Extended therapeutic window for caspase inhibition and synergy with MK-801 in the treatment of cerebral histotoxic hypoxia

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Abstract

In rats, striatal histotoxic hypoxic lesions produced by the mitochondrial toxin malonate resemble those of focal cerebral ischemia. Intrastratial injections of malonate induced cleavage of caspase-2 beginning at 6 h, and caspase-3-like activity as identified by DEVD biotin affinity-labeling within 12 h. DEVD affinity-labeling was prevented and lesion volume reduced in transgenic mice overexpressing BCL-2 in neuronal cells. Intrastratial injection of the tripeptide, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk), a caspase inhibitor, at 3 h, 6 h, or 9 h after malonate injections reduced the lesion volume produced by malonate. A combination of pretreatment with the NMDA antagonist, dizocilpine (MK-801), and delayed treatment with zVAD-fmk provided synergistic protection compared with either treatment alone and extended the therapeutic window for caspase inhibition to 12 h. Treatment with cycloheximide and zVAD-fmk, but not with MK-801, blocked the malonate-induced cleavage of caspase-2. NMDA injections alone resulted in a weak caspase-2 cleavage. These results suggest that malonate toxicity induces neuronal death by more than one pathway. They strongly implicate early excitotoxicity and delayed caspase activation in neuronal loss after focal ischemic lesions and offer a new strategy for the treatment of stroke.

Keywords: Caspases; malonate; apoptosis; excitotoxicity; therapeutic window; Nedd2

Abbreviations: DMSO, dimethyl-sulfoxide; MK-801, dizocilpine; NMDA, N-methyl-D-aspartate; PFA, paraformaldehyde; TTC, 2,3,5-triphenyl-tetrazolium chloride monohydrate; TUNEL, terminal transferase dUTP nick end-labeling; zVAD-fmk, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

Introduction

Excitotoxic and apoptotic cell death may contribute to neuronal loss in human neurodegenerative diseases and cerebral ischemia. Depending on the severity of the insult, excitotoxic cell death may be either necrotic or apoptotic (Ankarcrona *et al*, 1995; Bonfoco *et al*, 1995). Evidence that ischemic cell death involves apoptosis comes from *in situ* DNA end-labeling (TUNEL) and laddering on DNA gel electrophoresis in global and focal experimental ischemia (Choi, 1996). Ultrastructural morphological evidence for apoptosis in ischemic neuronal tissue is limited (Li *et al*, 1995b) or lacking (Campagne and Gill, 1996).

Studies in *Caenorhabditis elegans* have led to the identification of three cell death genes, *ced-3*, *ced-4*, and *ced-9*. Mammalian homologs of *ced-9* and *ced-3* genes include proteins of the BCL-2 and caspase (formerly called ICE-like protease) families (Alnemri *et al*, 1996). A dominant role for caspase-3 in neuronal apoptosis has been suggested in studies using transgenic animals. The nervous system of caspase-1-deficient mice develops normally (Li *et al*, 1995a), but caspase-3-deficient mice show decreased apoptosis primarily in neurons (Kuida *et al*, 1996). Yet, most human central nervous system neurons exhibit little or no caspase-3 immunoreactivity except in ischemic degeneration (Krajewska *et al*, 1997). Overexpression of BCL-2 in neurons (Martinou *et al*, 1994) protects against ischemia. Repeated intracerebroventricular administration of an irreversible caspase inhibitor, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) (Hara *et al*, 1997), or transgenic expression of a mutant caspase-1 inhibitory protein, reduces damage in focal cerebral ischemia (Friedlander *et al*, 1997).

Malonate is a reversible succinate dehydrogenase inhibitor which produces secondary excitotoxic lesions similar to those of focal ischemia when administered intrastratially. As in the penumbral region of focal ischemic lesions, malonate lesions are associated with metabolic compromise, sparing of NADPH diaphorase-positive neurons and somatostatin concentrations, and are attenuated by NMDA antagonists, glutamate release inhibitors, free radical scavengers, and inhibitors of neuronal nitric oxide synthase (Beal *et al*, 1993; Greene *et al*, 1993; Henshaw *et al*, 1994; Schulz *et al*, 1995a,b). In the present study, we show that delayed treatment with the caspase inhibitor

zVAD-fmk or overexpression of BCL-2 protects against malonate-induced lesions. We present a new therapeutic strategy for the treatment of stroke that is based on the synergistic combination of zVAD-fmk and MK-801.

Results

Malonate-induced neuronal cell death: insignificant role of apoptosis but attenuation by BCL-2

The first series of experiments was designed to characterize the type of neuronal cell death induced by malonate. Brain sections were stained to detect TUNEL-positive cells at 24 h after striatal injection of 3 μ mol malonate in rats. A limited number of TUNEL-positive cells with signs of chromatin condensation or fragmentation were detected in the transition zone but not in the core of the lesion. These accounted for only 2% of cells in the striatum. (Figure 1A). When the same brain areas were analyzed by electron microscopy, we observed numerous neurons with an increased density of cytoplasm, damaged membranes, swollen organelles and irregularly shaped nuclei with only slightly condensed heterochromatin and intact nucleolus (Figure 1B and C). Ultrastructural examination therefore did not show typical morphological features of apoptosis. Next, we asked whether overexpression of BCL-2 protected against striatal lesions produced by malonate. The lesion volume induced by striatal injection of 750 nmol malonate was significantly reduced in bcl-2 transgenic mice as compared with the lesion volume in littermates when measured at 7 days (Figure 2). Thus, malonate-induced neurotoxicity is a non-apoptotic type of cell death that is inhibited by the classical antiapoptotic gene product, BCL-2.

Bcl-2-sensitive activation of caspases by malonate

Prevention of apoptosis by bcl-2 involves, amongst others, inhibition of activation of caspases (Armstrong *et al*, 1996; Schulz *et al*, 1997). Therefore, we looked for evidence of caspase activation in malonate-lesioned brain tissue. Caspase activation requires partial proteolytic cleavage of full length procaspases (32–51 kDa) into active subunits. Caspase-1 (ICE) rather specifically cleaves YVAD whereas caspase-3-like proteases cleave DEVD. Labeling of striatal extracts with biotinylated DEVD-CHO revealed the induction of a ~20 kDa fragment (p20) at 12 and 24 h, but not at earlier time points, after striatal malonate injections in rats (Figure 3A, upper panel). p20 was not detected in the contralateral hemisphere that received saline control injections. The fragment corresponds to the larger subunit of an activated caspase family member. No time-dependent increase in labeling was observed with biotinylated YVAD-CHO at the same time points in rats (Figure 3A, lower panel). Therefore, our results suggest that caspase-3-like proteases but not caspase-1, are activated in the process of malonate toxicity. At 24 h after malonate injection, a p20 fragment labeled with biotinylated DEVD-CHO was also detected in the striatum of mice (Figure 3B). This fragment was not detected in littermates engineered to express high levels of BCL-2 in

neurons, suggesting that BCL-2 acts upstream from caspases *in vivo*.

Malonate induces caspase-2 activation

To identify the specific caspase which is cleaved after striatal malonate injections we used several antibodies (see Materials and Methods) against caspase-1, -2, and -3. Although all antibodies used recognized the respective procaspase (data not shown), only the Nedd-2 antibody directed against the N-terminus of Nedd-2 recognized a cleaved fragment of ~24 kDa (Figure 4A). This fragment first appeared with a weak signal at 6 h, and with a stronger signal at 9, 12, and 24 h. This fragment was also detected at 24 h after malonate lesions in wild-type mice. Cleavage was not detected in bcl-2 transgenic littermates (data not shown). Further, intraperitoneal treatment of animals with cycloheximide (10 mg/kg) at the timepoint of striatal malonate injection, or striatal co-injection of zVAD-fmk blocked the activation of the ~24 kDa fragment. No such effects were seen after striatal co-injection of DEVD-CHO or systemic treatment with MK-801 (5 mg/kg) at the time of striatal malonate injections (Figure 4B). Compared with malonate, striatal injection of 300 nmol NMDA which induce a comparable lesion size (Schulz *et al*, 1995a), only induced a weak signal of the activated 24 kDa fragment at 12 h (Figure 4C).

Time course of malonate histopathology

Next, we studied the temporal profile of malonate histopathology to correlate the development of malonate-induced neuronal death with the time course of caspase activation. There was a gradual increase of the lesion volume from 3–24 h (Figure 5 and 6). At 6, 9 and 12 h the brain region involved could be separated into two different areas. The core of the lesion presented as infarction (a region of parenchymal pan-necrosis due to death of astrocytes as well as neurons). At 6, 9 and 12 h a transition zone was apparent next to the infarcted area in which some neurons appeared morphologically affected as red neurons but were still viable. In this zone, there also was a certain degree of myelin pallor. At 12 and 24 h after malonate injection, this transition zone was found to be included in the demarcated lesion area. At 7 days there was a massive glial response with proliferation of GFAP-positive astrocytes (not shown) and microglia. Thus, the onset of caspase activation coincides with the development of a transition zone and precedes the development of the final infarction zone.

zVAD-fmk and MK-801 attenuate malonate-induced striatal lesions

Next, we sought to clarify whether the activation of caspases played a role in malonate-induced lesion formation. We examined whether pharmacological inhibitors of caspases reduced malonate-induced lesion volumes. Intrastriatal injection of 200 μ g zVAD-fmk (~400 μ mol dissolved in 2 μ l DMSO) 1 h before intrastriatal injection of malonate, via chronically implanted guide cannulas, significantly attenuated the lesion volume as compared with DMSO-treated controls

(Figure 7A). Intrastriatal control injections of 2 μ l DMSO alone did not cause any damage (data not shown). Pretreatment with zVAD-fmk also decreased the number of TUNEL-positive neurons at 24 h after malonate injection (Figure 7B). The slightly larger lesion volume produced by 3 μ mol malonate in vehicle-pretreated animals in this study, as compared with

earlier reports (Henshaw *et al*, 1994; Schulz *et al*, 1995a,b,c), is most likely due to the increased penetrance of malonate into neurons after pretreatment with DMSO. In subsequent experiments (Figure 8 and 9), only 1 μ g zVAD-fmk dissolved in 2 μ l of 1% DMSO was injected into the striatum which was as effective as the higher dose.

Figure 1 Morphology of cell death induced by malonate. (A) Intrastriatal malonate injections induce DNA breaks in a minority of neurons. Rat brain striatal sections were studied by *in situ* DNA end labeling at 24 h after malonate-induced striatal lesions. In the transition zone, only few cells are labeled and show margination, condensation and fragmentation of chromatin (arrows). (B) Electron microscopy in the transition zone of malonate-induced striatal lesions at 24 h shows numerous cells with increased density of cytoplasm, damaged membranes, swollen organelles and irregularly shaped nucleus with only slightly condensed heterochromatin and intact nucleolus, compared with (C) the contralateral, unaffected striatum

Since the activation of caspases was not detectable earlier than 6 h after striatal malonate injection, we examined whether delayed treatment with zVAD-fmk also

Figure 2 Bcl-2-transgenic mice are protected from malonate neurotoxicity. Transgenic BCL-2 overexpressing mice were injected with 0.75 μ mol of malonate and lesion volumes assessed by cresyl violet staining at 7 days (mean $\pm$ S.E.M., $n=12$, * $P<0.05$)

Figure 3 Activation of BCL-2-sensitive caspases in striatal malonate lesions. (A) Time course of $\sim$ p20 induction as detected by immunoblot analysis of striatal extracts labeled with biotinylated DEVD-CHO. There is no induction of a subunit labeled with biotinylated YVAD-CHO. (B) The induction of a $\sim$ p20 fragment is blocked (top) in mice with forced neuronal expression of BCL-2 (bottom). p20 was detected as in (A), upper panel. BCL-2 protein was detected by immunoblot analysis. Note that the transgene-derived BCL-2 protein migrates somewhat faster than the endogenous BCL-2 protein

provided protection. We previously showed that the glutamate release inhibitor, lamotrigine, and the NMDA antagonist, MK-801, exert neuroprotective effects against malonate toxicity when administered within 1 and 2 h, respectively (Schulz *et al*, 1995b). Treatment with 1 μ g zVAD-fmk attenuated the lesion volume produced by malonate when injected at 3, 6 and 9 h but not at 12 h after malonate injections (Figure 8A). Interestingly, treatment at 3 h was not superior to treatment at 9 h. Treatment with 5 mg/kg MK-801 i.p. 1 h before malonate injection was similarly effective, whereas treatment with MK-801 at 3 h after malonate injection was not protective (Figure 8B). The combination of pretreatment with MK-801 and posttreatment with zVAD-fmk at 3, 6 and 12 h was superior to either treatment alone (Figure 9). In the presence of MK-801, posttreatment with zVAD-fmk was still effective at 12 h. Moreover, following MK-801 pretreatment, zVAD-fmk administration at 12 h was almost as effective in terms of neuroprotection as administration at 3 h. According to the

Figure 4 Malonate-induced cleavage of caspase-2 (NEDD2). (A) Time course of $\sim$ p24 cleavage as detected by immunoblot analysis of striatal extracts using an anti caspase-2 N-terminal specific antibody. (B) Effects on malonate-induced caspase-2 cleavage by intraperitoneal treatment with cycloheximide (10 mg/kg) or MK-801 (5 mg/kg), or striatal co-administration of zVAD-fmk or DEVD-CHO. (C) At 12 h after striatal injection, NMDA leads only to a weak signal of caspase-2 cleavage

fractional product method (see Methods), the observed effect moderately exceeded the predicted independent effects of combination therapy, suggesting that these treatments act in an additive manner to provide neuroprotection, or even in synergy.

Putative synergistic effects of drugs can be studied best when subthreshold dosages of these therapeutics are combined, which itself have no or only a weak beneficial effect (Greco *et al*, 1995). Therefore, we first studied the dose response curves for systemic treatment of MK-801

Figure 5 Time course of malonate histopathology. Representative micrographs show cresyl-violet stained sections of the striatum at 3 h (A), 6 h (B), 9 h (C), 12 h (D), 24 h (E) and 7 days (F) after striatal injections of 3 μ mol malonate. The core of the lesion presented as infarction and was surrounded by a transition zone. In the transition zone, there was a certain degree of myelin pallor and some neurons appeared morphologically affected but most neurons were still viable

Figure 6 Temporal profile of malonate-induced lesion volumes. The lesion volumes were separated into an area of complete infarction and a transition zone (mean $\pm$ S.E.M., $n=4$)

administered i.p. 0.5 h before striatal malonate injections (Figure 10A) and for intrastratial injection of zVAD-fmk at 3 h after malonate treatment (Figure 10B). Both drugs provided dose-dependent protection. A combination of treatment with 0.5 mg/kg MK-801 i.p. at 0.5 h before malonate injections and 20 ng zVAD-fmk at 3 h provided strong synergistic effects as calculated by the fractional product method (Figure 10C). At these concentrations, both drugs on its own provide only marginal effects. This protection was as good as the effects observed after combination of 0.5 mg/kg MK-801 with 1000 ng zVAD-fmk. At these concentrations MK-801 did not cause hypothermia in the free moving, non-anesthetized animals.

Discussion

The hypothesis that ischemia-induced neuronal cell death involves apoptosis and is genetically regulated raises the possibility of specific interventions that could act in synergy with anti-excitotoxic therapies and allow for a prolonged therapeutic window (Choi, 1996). Here, we report that malonate induces an active type of cell death depending on cycloheximide-sensitive activation of caspases. Inhibition of caspase activation by BCL-2 expression or pharmacological agents heralds new therapeutic approaches that are shown here to protect neurons in synergy with anti-excitotoxic treatment. Our results confirm earlier reports of protection in focal ischemia by pharmacological caspase inhibition (Hara *et al*, 1997) or expression of a dominant negative mutant of caspase-1 in transgenic mice (Friedlander *et al*, 1997).

There was no major role of apoptosis in malonate-induced histochemical hypoxia. In the transition zone, only 2% of cells were TUNEL-positive and showed features of

Figure 7 zVAD-fmk attenuates striatal lesions produced by malonate. 200 μ g zVAD-fmk dissolved in 2 μ l DMSO or 2 μ l DMSO alone (vehicle-treated control) were injected into the striatum 30 min before striatal malonate injections. (A) Analysis of lesion volume 7 days after striatal malonate injections. (B) Analysis of TUNEL-positive neurons expressed as percentage of striatal neurons (mean $\pm$ S.E.M., $n=3$, * $P<0.05$, ** $P<0.01$)

apoptosis. Since the number of TUNEL-positive cells was small, we may have missed neurons with ultrastructural features of apoptosis in the electron microscopic studies. Yet, interfering with a genetic pathway typical of apoptosis, by means of overexpression of BCL-2 or pharmacological inhibition of caspases, protected from malonate-induced histotoxic hypoxia. Similarly, hypoxia-induced cell death by cyanide, rotenone, or antimycin A in naive PC12 cells *in vitro* did not show morphological features of apoptosis but is prevented by BCL-2 and caspase inhibitors (Shimizu *et al*, 1996). Further, BCL-2 has been shown to inhibit necrotic cell death of GT1-7 cells induced by glutathione depletion or chemical hypoxia (Kane *et al*, 1993; Myers *et al*, 1995). The morphologic demise of human T cells caused by staurosporine and CD95 stimulation, two classic apoptotic

Figure 8 Effects of delayed treatment with zVAD-fmk and MK-801 on lesions produced by malonate. Treatment with (A) zVAD-fmk (1 µg zVAD-fmk dissolved in 2 µl of 1% DMSO intrastratially) or (B) MK-801 (5 mg/kg i.p.). Controls in (A) received 1% intrastratial DMSO injections. Controls in (B) received intraperitoneal saline injections. Lesion volumes were determined 7 days after the malonate lesion (mean ± S.E.M., $n=7-10$, ** $p<0.01$)

triggers, changes from apoptosis to necrosis when cells are depleted of ATP by treatment with oligomycin. Therefore, the intracellular ATP concentration may be a switch in the morphologic decision between apoptosis and necrosis. Rapid depletion of ATP (Henshaw *et al*, 1994) after striatal malonate injection may explain the lack of apoptosis. However, interfering with the apoptotic cell death pathway provided protection in this model of energy depletion, even though morphologic evidence of apoptosis may be weak.

Caspases are synthesized as inactive proenzymes which require cleavage at specific aspartate residues to generate active proteases containing a large (17–20 kDa) and a small (1–12 kDa) subunit (Cohen, 1997). Here, we report that malonate induces proteolytic activation of NEDD2 (caspase-2), but not caspase-1 and caspase-3. During embryonic development, caspase-2 mRNA is expressed at relatively high levels in various tissues, including brain, liver, kidney and lungs (Kumar *et al*, 1994). NEDD2 is also expressed in several adult tissues, including post-mitotic

Figure 9 Synergistic effects of a zVAD-fmk and MK-801. Effects of pretreatment with MK-801 (5 mg/kg i.p.) and posttreatment with zVAD-fmk (1 g dissolved in 2 µl 1% DMSO intrastratially) at 3, 6 and 12 h, or their combination on striatal malonate lesions. The calculated independent effect (see Materials and Methods) is shown as open bar, the observed effect as hatched bar. Controls were injected 2 µl of 1% DMSO intrastratially. (mean ± S.E.M., $n=8-16$, ** $P<0.01$, *** $P<0.001$ compared with controls, # $P<0.05$ compared with MK-801 or zVAD-fmk treatment alone, ## $P<0.001$ compared with zVAD-fmk treatment alone, not significant different to MK-801 treatment alone)

neurons (Kumar *et al*, 1994). Upregulation of caspase-2 mRNA has been observed in response to ischemia-induced cell death in gerbils (Kinoshita *et al*, 1997). Activation of caspase-2 may require the cleavage of the 51 kDa precursor molecule into subunits of 19 and 12 kDa (Harvey *et al*, 1997). However, the cleavage site between the pro-domain and the larger subunit is not exactly known (Cohen, 1997). Our data indicate, that the size of the larger subunit cleaved may be 24 kDa in rodents. Cleavage of caspase-2 has been reported in other neuronal cell death paradigms, e.g. in the trophic factor withdrawal-induced apoptosis of differentiated PC12 cells (Troy *et al*, 1997) and staurosporine-induced apoptosis of neuronal GT1-7 cells (Srinivasan *et al*, 1996).

Using affinity labeling with the biotinylated caspase inhibitor DEVD-CHO we found activation of a ~20 kDa caspase subunit (p20) at 12 h after injection of malonate. p20 recognizes DEVD as a substrate and is likely to correspond to a larger caspase subunit. DEVD is a substrate for active caspase-3-like proteases, including caspase-3, -6, -7, -8, and -10, but not for caspase-2 (Talanian *et al*, 1997). Here, the p20 fragment was induced later than cleavage of caspase-2 occurs (Figure 3A and 4A). Therefore, this protein is unlikely to be identical with the cleaved ~24 kDa fragment of caspase-2. The p20 protein has been considered the cleavage product of full length pro-caspase-3 which is further processed to a p19 and p17 protein (Fernandes-Alnemri *et al*, 1996). Since we were not able to detect either caspase-3 cleavage with 2 antibodies nor cleavage of the putative substrates PARP and protein kinase Cδ (data not shown), the p20 fragment may correspond to another activated caspase-3-like caspase.

Figure 10 Synergy of subthreshold dosages of MK-801 and zVAD-fmk. Effects of MK-801 administered i.p. 30 min prior to and zVAD-fmk (respective dose dissolved in 2 μ l 1% DMSO) intrastratially injected at 3 h after striatal malonate lesions. MK-801 (A) and zVAD-fmk (B) provide dose-dependent protection against lesions induced by 3 μ mol malonate. The combination of treatment of 0.5 mg/kg MK-801 with 20 or 1000 ng zVAD-fmk provides synergistic effects (C). The calculated independent effect (see Materials and Methods) is shown as open bar, the observed effect as hatched bar. (mean $\pm$ S.E.M., $n=9-10$, ** $P<0.01$, *** $P<0.001$ compared with controls, ## $P<0.01$, ### $P<0.001$ compared with MK-801 or zVAD-fmk treatment alone)

Activation of a caspase recognizing DEVD is blocked in bcl-2-transgenic mice (Figure 3B). This is the first report to show that BCL-2 blocks the mammalian cell death pathway upstream of caspases *in vivo*. Malonate-induced activation of caspases occurred in neurons since the increased expression of human BCL-2 in the transgenic mice used is under control of the neuron-specific enolase promoter (Martinou *et al*, 1994). There is also no evidence for release of BCL-2 and uptake by neighboring cells.

zVAD-fmk is a potent, non-specific inhibitor of caspases, but is a poor inhibitor of other cysteine proteases (Armstrong *et al*, 1996). It has been questioned whether anti-apoptotic therapies that lack neuronal specificity may have unwanted effects on other types of cells, e.g. macrophages or microglia. Ischemia induces expression of caspase-1 in microglia (Bhat *et al*, 1996). The preferred substrate for caspase-1 is pro-interleukin-1 β that is proteolytically cleaved

to its active form. Blocking the ability of interleukin-1 to bind to its receptor with antibodies or inhibiting its activity have been shown to attenuate ischemic and excitotoxic neuronal damage and edema formation (Relton and Rothwell, 1992; Garcia *et al*, 1995). Although zVAD-fmk also blocks caspase-1, it is unlikely that this contributed to neuroprotection, since we did not find any increase in binding of the biotinylated caspase-1 specific inhibitor YVAD-CHO to activated caspase-1 for up to 24 h.

A critical issue in the treatment of stroke is the time window for administration of neuroprotective agents. After striatal injections of malonate, the therapeutic window for treatment with lamotrigine and MK-801 was 1 and 2 h, respectively, whereas a free radical spin trap showed efficacy when administered up to 6 h after malonate injections (Schulz *et al*, 1995b). zVAD-fmk was effective when administered up to 9 h after striatal malonate injection. At this time point, NMDA antagonists and glutamate release inhibitors were no longer protective. zVAD-fmk was no longer effective at the time point of 12 h at which a p20 fragment labeled with DEVD was first detected (Figure 3A).

Malonate toxicity is believed to involve secondary or slow excitotoxicity (Beal *et al*, 1993; Greene *et al*, 1993). zVAD-fmk does not block glutamate-induced death in cerebellar granule neurons *in vitro* at higher glutamate concentrations (Armstrong *et al*, 1997) and is only marginally effective in protecting against NMDA toxicity *in vivo* (Hara *et al*, 1997). Intense excitotoxic insults may produce necrosis in cultured cortical neurons whereas milder excitotoxic insults induce apoptosis (Bonfoco *et al*, 1995). Glutamate induces early necrosis in a subpopulation of cultured cerebellar granule neurons and delayed apoptosis in neurons surviving the initial death phase (Ankarcrona *et al*, 1995). These results and the present data suggest that malonate toxicity involves neurons dying by more than one pathway.

The present study suggests, that caspase-2 may be an important protease in cerebral hypoxia/ischemia. We show that there may be an extended therapeutic window to treat ischemia with caspase inhibitors. Furthermore, a combination of anti-excitotoxic and anti-caspase therapy exerts synergistic effects as well as an even more prolonged therapeutic window. This may provide a novel therapeutic strategy for treatment of stroke.

Material and Methods

Surgical procedure

Studies were done in accordance with the European Convention for Animal Care and Use of Laboratory Animals and were approved by the local Animal Care Committee. Male Sprague-Dawley rats weighing 350–375 g were anesthetized with pentobarbital (50 mg/kg i.p.) and positioned in a David Kopf stereotaxic instrument with the incisor bar set at 3.3 mm below the interaural line. A 1 mm burr hole was made in the skull over the left striatum and a 1 mm guide cannulas affixed with dental acrylic vertically with the tip on top of the brain surface. Malonate (Sigma, St. Louis, MO) was dissolved in 0.1 M PBS and

zVAD-fmk (Bachem, Heidelberg, Germany) in dimethyl-sulfoxide (DMSO). After recovering for 48 h, injections were made through the guide cannula into the left striatum (coordinates: bregma, 2.6 mm laterally, 4.5 mm below dura) in a volume of 1.5 μ l (3 μ mol malonate) or 2 μ l (DMSO, zVAD-fmk) using a blunt-tipped 26-gauge Hamilton syringe. All injections were made over 1 min. The needle was left in place for 1 min before being slowly withdrawn. The advantage of implanting a guide cannula for striatal injections were twofold: (1) Drugs can be exactly injected into the same region at different time points and (2) no anesthesia that might cause neuroprotective hypothermia is needed at the time of striatal injection.

Transgenic mice engineered to express human BCL-2 (Martinou *et al*, 1994) from the neuron specific enolase (NSE) promoter have been described (Martinou *et al*, 1994). We bred male founders (strain NSE73A) with the same strain female mice to obtain heterozygous transgenic offsprings, as assessed by PCR analysis of tail DNA. Wild-type littermates were used as controls. Twelve 2–3 month old male mice weighing 20–25 g in each group were anesthetized with methoxyflurane, and malonate (0.75 μ mol in 0.75 μ l) was injected stereotactically into the striatum. This is equivalent to the dose used in rats that lesions about 2/3 of the striatum. To study the activation of caspases, 0.75 μ mol of malonate or saline were injected into the striatum of bcl-2-positive mice or wild-type littermates.

Experimental design and treatment groups

For pharmacological protection studies, 8–12 Sprague-Dawley rats were examined per group. In a first study, 200 μ g zVAD-fmk dissolved in 2 μ l DMSO or 2 μ l DMSO were injected in the left striatum 30 min before injection of 3 μ mol malonate. In a second study, MK-801 (0.5 or 5 mg/kg) or saline (vehicle-treated controls) were administered intraperitoneally 30 min before striatal injection of malonate. 20 ng, 100 ng, 500 ng, 1000 ng or 2000 ng zVAD-fmk dissolved in 2 μ l of 1% DMSO or 2 μ l of 1% DMSO were injected in the striatum at various time points after striatal injection of malonate. In the awake animals MK-801 did not reduce body temperature at dosages used.

Quantification of lesion volume

For pharmacological protection studies rats were killed by decapitation 1 week after striatal malonate injections. The brains were rapidly removed, placed in ice-cold saline for 5 min, and sectioned coronally into slices at 2 mm intervals. Slices were stained in 2% 2,3,5-triphenyl-tetrazolium chloride monohydrate (TTC, Sigma) solution at room temperature in the dark for 30 min followed by fixation in phosphate-buffered 4% paraformaldehyde (PFA). The lesioned area (noted by pale staining) was measured on the posterior surface of each section using an image processing system (MCID M4, Imaging Research) by an observer blinded to the experimental conditions. We previously verified the reliability of the TTC measurements in animals injected with malonate on adjacent sections stained with either TTC or Nissl stain (Schulz *et al*, 1995a). To study the time course of malonate histopathology rats were transcardially perfused with 4% PFA at 3, 6, 9, 12, 24 h and 7 days after striatal malonate injections, paraffin-embedded, and 20 μ m sections at 240 μ m distance processed for cresyl violet. Four animals at each time point were studied. Bcl-2-transgenic mice or littermates were anesthetized and perfused transcardially with 4% PFA at 7 days after striatal malonate injections, paraffin-embedded, and 20 μ m sections at 200 μ m distance processed for cresyl violet. Lesion volumes were measured using an image processing system.

TUNEL staining

For terminal transferase dUTP nick end-labeling (TUNEL), rats treated with DMSO or zVAD-fmk 30 min before striatal injection of malonate were deeply anesthetized and perfused with ice-cold 0.9% saline followed by 4% PFA. The brains were removed and fixed in 4% PFA, paraffin-embedded, and 20 μ m sections processed for TUNEL. The sections were counterstained with cresyl violet for histological evaluation. Quantification of striatal apoptotic neurons was carried out in 350 μ m²-fields centered around the lesion at a magnification of 250 $\times$, using an eyepiece graticule. For each animal, counts were made in five sections at +0.4, +0.2, 0, –0.2, –0.4 mm to bregma. Eight to ten fields per section were averaged. The data were analyzed as the fraction of the total number of neurons detected in the same location in the contralateral unlesioned striatum.

Electron microscopy

Twenty-four hours after striatal malonate injection, rats were perfused transcardially under deep pentobarbital anesthesia with a fixation solution containing 2% glutaraldehyde plus 0.8% PFA solved in 0.05 M cacodylate buffer (pH 7.2, 330 mOsm/l). The forebrains were removed and postfixed for 12 h with the same solution. The specimens were then osmified in an aqueous solution of 1% OsO₄, dehydrated in an alcohol series and embedded into epon. Ultrathin sections (approx. 20 nm) were contrasted with 4% uranyl acetate and 2.6% lead citrate (both solved in water) and studied with a CM 100 (Philips).

Labeling of activated caspases with biotinylated DEVD-CHO

Animals were sacrificed after striatal malonate or saline injections at the time points indicated. The striatum was dissected from a 2 mm-thick slice and lysed on ice in 50 mM Tris/HCl, pH 8, containing 120 mM NaCl, 0.5% NP 40, 0.1 mM EDTA, 100 μ g/ml PMSF, 2 μ g/ml aprotinin, 10 μ g/ml leupeptin, 50 mM sodium fluoride, and 200 μ M sodium vanadate followed by centrifugation at 10000 $\times$ g for 10 min. We performed reactions in 50 μ l volume containing 2 μ g of protein per μ l. Samples were incubated for 25 min at room temperature with biotinylated DEVD-CHO at 2 μ M. Reactions were stopped by adding an equal volume of denaturing sample buffer. The samples were subjected to SDS–PAGE on 12% polyacrylamide gels and electroblotted to nitrocellulose membranes for 60 min. After blocking (1 h at room temperature), sheets were incubated for 1 h with streptavidine alkaline phosphatase conjugate (1:2500). Blotted proteins were visualized by chemiluminescence detection.

Immunoblotting with ICE (caspase-1), Nedd2 (caspase-2), and CPP32 (caspase-3) antibodies

Mice and rats were sacrificed after striatal malonate or saline injections at the time points indicated. Proteins were extracted as described above. Samples were diluted to 1 μ g/ μ l protein and 20 μ g per lane subjected to SDS–PAGE on 12% polyacrylamide gels. After electrophoresis and electroblotting to nitrocellulose membranes, the blots were blocked in 250 mM Tris-HCl, pH 8.0, 120 mM NaCl, 10% non fat dry milk, 5% BSA, 1% of serum corresponding to the secondary antibody, 0.5% Tween 20 and 0.1% azide for 30 min. The blots were incubated with the first antibody at 4°C overnight. The following antisera were used: Caspase-1 p10 (M20) rabbit polyclonal antibody raised against the p10 subunit of murine caspase-1 (Santa Cruz, Heidelberg, Germany), antiserum against a C-terminal peptide of Caspase-2 (Nedd2 p12 C20, Santa Cruz), a monoclonal mouse-anti-

human Nedd-2 antibody raised against an epitope corresponding to amino acids 225-401 (Transduction Laboratories, Manhead Castle, Exeter, U.K.), an N-terminal rabbit-anti-human anti-CASPASE-3 monoclonal antibody (Upstate, Lake Placid, NY, USA), polyclonal caspase-3 rabbit-anti human X-terminal antibody (kindly provided by Dr. W. Fiers). After three washes in PBS containing 0.05% Tween 20, the membranes were incubated with secondary alkaline phosphatase-conjugated antibody for 1 h, washed three times in PBS, and stained with 0.2 mg/ml nitroblue tetrazolium chloride and 0.3 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate in 0.1 M Tris HCl, pH 9.5, containing 50 mM MgCl₂ and 100 mM NaCl.

Statistical analysis

Data are expressed as means $\pm$ S.E.M. values. Tests of variance homogeneity, normality and distribution were performed to ensure that the assumptions required for standard parametric analysis of variance were satisfied. Statistical analysis was performed by ANOVA followed by Fisher's PLSD *post hoc* test to compare group means. To study whether treatment with MK-801 and zVAD-fmk has synergistic effects, we used the fractional product method (Greco *et al*, 1995). In the fractional product method, the effect of two independently acting agents is defined as the product of the unaffected fractions after treatment with either drug alone: $fu(1,2)=fu(1) \times fu(2)$. This formula allows to calculate the predicted effect of cotreatment, based on the assumption that two agents do not interact or cooperate in inducing their effects. If the unaffected fraction, that is, the remaining lesion volume, is below the calculated product $fu(1,2)$ after cotreatment, then the two drugs show synergy.

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