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Synergistic effects of caspase inhibitors and MK-801 in brain injury after transient focal cerebral ischaemia in mice

Jianya Ma, Matthias Endres & [1]Michael A. Moskowitz

Stroke and Neurovascular Regulation Laboratory, Neurology and Neurosurgery Services, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA, U.S.A.

1 Excitotoxic and apoptotic mechanisms have been implicated in the pathophysiology of cerebral ischaemia. Both MK-801, an NMDA receptor antagonist, or peptide inhibitors of the caspase family (z-VAD.FMK and z-DEVD.FMK), protect mouse brain from ischaemic cell damage. In this study, we examined whether these drugs which act via distinct mechanisms, afford even greater neuroprotection when given in combination following 2 h MCA occlusion (filament model) and 18 h reperfusion.

2 Given alone as pretreatment, MK-801 (1, 3 and 5 mg kg^{-1}, but not 0.3 mg kg^{-1}, i.p.) decreased infarct size by 34–75%. When injected 1 h after occlusion and before reperfusion, 3 mg kg^{-1} reduced injury but not when administered 1 h after reperfusion.

3 Pretreatment with a subthreshold dose of MK-801 (0.3 mg kg^{-1}) plus a subthreshold dose of z-VAD.FMK (27 ng) or z-DEVD (80 ng) significantly decreased infarct size by 29 and 30%, respectively, and enhanced neurological function.

4 Administering a subthreshold dose of z-VAD.FMK (27 ng) or z-DEVD.FMK (80 ng) as pretreatment extended the time window for MK-801 (3 mg kg^{-1}) by 2 h from 1 h before reperfusion to at least 1 h after reperfusion.

5 Pretreating with a subthreshold dose of MK-801 (0.3 mg kg^{-1}) extended the time window for z-DEVD.FMK (480 ng) from 1 h after reperfusion to at least 3 h after reperfusion.

6 We conclude that caspase inhibitors which putatively block apoptotic cell death and inhibit cytokine production and the NMDA antagonist MK-801 act synergistically and prolong their respective therapeutic windows in cerebral ischaemia.

Keywords: MK-801; NMDA receptor; ICE-family caspases; focal cerebral ischaemia; apoptosis

Introduction

Mounting evidence suggests that certain excitotoxins cause cell death in ischaemia by overactivation of receptors such as the N-methyl-D-aspartate (NMDA) receptor subtype. Several NMDA receptor antagonists protect against ischaemic cell death including MK-801 (dizocilpine), dextrorphan, dextromethorphan or newer glycine site-specific antagonists, like ACEA 1021 (Park *et al*., 1988a; Dirnagl *et al*., 1990; Steinberg *et al*., 1991; Buchan *et al*., 1992; Hatfield *et al*., 1992; Margaill *et al*., 1996; Hawkinson *et al*., 1997). Ischaemia is characterized by cell swelling and lysis, disruption of membranes and subcellular organelles and associated inflammation consistent with necrotic cell death (Wyllie *et al*., 1980). Necrosis also characterizes cell death after NMDA receptor activation (Choi, 1992). Over the last few years, apoptosis has become recognized as an additional mechanism of ischaemic and excitotoxic cell death based on morphological, histochemical, molecular and pharmacological evidence (Li *et al*., 1995a,b,c; McManus *et al*., 1995a,b; Charriaut-Marlangue *et al*., 1996; Hara *et al*., 1997a,b). Apoptosis is mediated by activation of caspase family members (Yuan *et al*., 1993; Alnemri *et al*., 1996), the human homologues of the *C. elegans* CED-3 (Yuan & Horvitz, 1990; Ellis *et al*., 1991).

Caspase-1 and caspase-3 are activated following 2 h ischaemia and reperfusion (Hara *et al*., 1997b; Namura *et al*., 1997; 1998). Peptide caspase inhibitors, such as N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD.FMK), a non-selective inhibitor, or N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone (z-DEVD.FMK) which is reportedly more specific for caspase-3 (CPP32), reduce focal cerebral ischaemic injury in mice and rats (Hara *et al*., 1997b). These drugs penetrate brain tissue after i.c.v. administration as z-VAD.FMK reduces brain interleukin-1β (IL-1β) levels and z-DEVD.FMK inhibits DEVDase activity after *in vivo* administration (Hara *et al*., 1997b; unpublished observations). Moreover, transgenic animals expressing a dominant negative mutation have reduced brain damage after ischaemic infarction (Friedlander *et al*., 1997; Hara *et al*., 1997a). Using a mouse model of mild focal cerebral ischaemia adapted from the rat (Du *et al*., 1996a), Endres *et al*. (1998) found that caspase inhibitors reduce infarct volume by 50–70%, exhibit an extended treatment window (6–9 h) and decrease DNA laddering. MK-801 on the other hand, protects by a different mechanism in this model, as we have observed that the treatment window is not extended, and apoptotic markers do not decrease after MK-801 pretreatment. Not surprisingly then, pretreatment with caspase inhibitors after intrastriatal NMDA injections only weakly blocked excitotoxic damage developing days after intrastriatal injection (Hara *et al*., 1997b).

Because NMDA antagonists and caspase inhibitors appear to protect brain tissue by distinct mechanisms in acute cerebral ischaemia, we examined for possible therapeutic interactions between the two drugs in a well-defined mouse model of reversible focal cerebral ischaemia. To do so, we administered them in doses which when given individually provided no tissue protection against ischaemic injury. Furthermore, we examined whether or not pretreatment with either

[1] Author for correspondence at: Massachusetts General Hospital, 149 13th Street, Room 6403, Charlestown, MA 02129, U.S.A.

z-VAD.FMK or z-DEVD.FMK extended the time window for MK-801, beyond 1 h after middle cerebral artery (MCA) occlusion (Margaill *et al*., 1996) and whether pretreatment with MK-801 prolonged the time window for caspase inhibition (Hara *et al*., 1997b).

Methods

Physiology

Adult male 129/S/vEvTacfBR mice ($n = 120$, Taconic Farms, Germantown, NY, U.S.A.), weighing 18 to 23 g, were allowed free access to food and water *ad libitum*. All animals were kept under diurnal lighting conditions. Anaesthesia was induced and maintained by 2% and 1% halothane, respectively, in 70% N_2O and 30% O_2 using a Fluotec 3 vaporizer (Colonial Medical, Amherst, NH, U.S.A.). Rectal temperature was maintained at approximately 36.5–37°C with a thermostatically controlled blanket (FHC, Brunswick, ME, U.S.A.) and a heating lamp. Arterial blood pressure was measured in randomly selected mice via a PE-10 polyethylene cannula placed in the right femoral artery (Gould, Oxnard, CA) and blood gas determination using a blood gas/pH analyser (248 pH/Blood Gas Analyzer, Ciba Corning Diag. Medford, MA), and rectal temperature was measured using a thermostat (BAT-12, Physitemp, Clifton, NJ, U.S.A.). Care of animals was in strict accordance to guidelines by the National Institutes of Health and the Division of Animal Care, Massachusetts General Hospital.

Ischaemia model (2 h MCA occlusion/18 h reperfusion)

Focal cerebral ischaemia was induced by occlusion of the left middle cerebral artery (MCA) with an 8-0 nylon monofilament (Ethicon, Somerville, NJ, U.S.A.). coated with a mixture of silicone resin (Xantopren, Bayer Dental, Osaka, Japan) and a hardener (Elastomer Activator, Bayer Dental) as described previously (Hara *et al*., 1996).

This coated filament was introduced into the external carotid artery, up to the origin of the anterior cerebral artery via the internal carotid artery. By so doing, the MCA and anterior choriodal arteries were occluded. After surgery the wound was cleaned with betadine solution. Xylocaine (1 mg kg^{-1}) was injected s.c. into the cervical area to alleviate the possibility of incisional pain. In the first postoperative hours the animals were closely monitored. No major infections occurred after animal surgery. Because hypothermia is an acknowledged effect of MK-801 treatment, mice were kept in an incubator (ThermoCare System, Incline Village, NV, U.S.A.) at 31°C for the time of ischaemia and reperfusion. In randomly selected mice, rectal temperatures were measured using a thermometer (BAT-12, Physitemp, Clifton, NJ, U.S.A.) 3, 6, and 18 h after reperfusion. For filament withdrawal, mice were briefly reanaesthetized with halothane after 2 h of ischaemia. Animals were killed with an overdose of pentobarbitone (200 mg kg^{-1}, i.p.) 18 h after reperfusion.

Neurological deficits

Mice were rated for behavioural changes and ranked by an observer naive to the treatment group as described by Bederson *et al*. (1986) with minor modifications as follows (Hara *et al*., 1996): 0: no observable neurological deficits (normal), 1: failure to extend right forepaw (mild), 2: circling to the contralateral side (moderate); 3: loss of walking or righting reflex (severe).

Infarct measurement

The brains were removed and sectioned coronally into five 2 mm sections in a mouse brain matrix (RBM-2000C, Activational System, MI, U.S.A.). Slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, St. Louis, MO, U.S.A.) in PBS, followed by 10% formalin overnight. The infarction areas on each slice (mm^2) were measured by an image-analysis system (M4, Imaging Research, St. Catherines, Ont, Canada) on the posterior surface of each section, and the total infarction volume was calculated by summing the volumes as described by Huang *et al*. (1994). White or pink was considered ischaemic whereas red tissue was considered viable.

Experimental protocol

Dose and time-dependent effects of MK-801 Mice were injected with MK-801 (0.3, 1.0, 3.0, 5.0 mg kg^{-1}, intraperitoneally (i.p.) or an equivalent volume of saline 15 min before MCA occlusion. To determine the therapeutic window, mice were injected with MK-801 (3.0 mg kg^{-1}, i.p.) or saline 15 min preischaemia or 1 h after MCA occlusion. Other groups were injected 1 h or 4 h after the onset of reperfusion.

Dose and time-dependent effects of caspase inhibitors Mice were injected with z-DEVD.FMK (80, 160 ng, 480 ng, i.c.v.) or 0.4% DMSO 15 min before MCA occlusion. To determine the therapeutic window, mice were injected with z-DEVD.FMK (480 ng, i.c.v.) or DMSO 15 min preischaemia or 1 h after MCA occlusion or 1 h or 4 h after reperfusion. Dose and time-dependent effects of z-VAD.FMK were determined in a study described earlier: pretreatment with 80 or 160, but not 27 ng z-VAD.FMK i.c.v. decreased infarct size; 80 or 160 ng was protective until the onset of reperfusion but not later (Hara *et al*., 1997b).

Effects of combining subthreshold doses of both caspase inhibitors and MK-801 administered before ischaemia z-VAD.FMK (27 ng), z-DEVD.FMK (80 ng) or DMSO vehicle were injected i.c.v. 15 min preischaemia along with MK-801 (0.3 mg kg^{-1}, i.p.) or vehicle (saline).

Effects of pretreatment with caspase inhibitors on the therapeutic window for MK-801 given after reperfusion Fifteen minutes before occlusion, z-VAD.FMK (27 ng or 160 ng), z-DEVD.FMK (80 ng or 480 ng) or vehicle (DMSO) were injected i.c.v. MK-801 (3.0 mg kg^{-1}, i.p.) or saline were then injected 1 h or 4 h after reperfusion.

Effects of subthreshold doses of MK-801 on the therapeutic window for caspase inhibitors after reperfusion Fifteen minutes before occlusion, MK-801 (0.3 mg kg^{-1}) or vehicle (saline) was injected i.p. z-DEVD.FMK (480 ng) or vehicle (DMSO) was then injected i.c.v. 3 h after reperfusion.

Drugs

N - Benzyloxycarbonyl - Val - Ala - Asp(OMe)-fluoro-methylketone (z - VAD.FMK) and N - benzyloxycarbonyl - Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone (z-DEVD.FMK) were obtained from Enzyme Systems Products (Dublin, CA, U.S.A.). Compounds were dissolved in 0.4% dimethylsulph-

oxide (DMSO, MC/B, Norwood, OH, U.S.A.) in 0.1 M phosphate-buffered saline (PBS; pH 7.4). z-VAD.FMK (2 μl) or z-DEVD.FMK (2 μl), or 0.4% DMSO (2 μl) were injected intracerebroventricularly (i.c.v.) using a Hamilton syringe (needle diameter: 0.508 μm; total volume 10 μl). The syringe was placed perpendicular to the skull at the coordinates: bregma -0.9 mm lateral, -0.1 mm posterior, -3.1 mm deep and free-handed injections were made 15 min before ischaemia. MK-801 ([+]-MK-801 hydrogen maleate) was obtained from RBI (Natick, U.S.A.) and dissolved in PBS (Abbott Laboratories, North Chicago, IL, U.S.A.).

Statistical analysis

Data are expressed as mean$\pm$s.e. Statistical analysis was performed by one-way (protocols 1 and 2) or two-way (physiology, protocols 3, 4 and 5) ANOVA followed by Bonferroni's test. For neurological deficits Kruskal-Wallis nonparametric ANOVA was used. The software INSTATE 2.0 (Graph Pad Software, San Diego, CA, U.S.A.) or super ANOVA (Abacus, Berkeley, CA, U.S.A.) was used for statistical analysis. $P<0.05$ was considered to indicate statistical significance.

Results

Physiology

There were no significant differences in mean arterial blood pressure, rectal temperature, arterial pH or blood gases (Pa_{O_2} and Pa_{CO_2}) between groups at any single time point. Over time, mean arterial blood pressure, pH values and core temperature were lower than preischaemic values for each group, but the groups did not differ from each other (Table 1).

Dose- and time-response to MK-801

MK-801 decreased infarct volume and decreased neurological deficits in a dose-dependent manner when administered above 0.3 mg kg^{-1} ($P<0.05$). Infarct volumes were 99 ± 9.5 mm^3 (control, $n=9$), 84 ± 4.0 mm^3 (0.3 mg kg^{-1}, $n=7$), 65 ± 5.8 mm^3 (1.0 mg kg^{-1}, $n=8$), 31.6 ± 6.7 mm^3 (3.0 mg kg^{-1}, $n=7$), and 24.2 ± 2.7 mm^3 (5.0 mg kg^{-1}, $n=4$). Infarct size was reduced after MK-801 (3 mg kg^{-1}) - pretreatment and after injection during MCA occlusion (Figure 1), although no significant effect was obtained when MK-801 was given 1 or 4 h after reperfusion (Figure 1).

Dose- and time-response to caspase inhibitors

z-DEVD.FMK pretreatment significantly decreased infarct size at 160 ng and 480 ng ($P<0.05$) but not 80 ng (infarcts: 95.2 ± 4.7 mm^3 (DMSO); 85 ± 5.2 mm^3 (80 ng); 57.0 ± 6.2 mm^3 (160 ng); 56.6 ± 6.7 mm^3 (480 ng), $n=6-8$ per group). Infarct reduction was accompanied by functional improvements (2.0 ± 0.0 (DMSO), 1.4 ± 0.2 (80 ng), 1.2 ± 0.3 (160 ng), 0.9 ± 0.3 (480 ng, $P<0.05$)). Infarct size was also reduced after z-DEVD.FMK (480 ng) was given at 1 h during 2 h of MCA occlusion (61.2 ± 7.6 mm^3 (drug) *vs* 92.5 ± 7.2 mm^3 (vehicle), $n=5-6$ per group, $P<0.05$) and 1 h after reperfusion (58.5 ± 7.8 mm^3 (drug) *vs* 92.3 ± 3.8 mm^3 (vehicle), $n=6-7$ per group, $P<0.05$), but not 4 h after reperfusion (100.7 ± 9.0 mm^3 (drug) *vs* 97.5 ± 10.2 mm^3 (vehicle), $n=6-7$ per group).

Pretreatment with subthreshold doses of caspase inhibitors plus MK-801 decreased infarct size

z-VAD.FMK (27 ng) plus MK-801 (0.3 mg kg^{-1}) administered 15 min preischaemia reduced infarct volume by 29% (Figure 2) and also neurological deficits (1.1 ± 0.2 (MK-801 + z-VAD.FMK) *vs* 1.7 ± 0.2 (saline + z-VAD.FMK) and 1.9 ± 0.4 (MK-801 + DMSO), $P<0.05$). Similarly, z-DEVD.FMK (80 ng) plus MK-801 (0.3 mg kg^{-1}) decreased infarct size by 30% when given 15 min preischaemia (Figure 2). Also neurological deficits were significantly improved compared to their controls (0.8 ± 0.3 (MK-801 + z-DEVD.FMK) *vs* 1.7 ± 0.1 (saline + z-DEVD.FMK) and 1.9 ± 0.4 (MK-801 + DMSO), $P<0.05$).

A subthreshold dose of caspase inhibitor prolonged the therapeutic window for MK-801

z-VAD.FMK (27 ng, 15 min preischaemia) plus MK-801 (3 mg kg^{-1}, 1 h after reperfusion) reduced both infarct volume

Table 1 Physiology parameters

	DMSO + saline	*DMSO + MK-801*	*z-VAD + saline*	*z-VAD + MK-801*
MABP (mmHg)				
15 min before MCAo	94.9 ± 2.0	95.8 ± 1.7	100.4 ± 2.1	102.0 ± 1.8
15 min after MCAo	96.1 ± 1.5	98.2 ± 3.2	100.0 ± 3.3	99.3 ± 0.4
20 h after MCAo	**84.7 ± 2.4**#	**86.7 ± 2.1**#	**82.4 ± 1.9**#	**84.8 ± 2.0**#
Core temperature (°C)				
15 min before MCAo	36.9 ± 0.04	36.9 ± 0.06	36.9 ± 0.03	36.9 ± 0.04
3 h after MCAo	36.6 ± 0.05	36.6 ± 0.05	36.5 ± 0.07	36.6 ± 0.07
6 h after MCAo	36.5 ± 0.03	36.6 ± 0.08	36.6 ± 0.06	36.6 ± 0.10
20 h after MCAo	**36.4 ± 0.05**#	**36.2 ± 0.21**#	**36.3 ± 0.06**#	**36.2 ± 0.09**#
Blood gas analysis				
15 min before MCAo				
pH	7.34 ± 0.02	7.34 ± 0.01	7.39 ± 0.06	7.36 ± 0.03
Pa_{CO_2} (mmHg)	38.3 ± 1.4	38.3 ± 1.4	39.3 ± 1.7	41.6 ± 2.3
Pa_{O_2} (mmHg)	115.5 ± 5.0	133.3 ± 9.2	121.4 ± 10.5	114.1 ± 4.6
20 h after MCAo				
pH	**7.27 ± 0.02**#	**7.28 ± 0.07**#	**7.28 ± 0.06**#	**7.26 ± 0.04**#
Pa_{CO_2} (mmHg)	42.1 ± 1.0	43.0 ± 1.0	42.0 ± 0.9	42.7 ± 0.9
Pa_{O_2} (mmHg)	126.9 ± 4.2	125.7 ± 4.1	111.6 ± 6.7	112.7 ± 7.8

Data are expressed as mean$\pm$s.e.mean, $n=5-7$ in each group. #$P<0.05$ with respect to those of 15 min before ischaemia.

(Figure 3) and neurological deficits. The neurological deficits were decreased to a score of 1.3 ± 0.2 (z-VAD.FMK + MK-801) *vs* $1.8 + 0.3$ (z-VAD.FMK + saline) and 2.0 ± 0.1 (DMSO + MK-801) ($P < 0.05$). No significant protection was observed when z-VAD.FMK (27 ng, 15 min preischaemia) was combined with MK-801 (3 mg kg^{-1}) given as late as 4 h after reperfusion (Infarct size 92.3 $mm^3 \pm 0.6$ (DMSO $\pm$ saline) *vs* 93.9 ± 1.1 (DMSO + MK-801) *vs* 94.9 ± 11.4 (z-VAD.FMK + saline) *vs* 96.8 ± 12.4 (z-VAD + MK-801), $n = 3-4$). A higher dose of z-VAD.FMK (160 ng, preischaemia) plus MK-801 (3 mg kg^{-1}, 1 h after reperfusion) reduced infarction volume by 38% compared to z-VAD.FMK (160 ng) alone, or by 54% compared to MK-801 alone (Figure 3).

When a subthreshold dose of z-DEVD.FMK (80 ng i.c.v., 15 min preischaemia) was given before MK-801 (3 mg kg^{-1}, i.p., 1 h after reperfusion), infarct volume was reduced by 37% compared to control (Figure 4); neurological scores were also improved (1.1 ± 0.1 (z-DEVD.FMK + MK-801) *vs* 1.7 ± 0.2 (z-DEVD.FMK + saline) and 2.0 ± 0.1 (DMSO + MK-801), $P < 0.05$). However, no additional protective effect was seen when MK-801 was combined with a higher dose of z-DEVD.FMK (480 ng) (Figure 4).

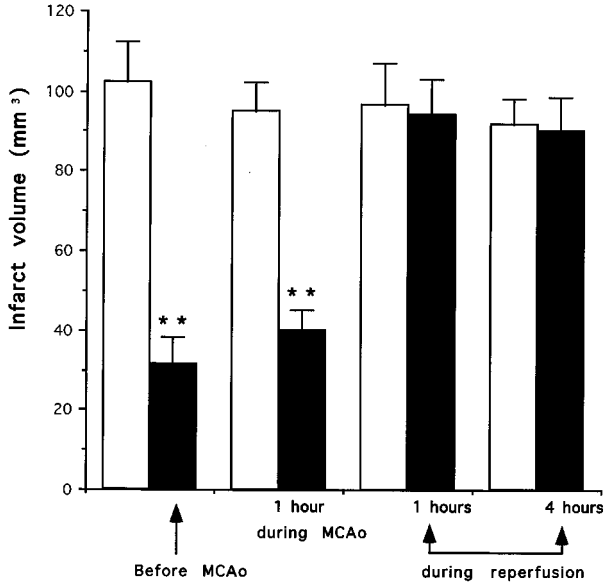

Figure 1 MK-801 (3 mg kg^{-1}, i.p.) decreased infarct volume when administered either as pretreatment (15 min before 2 h MCA occlusion (filament)) or 1 h before reperfusion but not when given as a single dose 1 or 4 h after reperfusion in halothane-anaesthetized SV/129 mice. Animals were killed after 20 h. Infarct volume was determined as described in Methods. Open columns-saline; solid columns-MK-801; $n = 7-9$ animals per group. Mean $\pm$ s.e. are shown; $^{**}P < 0.01$ as compared to controls.

A subthreshold dose of MK-801 prolonged the therapeutic window for caspase inhibitors

When a subthreshold dose of MK-801 (0.3 mg kg^{-1}, i.p.) was given 15 min before ischaemia, the treatment window for z-DEVD.FMK (480 ng) extended for 1 h after reperfusion to 3 h after reperfusion. Infarct size was reduced by 55% (Figure 5) and also neurological deficits were significantly improved (1.3 ± 0.2 (MK-801 $\pm$ z-DEVD.FMK) *vs* 1.7 ± 0.2 (saline $\pm$ z-DEVD.FMK) *vs* 2.0 ± 0.0 (MK-801 + DMSO) *vs* 2.0 ± 0.0 (saline + DMSO), $P < 0.05$).

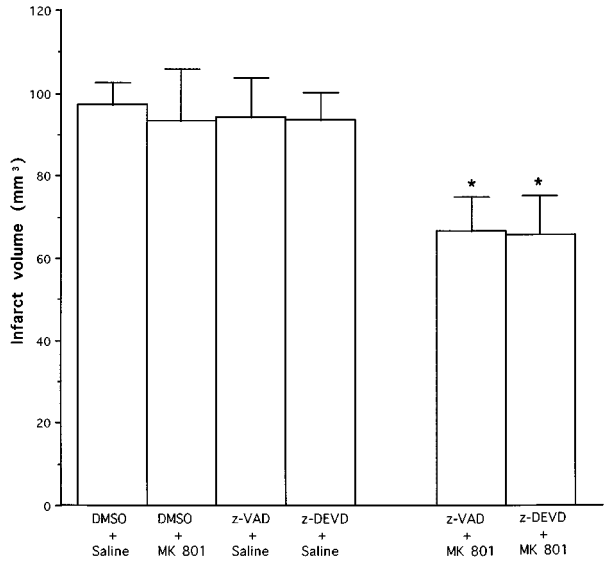

Figure 2 Combined pretreatment with subthreshold doses of MK-801 plus z-VAD.FMK, or MK-801 plus z-DEVD.FMK reduced infarct size after 2 h MCA occlusion plus 18 h reperfusion. Pretreatment with MK-801 alone (0.3 mg kg^{-1}, i.p.) (15 min before occlusion) or z-VAD.FMK alone (27 ng, i.c.v.) or z-DEVD.FMK alone (80 ng, i.c.v.) did not decrease ischaemic injury compared to vehicle. However, combining the above doses of MK-801 plus z-VAD.FMK or MK-801 plus z-DEVD.FMK reduced infarct size by 29% and 30%, respectively ($n = 8-13$ animals per group). Mean $\pm$ s.e. are shown; $^{*}P < 0.05$ as compared to controls.

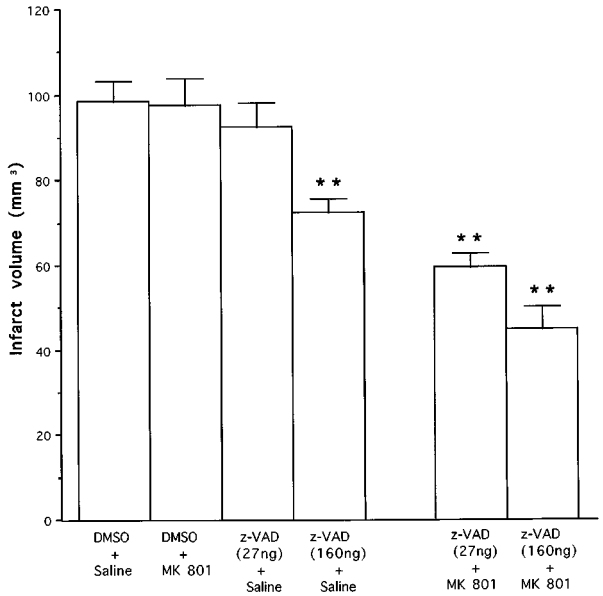

Figure 3 Pretreatment with a subthreshold dose of z-VAD.FMK extended the therapeutic window for MK-801 by 2 h from 1 h before (see Figure 1) to 1 h after reperfusion compared to z-VAD.FMK or MK-801 alone. z-VAD.FMK (27 ng) was given i.c.v., 15 min before occlusion and MK-801 was given 1 h after reperfusion. Protection was also enhanced after combining pretreatment with larger doses of z-VAD.FMK (160 ng) plus treatment with MK-801 given 1 h after reperfusion. z-VAD.FMK (160 ng) plus MK-801 (3 mg kg^{-1}, 1 h after reperfusion) reduced infarct volume compared to z-VAD.FMK, MK-801 or vehicle alone. Mean $\pm$ s.e. are shown, $n = 8-13$; $^{**}P < 0.001$.

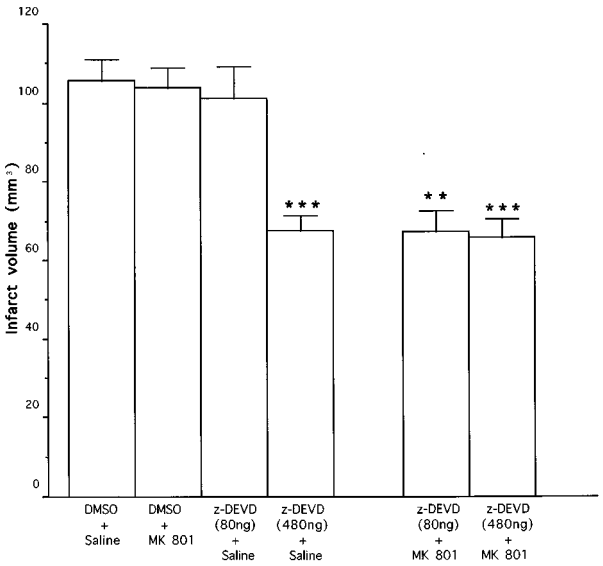

Figure 4 z-DEVD.FMK pretreatment extended the therapeutic window for MK-801 by 2 h from 1 h before to 1 h after reperfusion compared to z-DEVD.FMK or MK-801 alone, or vehicle. z-DEVD.FMK was given i.c.v. using a single subthreshold dose (80 ng), whereas MK-801 (3 mg kg^{-1}, i.p.) was given 1 h after reperfusion. When a maximally effective dose of z-DEVD.FMK (480 ng) was combined with MK-801 posttreatment (3 mg kg^{-1}, i.p.), no additional protection was seen. Mean ± s.e. are shown, $n = 9-11$; $^{**}P < 0.01$.

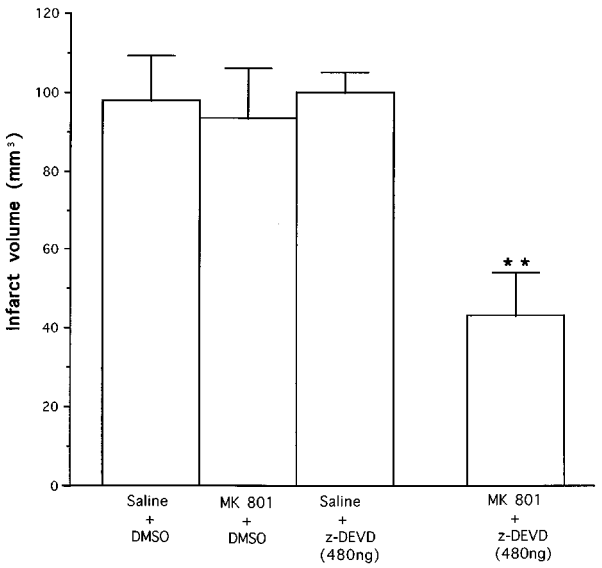

Figure 5 Pretreatment with a subthreshold dose of MK-801 extended the treatment window for z-DEVD.FMK from 1 to 3 h after reperfusion. MK-801 was injected i.p., 15 min before 2 h of MCA occlusion. When administered alone, MK-801 (0.3 mg kg^{-1}) was not protective compared to vehicle. Similarly, z-DEVD.FMK (480 ng, i.c.v.) was not protective given 3 h after reperfusion. Combining the two as above reduced infarct volume compared to z-DEVD.FMK or MK-801 alone. Mean ± s.e. are shown, $n = 6-7$; $^{**}P < 0.01$.

Discussion

We provide evidence for synergistic effects of the NMDA receptor antagonist, MK-801 and caspase inhibitors. When z-VAD.FMK or z-DEVD.FMK pretreatment was administered at doses that did not provide protection when given alone, the window for MK-801 was extended from 1 h before to 1 h after reperfusion. Similarly, subthreshold doses of MK-801 given before ischaemia extended the treatment window of z-DEVD.FMK from 1 to at least 3 h after reperfusion. In both paradigms, a clear neuroprotective effect was achieved along with improved neurological scores, suggesting that combination treatment not only reduces tissue injury but preserves neurological function. In preliminary experiments, we determined that pretreatment with maximally effective doses of both z-VAD.FMK and MK-801 did not reduce ischaemic damage beyond 60%, probably because this level of protection is close to the maximum possible protection (ceiling effect) observed in the ischaemia model (65–70%). Infarct sparing was not due to obvious effects on core temperature, blood pressure or blood gases as these parameters did not differ between groups (Table 1).

Glutamate and especially NMDA-related excitotoxicity contribute to injury after brain ischaemia, presumably because of linkage to augmented Ca^{2+} ion flux (MacDermott *et al.*, 1986). Excitotoxicity is a very early event and in the traditional view, mediates necrotic cell death, as brain cells die rapidly after ischaemia (Choi, 1992). In fact, NMDA receptor antagonists reliably afford protection against focal ischaemic injury (Dirnagl *et al.*, 1990; Buchan *et al.*, 1992; Margaill *et al.*, 1996), but the effects depend upon the strain of animal (Oliff *et al.*, 1996) and on the stroke model (Buchan *et al.*, 1991; Yao *et al.*, 1993). The treatment window of MK-801 in focal ischaemia ranges from 5 min after the onset of ischaemia (Gotti *et al.*, 1990) to 30 min and 2 h post occlusion (Park *et al.*, 1988b; Dezsi *et al.*, 1992). Here, we demonstrate efficacy 1 h after MCA occlusion in a mouse reperfusion model which can be extended to 1 h after reperfusion by pretreatment with subthreshold doses of caspase inhibitors. In the last few years, mounting evidence suggests an additional role for apoptosis in cerebral ischaemia, especially in the borderzone of an infarct (Li *et al.*, 1995a,b,c; McManus *et al.*, 1995a,b; Charriaut-Marlangue *et al.*, 1996). Protein synthesis inhibition by cycloheximide reportedly protects from focal cerebral ischaemia (Linnik *et al.*, 1995; Du *et al.*, 1996a,b; Endres *et al.*, 1998). Recent studies in our laboratory showed that caspase-inhibitors (z-VAD.FMK, YVAD.FMK, z-DEV.FMK) decrease ischaemic injury after focal cerebral ischaemia in mice and rats (Hara *et al.*, 1997a,b). Key enzymes like caspase-1 (ICE) and caspase-3 (CPP32) which are essential for the execution of apoptosis, are activated within min to h after cerebral ischaemia and mediate cell death, as evidenced by TUNEL stained cells containing caspase-3p20 cleavage products (Namura *et al.*, 1997; 1998).

Pathways mediating ischaemia-induced necrosis and apoptosis seem to be mechanistically distinct, although they may be difficult to distinguish. *In vitro*, the same triggers can mediate necrosis and/or apoptosis, depending upon the severity of the insult (Bonfoco *et al.*, 1995). After intrastriatal injections of the excitotoxin NMDA, cell death develops within min to h, whereas apoptosis appears very late, indicating that apoptosis is probably not an immediate consequence of NMDA-receptor activation (Ayata *et al.*, 1997). In focal cerebral ischaemia, cells die due to necrosis and/or apoptosis, perhaps depending upon energy status, redox state or production of oxygen free radicals (Choi, 1996). In mild ischaemia, the distinction between putative apoptotic and excitotoxic mechanisms becomes more pronounced as cell death is delayed and apoptosis is prominent (Du *et al.*, 1996a; Endres *et al.*, 1998). In this model, caspase inhibitors decrease biochemical markers of apoptosis, reduce

infarct size, and remain therapeutically active if administered hours after ischaemic injury. By comparison, the treatment window for MK-801 remains short and markers of apoptosis do not change as a consequence of treatment (Endres *et al*., 1998). The present study strengthens the argument that MK-801 and caspase inhibitors block mechanistically distinct pathways.

The fact that pretreatment with subthreshold doses of caspase inhibitors extended the MK-801 treatment effect from 1 h before to 1 h after reperfusion is a unique finding, because excitotoxic events are believed to develop early after the onset of ischaemia. Arguably, even within individual cells, doses of caspase-inhibitors which do not inhibit infarct size might slow pathological events enabling lower doses and delayed administration of MK-801 to block excitotoxic cell death.

The current literature provides some evidence that combination treatment with an NMDA receptor antagonist can enhance protection in focal ischaemia. For example, the administration of citicoline, a precursor of phosphatidylcholine, plus MK-801 (Önal *et al*., 1997) or kynurenic acid plus MK-801 (Gill & Woodruff, 1990) synergistically reduced infarct size. Du and colleagues showed additive neuroprotective effects of dextrorphan and cycloheximide (Du *et al*., 1996b). Recently, Schulz *et al*. provided preliminary evidence that MK-801 and caspase inhibitors act synergistically in a model of histotoxic hypoxia *in vivo* (Schulz *et al*., 1997).

In this study we did not determine if the protective effects of the different protocols sustain for longer than 24 h, although we and others observed that the protective effects of caspase inhibitors or MK-801 sustains for at least 7 days. Nevertheless, tissue protection for even 24 h affords the opportunity to implement additional strategies when cell death is delayed. Combining subthreshold doses of MK-801 may provide a useful strategy to avoid untoward effects such as pathological vacuolization in specific populations of brain neurones, e.g. in the posterior cingulate and retrosplenial cortices (Olney *et al*., 1989; Fix *et al*., 1993), psychotomimetic effects as well as depressed ventilation and hypotension (Deutsch *et al*., 1989; Albers *et al*., 1995).

In conclusion, our results suggest the potential use of caspase inhibitors as candidates for combination therapy to extend the therapeutic window for cytoprotection via mechanisms distinct from MK-801. Of course, it would be important to develop non-peptide caspase inhibitors which cross the blood-brain barrier and could be administered systemically.

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