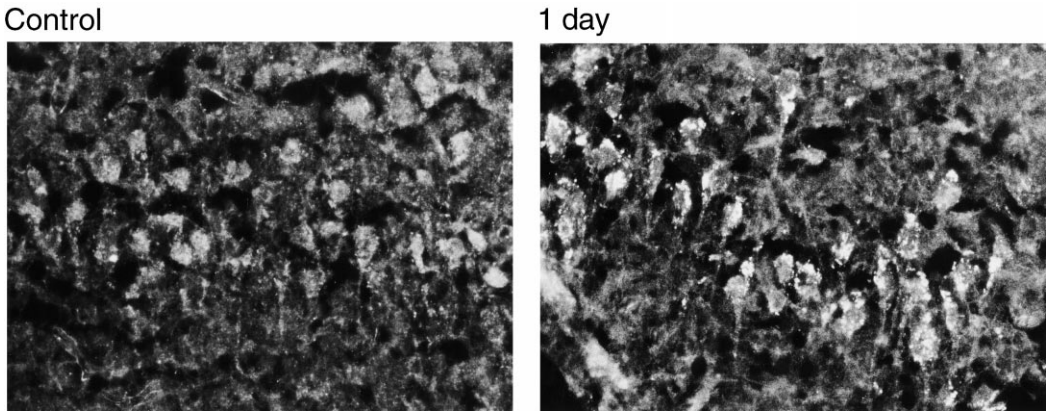


Fig. 2. Immunolocalization of caspase-3 in hippocampal CA1 neurons of a control rat brain (left panel) and of a brain one day after transient ischemia (right panel). Granular caspase-3-like immunoreactivity becomes apparent in the cytoplasm of post-ischemic CA1 neurons. Immunostaining was performed in brain cryosections using a polyclonal rabbit anti-caspase-3 antibody (1:500; Pharmingen) followed by biotinylated secondary antibody and streptavidin–Cychrome-3 conjugate (Vector Labs), as published elsewhere.[11] The anti-caspase-3 antibody reacts exclusively with the 32,000 mol. wt caspase-3 proenzyme and the 17,000 mol. wt active subunit in immunoblot analysis.[11]

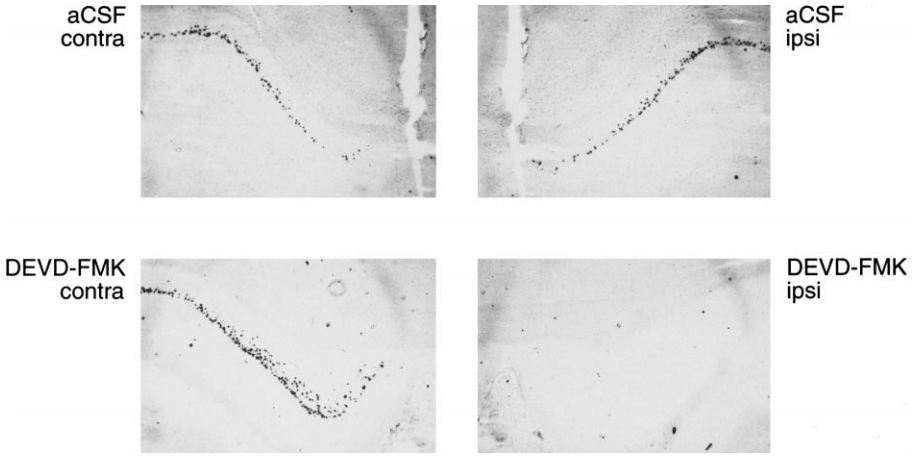
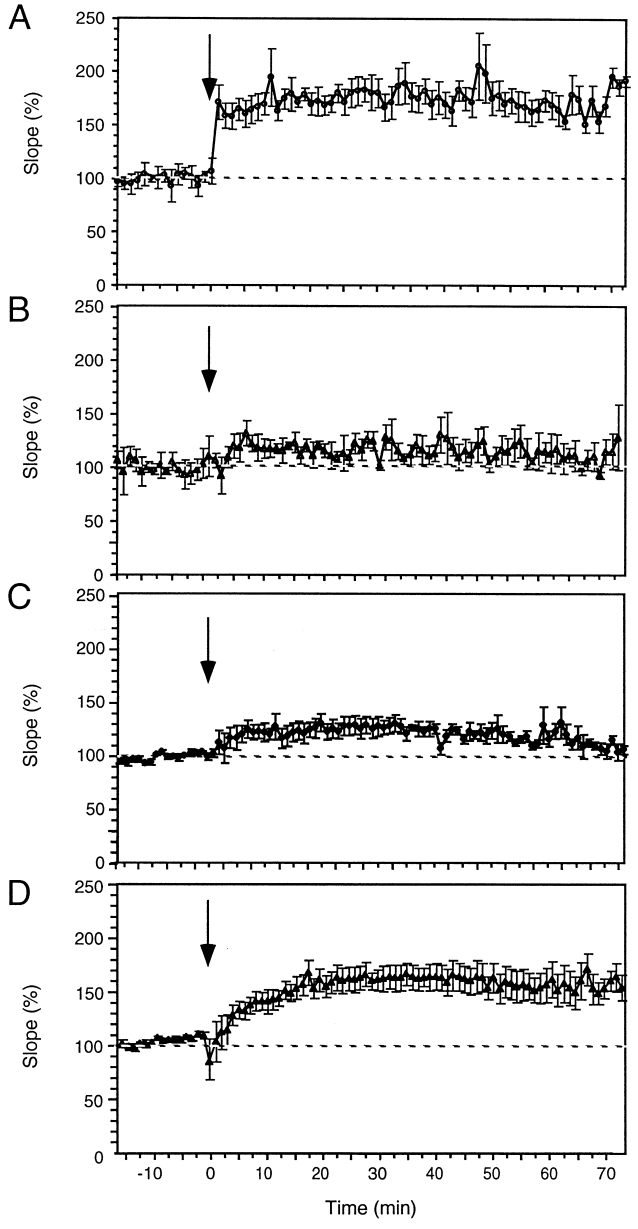


Fig. 3. Detection of nuclear DNA fragmentation in hippocampal CA1 neurons seven days after transient forebrain ischemia. Animals received i.c.v. infusion of either ACSF or Z-DEVD-FMK into the right (ipsi)lateral ventricle. DNA cleavage is completely prevented in the ipsilateral hippocampus of Z-DEVD-FMK-treated rats. DNA fragments were identified in brain cryosections by *in situ* end-labeling using terminal deoxynucleotidyl transferase and biotin-16-dUTP as substrate (TUNEL).[7]



Histological examination of Cresyl Violet-stained brain sections after electrophysiological recordings revealed morphological preservation of hippocampal CA1 neurons by i.c.v. Z-DEVD-FMK infusion up to day 14 after ischemia (Table 1).

The present study indicates an activation of caspase-3 selectively in vulnerable hippocampal CA1 neurons 24 h after transient four-vessel occlusion. *XIAP*, an endogenous inhibitor of caspase-3, was expressed at similar levels in ischemia-resistant granule cells of the dentate gyrus and in vulnerable CA1 neurons, and therefore does not seem to contribute to selective neuronal vulnerability.

More importantly, i.c.v. infusion of the synthetic caspase inhibitor Z-DEVD-FMK demonstrated neuroprotective effectiveness, as indicated by the complete inhibition of nuclear DNA fragmentation in post-ischemic CA1 neurons. There is some evidence that caspase-3-mediated cell death can be executed in the absence of DNA fragmentation.[14] In our study, however, TUNEL-negative CA1 neurons in

Fig. 4. Effect of i.c.v. infusion of Z-DEVD-FMK on ischemia-induced impairment of hippocampal LTP.[9,13] The mean slope of fEPSPs are shown from sham-operated, vehicle-treated rats (A), ischemic, vehicle-treated rats (B), ischemic, Z-DEVD-FMK-treated rats (C) and sham-operated, Z-DEVD-FMK-treated rats (D) (mean ± S.D., $n = 5–6$ per group). The arrows indicate the time-point of tetanic stimulation. High-frequency stimulation evoked hippocampal LTP in A and D. Animals were anesthetized 14 days after transient forebrain ischemia with pentobarbitone (40 mg/kg, i.p.). This time-point has been chosen to exclude any interference from Z-DEVD-FMK with excitatory synaptic transmission, although Z-DEVD-FMK does not appear to block ionotropic glutamate receptors in cultured neurons.[5] A tungsten recording microelectrode (A-M System, Everett, WA, U.S.A.) with an impedance between 3 and 5 MΩ was aimed at 5 mm posterior and 3.5 mm lateral to bregma. The concentric bipolar stimulation electrode (tip separation of 0.5 mm) was lowered into the Schaffer collateral pathway in the hippocampal CA3 region at 3 mm posterior to bregma and 1.5 mm lateral at a depth of 2.5 mm. The recording electrode was initially placed at the depth where spontaneous firing of CA1 pyramidal cells was best recorded. The population responses to stimulation of Schaffer collaterals were then recorded. Test stimuli consisted of cathodal square-wave single pulses of 0.1 ms duration and 2–5 mV amplitude given at 60-s intervals. The depth of the recording electrode was adjusted in order to obtain the largest positive fEPSP. LTP was induced by tetanic stimulation (four trains of 1 s duration, each consisting of 100 Hz of 2–5 mV amplitude, separated by 10 s) after at least 20 min of stable baseline recordings.

Table 1. Quantitative analysis of ischemia-induced impairment of hippocampal long-term potentiation and effect of i.c.v. infusion of Z-DEVD-FMK

Treatment	*n*	30 min	60 min
Sham operation/vehicle	6	172 ± 13	158 ± 14
Ischemia/vehicle	5	104 ± 4	109 ± 8
Ischemia/Z-DEVD-FMK	5	129 ± 10	120 ± 5
Sham operation/Z-DEVD-FMK	6	157 ± 10	155 ± 16

The mean fEPSP slopes were determined after various treatments.[10] The increase of slope (mean ± S.E.M.) at 30 and 60 min after tetanic stimulation compared to baseline before tetanus is given as a percentage. Statistical analysis was performed on pooled data from the 30- and 60-min time-points, respectively. Transient forebrain ischemia significantly reduced LTP at both time-points ($P < 0.01$, Mann–Whitney *U*-test). Post-ischemic administration of Z-DEVD-FMK attenuated LTP reduction which, however, did not reach statistical significance ($P > 0.05$). Moreover, Z-DEVD-FMK did not impair induction of LTP in sham-operated rats.

the Z-DEVD-FMK-treated hippocampus also appear morphologically intact as assessed by Cresyl Violet staining. Lack of effectiveness on the contralateral side may be explained by a slow accumulation of the inhibitor in CA1 cells after unilateral infusion due to the delay in minipump function and the rapid increase in caspase-3-like enzymatic activity after ischemia.[7] Furthermore, lateralized neuroprotection in our study is evidence against an effect due to systemic thermoregulatory or hemodynamic alterations. In two recent studies, i.c.v. injection of Z-DEVD-FMK reduced neuronal cell death after transient focal cerebral ischemia and fluid-percussion-induced traumatic brain injury, respectively.[8,17] Additionally, Z-DEVD-FMK-treated animals showed an acceleration in neurological recovery as assessed by different motor tests. In our study, however, electrophysiological examination of hippocampal LTP, which has been implicated in memory formation, clearly indicated that post-ischemic CA1 neurons, while rescued from degeneration by Z-DEVD-FMK, remain dysfunctional in synaptic plasticity. Similarly, block of neuronal cell death by caspase inhibitors *in vitro* does not prevent neuronal atrophy, neurite fragmentation and disturbances in protein synthesis.[3] In contrast to Z-DEVD-FMK, i.c.v. infusion of leupeptin, a non-specific inhibitor of cysteine and serine proteases, protected against both degeneration of CA1 neurons and impairment of LTP in the CA1 sector following transient cerebral ischemia in gerbils.[12] In this study, however, drug infusion was started three days before induction of ischemia, which may not be feasible in ischemic insults.

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(*Accepted* 25 *May* 1999)