Caspase Inhibitors Reduce Neuronal Injury After Focal but Not Global Cerebral Ischemia in Rats

Hui Li, MD; Frederick Colbourne, PhD; Ping Sun, MD; Zonghang Zhao, MD; Alastair M. Buchan, MD, FRCP

Background and Purpose—Studies show that blocking the activation of caspases by the caspase inhibitors z-VAD.FMK and z-DEVD.FMK can reduce ischemic neuronal injury after cerebral ischemia. Because the severity of ischemia was mild in some studies, we tested the efficacy of these caspase inhibitors on moderately severe but transient forebrain and focal ischemic insults in the rat.

Methods—Various regimens of z-VAD, z-DEVD, and control DMSO were given to rats subjected to either 4-vessel occlusion ischemia (4-VO, 10-minute occlusion, 7-day survival) or distal middle cerebral artery occlusion (MCAo, 90-minute occlusion, 22.5-hour survival). In global ischemia, treatments were given immediately after ischemia (experiment 1) or as preischemic and posts ischemic treatments (experiment 2). Three focal ischemia experiments were done. Injection times were 60 minutes into ischemia (experiment 1) and 60 minutes into ischemia plus 30 and 120 minutes after ischemia (experiment 2). Experiment 3 was identical to experiment 2 except that a 30-minute preischemia treatment was instituted. Core normothermia was maintained in all experiments during ischemia. However, in the last focal and global experiments, core and brain temperatures, respectively, were also measured after ischemia with telemetry probes. Because hyperthermia accompanied z-DEVD treatment, an extra z-DEVD–treated group (MCAo) was included with temperature clamped at normothermia.

Results—Neither z-VAD nor z-DEVD significantly reduced CA1 injury after global ischemia. In focal ischemia, both drugs significantly reduced infarction, but only in the third experiment, and the prevention of hyperthermia that accompanied z-DEVD treatment did not alter this.

Conclusions—These results suggest a detrimental role of caspases in moderately severe focal but not global cerebral ischemia. (Stroke. 2000;31:176-182.)

Key Words: hippocampus ▪ cerebral ischemia ▪ caspase ▪ neuronal death ▪ apoptosis ▪ rats

Global and focal cerebral ischemic insults result in significant brain injury that matures hours to days after the insult. Hippocampal CA1 sector injury occurs >2 days after untreated global ischemia in the rat, gerbil, and human. Infarction after a severe focal ischemic insult (eg, middle cerebral artery occlusion, MCAo) occurs more quickly with a time course of <1 day. In some cases, however, injury may be delayed for days after a mild insult (A.M.B., unpublished data). The mechanisms accounting for delayed ischemic injury have been repeatedly investigated without consensus on an exact cause of cell death. Controversy continues over the relative importance of the morphological mode of cell death (necrosis or apoptosis) versus the molecular and biochemical mechanisms.

Most ultrastructural studies of moderate to severe focal ischemia suggest that cell death occurs by necrosis. Similarly, most ultrastructural studies of hippocampal neuronal injury (ie, 2 to 4 days posts ischemia) provide evidence more consistent with but not completely typical of necrotic cell death. Furthermore, even when CA1 neuronal loss is further delayed, as occurs after very brief 4-vessel occlusion (4-VO) ischemia and in rats and gerbils treated with posts ischemic hypothermia, the injury still appears to have necrotic morphology.

In contrast to the conclusions from ultrastructural studies, evidence (genetic and pharmacological) is accumulating that ischemic injury after global and focal ischemia might occur by apoptosis. For example, several studies have shown elevations in apoptosis-promoting genes (eg, Bax) after global ischemia. Downstream apoptotic events, such as caspase (cysteinyl aspartate–specific proteinases) activation, have also been reported after global and focal ischemia. Furthermore, caspase inhibitors have shown promising effects after global and focal ischemia.
nally, transgenic studies in mice support the detrimental role of caspases in focal ischemia.\textsuperscript{31} We tested the hypothesis that the caspase inhibitors z-VAD.FMK and z-DEVD.FMK would reduce ischemic neuronal injury after moderately severe but brief global and focal ischemic insults. Global ischemia (10 minutes) was produced in the rat by the 4-VO method, whereas focal ischemia was produced for 90 minutes in spontaneously hypertensive rats. In some experiments, temperature was continuously measured to determine whether it confounded neuroprotection, because it is well known that postischemic hyperthermia is detrimental\textsuperscript{32} but postischemic hypothermia is neuroprotective.\textsuperscript{33} Furthermore, recent work in gerbils\textsuperscript{25} indicates that intraventricular administration of z-DEVD causes hypothermia.

**Materials and Methods**

**Animals**

These experiments complied with the guidelines of the Canadian Council on Animal Care and were approved by an Animal Care Committee of the University of Calgary. Male Wistar rats (150 to 175 g) and spontaneously hypertensive male rats (200 to 250 g) were obtained from Charles River (Montreal, Quebec, Canada), except for the first focal ischemia experiment, in which the hypertensive rats were obtained from Taconic Farms (Germantown, NY).

**4-VO Model of Global Ischemia**

Wistar rats were subjected to 4-VO ischemia as previously described.\textsuperscript{34} Briefly, rats were anesthetized with halothane (2% in 70% O\textsubscript{2}) while the vertebral arteries were electrocauterized. In addition, a 3-0 silk was placed around the carotid arteries, and a 2-0 silk was threaded through the neck posterior to the esophagus, trachea, jugular veins, carotid arteries, and vagal nerves but anterior to the cervical and paravertebral musculature. On the following day, rats were briefly anesthetized with halothane while the wound was reopened. The carotid arteries were then occluded for 10 minutes with clamps while the 2-0 silk was tightened. Rats typically became unresponsive after initial running behavior.

**MCAo Model of Focal Ischemia**

Male spontaneously hypertensive rats were anesthetized with a mixture of 2% halothane in 28% O\textsubscript{2} and 70% air.\textsuperscript{35} First, the tail artery was cannulated so that blood pressure could be monitored throughout anesthesia. Next, the right common carotid artery was isolated, and a ligature was looped around it. A 1-cm incision was then made perpendicular to and bisecting a line between the lateral canthus of the right eye and the external auditory canal. The underlying temporals muscle was retracted, and a hole 1 mm in diameter was drilled 2 to 3 mm rostral to the point of fusion of the zygomatic arch with the temporal bone. A hole in the dura was made over the proximal MCA, and a microclip (No. 1, Codman) was placed on the MCA at a site proximal to the point at which it crosses the inferior cerebral vein in the rhinal fissure. Then the ligature on the right common carotid artery was tightened; this was considered the start of ischemia. During ischemia, anesthesia was discontinued, and the animals regained consciousness. At the end of 90 minutes of ischemia, the rats were briefly reanesthetized, followed by removal of the MCA clip. Physiological variables, including blood pressure, blood gases, plasma glucose, and hematocrit, were recorded. Regional cerebral blood flow was measured at the onset and end of ischemia, the onset of reperfusion, and immediately before decapitation by use of a laser Doppler probe situated over the core and penumbral regions of the insult.

**Drug Treatments**

In all experiments, z-VAD.FMK and z-DEVD.FMK (Enzyme Systems Products) were dissolved in 0.3% dimethylsulfoxide (DMSO; MCB Chemical) prepared with 0.1 mol/L phosphate-buffered saline. These drugs (z-VAD, z-DEVD), or control solution (DMSO) were injected intracerebroventricularly under halothane anesthesia at a location 1.0 mm posterior to the bregma, 0.95 mm right of the sagittal line, 4.2 to 4.7 mm deep from the dorsal surface of the rat skull. The needle was left in place for \textasciitilde{}3 minutes after injection.

**Global Ischemia**

Three separate experiments, each with DMSO-, z-VAD-, and z-DEVD-treated groups, were carried out in global ischemia. In the first experiment, 2 \textmu L of solution, either DMSO control (n = 11), z-VAD (320 ng; n = 9, plus 1 that died on day 4), or z-DEVD (320 ng; n = 9) was injected immediately after ischemia. In the second experiment, during which brain temperature was measured in \textasciitilde{}50% of the rats, 1 \textmu L of solution, either DMSO control (n = 13 plus 1 that died during ischemia), z-VAD (200 ng; n = 17 plus 1 that died during ischemia), or z-DEVD (200 ng; n = 16 plus 1 that died during ischemia), was injected 15 minutes before ischemia and again at reperfusion. In a third experiment, a higher dose of z-DEVD (z-DEVD-HD, 1.5 \mu g \times 3; n = 9) at 30 minutes before ischemia and 2 hours and 24 hours after ischemia or DMSO (DMSO-HD, n = 4) was injected at similar time points. However, this experiment was terminated prematurely because of high mortality in the z-DEVD-HD group.

**Focal Ischemia**

Three separate experiments were done. In the first, 2 \mu L of DMSO (n = 7), z-VAD (320 ng; n = 7), and z-DEVD (320 ng; n = 7) was given at the time of reperfusion. In the second experiment, 1 \mu L of DMSO (n = 8), z-VAD (200 ng; n = 7), or z-DEVD (200 ng; n = 9) was injected 3 times: at 60 minutes after onset of ischemia and at 30 and 120 minutes after onset of reperfusion. In the third experiment, during which telemetry core temperature probes were used, a dosing regimen similar to that in the second experiment was used; in addition, a dose was given at 30 minutes before ischemia (DMSO, n = 7; z-VAD, n = 6; z-DEVD, n = 7, and treatment with z-DEVD with regulated normothermia [z-DEVD-REG], n = 9).

**Temperature Measurement/Control**

In both global ischemia experiments and the first 2 focal studies, core temperature was maintained near 37.0°C during ischemia with a feedback-controlled infrared lamp (model 73A, YSI). The third focal ischemia experiment used implanted core temperature probes (model XM-FH-BP, Mini-Mitter Co). The implantation of the temperature probes has been described.\textsuperscript{36,37} Briefly, temperature probes were implanted in the brain temperature probes was used, a dosing regimen similar to that in the second experiment was used; in addition, a dose was given at 30 minutes before ischemia (DMSO, n = 7; z-VAD, n = 6; z-DEVD, n = 7, and treatment with z-DEVD with regulated normothermia [z-DEVD-REG], n = 9).

**Assessment of Ischemic Injury**

**4-VO Model**

At 7 days after global ischemia, animals were reanesthetized with halothane and trancairdially perfused with heparinized saline fol-
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hematoxylin and eosin. Remaining viable neurons (not eosinophilic) bregma. These data were expressed as percentage dead based on between sections.

farcted area of all sections and multiplying by the interval thickness previous counts from normal animals.

significantly different (F 2,26 $\text{SD}$). Contrast was used after a significant main effect. All data are the data analysis. Data were examined with ANOVA, and specific

only by 4% buffered formaldehyde. Brains were left in situ at 2°C for 24 hours before extraction from the skull. After paraffin embedding, coronal sections 7 $\mu$m thick were cut and stained with hematoxylin and eosin. Remaining viable neurons (not cosinophilic) were counted in the hippocampal CA1 area $\approx$3.3 mm posterior to the bregma. These data were expressed as percentage dead based on previous counts from normal animals.

MCAo Model

Rats were decapitated after brief halothane anesthesia 24 hours after the onset of ischemia (ie, 22.5 hours of reperfusion). The brains were then immediately removed and frozen in $-80^\circ$C isopentane. Serial (every 25th) brain sections 20 $\mu$m thick were cut and later stained with hematoxylin and eosin. The area of infarcted neocortex was traced by use of image analysis (Image Pro II; Media Cybernetics). The infarct volumes (mm$^3$) were calculated by summing the infarcted area of all sections and multiplying by the interval thickness between sections.

Statistical Analysis

Only animals that survived to the scheduled time were included in the data analysis. Data were examined with ANOVA, and specific contrast was used after a significant main effect. All data are presented as mean±SD.

Results

Global Ischemia

Hippocampal CA1 sector cell counts (% dead) were not significantly different (F$\text{2,26}=1.86$, $P=0.1755$) among DMSO, z-VAD, and z-DEVD groups in experiment 1 (Figure 1). Likewise, these treatments had no significant effects in experiment 2 (F$\text{2,26}=1.93$, $P=0.1575$), and there were no notable differences between those with and without brain temperature probes. However, the z-DEVD group displayed a higher postischemic temperature than the other groups (Figure 2). Finally, a third experiment attempted to determine the efficacy of a much higher dose of z-DEVD. Because 4 of 8 DEVD-HD–treated rats died after ischemia compared with 0 of 3 DMSO-HD rats, this experiment was terminated. An additional 2 rats died during ischemia in this experiment, 1 in the DEVD-HD group and 1 because of artery rupture in the DMSO-HD group. Regardless, CA1 cell counts in the surviving animals showed no protective effect (z-DEVD-HD 87.9±6.5% versus DMSO-HD 89.3±3.4%, mean±SD).

Focal Ischemia

Data from the physiological measurements immediately before and after ischemia are presented in the Table; there are no notable differences between groups. Similarly, blood flow estimations with laser-Doppler flowmetry in both core and peripheral territories indicated severe ischemia ($\approx$10% of baseline), with similar recovery after ischemia in all groups. The inhibitors did not influence baseline blood flow.

The DMSO, z-VAD, and z-DEVD groups were not significantly different (volume of infarction; Figure 3) in experiment 1 (F$\text{2,18}=2.19$, $P=0.1409$) or experiment 2 (F$\text{2,21}=0.33$). In the third experiment, z-VAD (F$\text{1,25}=7.58$, $P=0.0108$) and z-DEVD (F$\text{1,25}=5.26$, $P=0.0305$) did significantly reduce the volume of infarction versus DMSO-treated rats. Notably, the z-DEVD rats were warmer after ischemia than either z-VAD– or DMSO-treated rats (Figure 4). z-DEVD-REG treatment revealed a significant protective effect versus DMSO (F$\text{1,25}=9.38$, $P=0.0052$), but this was not significantly better in than the z-DEVD group rats, which were allowed to become hyperthermic (F$\text{1,25}=0.40$).

Control (DMSO) groups had significantly less injury in experiment 1 than in the second ($P=0.0017$) and third studies ($P=0.0007$). There were no physiological differences (eg, cerebral blood flow, pH, etc) to account for this. Notably, rats in experiment 1 were obtained from Taconic Farms, whereas those in subsequent experiments were from Charles River.

Discussion

The irreversible caspase inhibitors z-VAD and z-DEVD significantly reduced cortical infarction at 1-day survival. This is consistent with studies in mice23,27,29,30 and rats26 that have found a reduction in infarction after MCA occlusion with these caspase inhibitors. Thus, the elevations in caspases that follow focal ischemia23,27,28 appear to contribute to ischemic injury after transient MCA occlusion. Protection occurred only with the highest total dose that was given as a preischemic and postischemic dosing regimen. Higher doses might also protect in this model when given solely in the...
Caspases are also activated after brief forebrain ischemia. However, we were unable to demonstrate any significant protective efficacy of z-VAD or z-DEVD when given before and/or after a moderate 4-VO ischemic insult despite the use of several dosing regimens, including a high dose of z-DEVD previously found to be maximally neuroprotective in the rat 4-VO model and to substantially block caspase-3 activity in the hippocampus. Himi et al also found that z-DEVD was protective in gerbils subjected to brief global ischemia.

The many differences between the studies by Chen et al and Himi et al and the present study make it difficult to explain why we did not observe protection. One could argue that other dosing regimens of z-VAD or z-DEVD might be neuroprotective in CA1 after this or especially after briefer 4-VO ischemia. However, the present failure to find any protection is in agreement with recent ultrastructural studies that failed to find CA1 cell apoptosis after 5 or 15 minutes of 4-VO ischemia in the rat and after untreated and hypothermia-treated ischemia (with some slowed CA1 cell death) in the gerbil. Thus, although z-VAD and z-DEVD might reduce CA1 injury after briefer ischemia (eg, 5 minutes’ duration), the morphological findings of necrosis after either 5 or 15 minutes of 4-VO ischemia suggest otherwise.

The novel finding that z-DEVD–treated rats experienced persistent hyperthermia after either global or focal ischemia was expected to dampen the efficacy of this compound. However, no additional protection was observed in z-DEVD–treated MCAo animals that had regulated normothermia. The method used to cool rats is not without side effects; it induces a stress response (shivering, etc) that may have counteracted any positive benefit of the mild temperature reduction. In addition, given the known aggravating effects of posts ischemic hyperthermia after global and focal cerebral ischemia, it is possible that hyperthermia may have confounded other studies. Surprisingly, z-DEVD given intracerebroventicularly after global ischemia causes hyperthermia in gerbils. Although hyperthermia occurred after our low-dose treatments, some rats that were repeatedly treated with a high dose of z-DEVD after 4-VO ischemia experienced

<table>
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<th>P_{O_2}, mm Hg</th>
<th>P_{O_2}, mm Hg</th>
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delayed hypothermia, often concomitant with other ill effects and eventual death. Perhaps the differences between these studies are due to both species and dosing effects. Importantly, Chen et al.21 did not rule out confounding protection by postischemic hypothermia, which, by itself, has repeatedly been found to attenuate CA1 injury.14,33,37

Administration of z-DEVD caused a greater rise in temperature than z-VAD. It is possible that both solutions invoked a fever response, but the greater inhibition of caspase 1 (interleukin-1β-converting enzyme) by z-VAD would be expected to attenuate this fever more than z-DEVD. Accordingly, nonselective caspase inhibitors, which have previously been shown to have greater efficacy over selective inhibitors,27 may exert better protection through anti-inflammatory and antipyretic mechanisms.

The anti-inflammatory effects (eg, reduced edema27) of these caspase inhibitors, and not necessarily antiapoptotic effects, might account for the protection offered by these compounds. A similar argument was made by Hara and colleagues31 in an experiment that showed that transgenic mice with reduced IL-1β levels had smaller infarcts and less edema. At this time, the fact that protection was found only in focal ischemia and not in forebrain ischemia argues for this, because inflammatory mechanisms (eg, edema, macrophages, etc) play a larger role after focal ischemia.

One limitation of our study was that we assessed only a 1-day survival after MCAo. Focal ischemic injury7,40 may not necessarily mature as quickly as initially thought. Thus, our findings of reduced cortical infarction at 1 day may have markedly overestimated true benefit. Although lasting protection (21 days) was achieved with z-DEVD given after brief focal ischemia in the mouse,23 this may not be the case in the more severe insult used here. Accordingly, studies using long survival times (eg, 2 months) are needed. Several studies2,13,41 also show that global ischemic injury can mature more slowly than initially thought. Thus, the early protection (7 to 8 days) observed with caspase inhibitors in global ischemia in the rat21 and gerbil25 may simply disappear at longer survival times.

In summary, our findings show that both z-VAD and z-DEVD can, at least temporarily, reduce ischemic injury after MCAo (90 minutes) but not after a moderate-length forebrain insult (10 minutes of 4-VO). Thus, caspase activation appears to contribute to ischemic injury after moderately severe focal ischemia in the rat clip model. It is unknown whether this is via an antiapoptotic effect or some other means (eg, anti-inflammatory). A surprising finding was that both z-VAD and z-DEVD can modify temperature, with, for example, hyperthermia occurring after lower doses of z-DEVD in global and focal ischemia animals and hypothermia observed in some 4-VO rats treated with a high dose. Thus, prolonged temperature measurement is necessary in future studies.

Acknowledgments

Research funding was provided by the Heart and Stroke Foundation of Canada and the Alberta Heritage Foundation for Medical Research in grants awarded to Dr Buchan.

References

Cerebral ischemic cell death has traditionally been considered a direct consequence of energy failure resulting in cell necrosis, a process characterized by cell swelling and membrane dissolution. However, evidence accumulated during the past decade suggests that this is not always the case. There are instances in which ischemic cell death results from an orderly sequence of molecular events driven by gene expression and protein synthesis, a process termed programmed cell death or apoptosis and best characterized in the nematode C. elegans. That programmed cell death may occur in cerebral ischemia was first hinted at by studies demonstrating that inhibition of protein synthesis protects hippocampal CA1 neurons after transient global ischemia. Subsequently, it was found that intraneuronal DNA fragmentation, one of the hallmarks of apoptosis, occurs after focal or global ischemic injury. Furthermore, transgenic mice that overexpressed gene prod-
ucts opposing programmed cell death were protected from cerebral ischemia (eg, Reference 5). Finally, inhibition of caspases, a family of cysteine proteases thought to be “executioners” of apoptotic cell death, reduces ischemic injury6–9 (see Reference 10 for a review). These observations, collectively, suggest that the mechanisms by which cerebral ischemia kills brain cells may also include processes resembling classical apoptosis.

In the accompanying paper, Li and colleagues examine the effect of caspase inhibition in rodent models of global and focal cerebral ischemia. Using a well-controlled experimental protocol, they found that caspase inhibitors reduce ischemic damage in focal but not in global cerebral ischemia. The results suggest fundamental differences in the role of caspases, and possibly apoptosis, in the mechanisms of cell death initiated by focal and global ischemia.

Studies on the role of programmed cell death in global cerebral ischemia have not been conclusive. While DNA fragmentation and caspase activation occur in vulnerable regions after transient global ischemia, typical morphological features of apoptotic cell death have not been observed at the ultrastructural level, despite intense efforts.11 The study of Li et al, by suggesting that the death of CA1 neurons is caspase independent, supports the notion that programmed cell death does not occur in the posts ischemic hippocampus. On the other hand, caspase inhibitors have been found to be protective in another careful study of global ischemia.7 The reasons for the discrepancy in the results of these investigations are not clear, and the issue requires further inquiry. Perhaps mutant mice lacking one of the caspases or other genes critical to the expression of apoptosis may be useful to provide additional experimental evidence for or against a role of programmed cell death in global cerebral ischemia.

A more general issue concerns the biological significance of apoptosis in the postischemic brain. Is apoptosis meant to eliminate dysfunctional neurons that are no longer viable? If so, rescuing apoptotic neurons would not help the long-term outcome of the ischemic brain. Furthermore, what is the relationship between apoptosis and functional recovery? If elimination of selected injured neurons is needed to “reprogram” the brain after injury, then apoptosis should not be seen as destructive but as a process necessary for recovery of function. Investigations addressing these issues are eagerly awaited.

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References
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Stroke. 2000;31:176-182
doi: 10.1161/01.STR.31.1.176

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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