Attenuation of Ischemia/Reperfusion Injury in Rats by a Caspase Inhibitor

Hiroyuki Yaoita, MD; Kazuei Ogawa, MD; Kazuhira Machara, MD; Yukio Maruyama, MD

Background—Z-Val-Ala-Asp(OMe)-CH₂F (ZVAD-fmk), a tripeptide inhibitor of the caspase interleukin-1β–converting enzyme family of cysteine proteases, may reduce myocardial reperfusion injury in vivo by attenuating cardiomyocyte apoptosis within the ischemic area at risk.

Methods and Results—Sprague-Dawley rats were subjected to a 30-minute coronary occlusion followed by a 24-hour reperfusion. An inert vehicle (dimethylsulfoxide; group 1, n=8) or ZVAD-fmk, at a total dose of 3.3 mg/kg (group 2, n=8), was administered intravenously every 6 hours starting at 30 minutes before coronary occlusion until 24 hours of reperfusion. At this 24-hour point, hemodynamics were assessed by means of cardiac catheterization; then, the rats were killed, and the left ventricle was excised and sliced. The myocardial infarct size/ischemic area at risk and the count of presumed apoptotic cardiomyocytes (terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling [TUNEL]-positive cells) within the ischemic area at risk were assessed through triphenyltetrazolium chloride staining and TUNEL methods, respectively. Peak positive left ventricular dP/dt was higher (P=.02) and left ventricular end-diastolic pressure was lower (P=.04) in group 2 than in group 1. The infarct size/ischemic area at risk of group 2 (52.4±4.0%) was smaller (P=.02) than that of group 1 (66.6±3.7%), and TUNEL-positive cells were fewer (P=.0002) (group 2, 3.1±0.9%; group 1, 11.1±1.0%). Agarose gel electrophoresis revealed DNA laddering in the border zone myocardium of group 1, but DNA ladder formation was attenuated in group 2.

Conclusions—ZVAD-fmk was effective in reducing myocardial reperfusion injury, which could at least be partially attributed to the attenuation of cardiomyocyte apoptosis. (Circulation. 1998;97:276-281.)

Key Words: myocardium • reperfusion • apoptosis • caspase

Attempts to reduce the extent of myocardial reperfusion injury have included lowering the risk posed by certain injurious factors and potentiating various aspects of cardioprotection relating to ischemic duration,1 oxygen free radicals,2,3 proinflammatory cytokines,4,5 and preconditioning.6–10 It has been reported that apoptosis is a significant contributor to myocardial cell death as a result of reperfusion injury.1 Therefore, it might be hypothesized that this type of injury could be attenuated if a portion of the injured myocardial cells could be rescued from an apoptotic death.

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The caspase inhibitors, that is, ICE-like protease inhibitors,11 interfere with apoptosis at a point subsequent to the initiation of the proapoptotic process in cells that have already received apoptosis-promoting signals. As opposed to reducing the exposure of cardiomyocytes to injurious stimuli, apoptosis of these cells is attenuated through modulation of the caspase-related proapoptotic process, and this may allow ischemic myocardium to survive even after receiving significant injury. ZVAD-fmk (fluoro-methylketone), a tripeptide inhibitor of the caspase, is reported to attenuate cardiomyocyte apoptosis in vitro.5 In the present study, we investigated whether ZVAD-fmk lowers the extent of experimental myocardial reperfusion injury in vivo by attenuating cardiomyocyte apoptosis. In a rat model for myocardial reperfusion injury, infarct size and the appearance of presumed apoptotic cardiomyocytes were assessed in two groups that were or were not administered this protease inhibitor.

Methods

This study was carried out under the supervision of the Animal Research Committee in accordance with the Guideline on Animal Experiments of Fukushima Medical College and Japanese Government Animal Protection and Management Law (No. 105).

Animal Model

Twenty-six of 36 adult male (290 to 310 g body weight) Sprague-Dawley rats were anesthetized through intraperitoneal administration of 30 mg/kg sodium pentobarbital. Under artificial ventilation with a rodent ventilator, a left thoracotomy was performed. The proximal portion of the left coronary artery was surgically occluded for 30 minutes through ligation with a suture (size 6.0) followed by coronary reperfusion through release of the tie. Coronary occlusion was confirmed through elevation of the ST segment on the ECG obtained from a limb lead. Transient ventricular arrhythmias were evoked in all...
After hemorrhagic shock, rats were anesthetized again through intraperitoneal administration of 30 mg/kg sodium pentobarbital (PE 50; Becton-Dickinson) and were inserted into the left ventricular cavity via the right carotid artery. ECG readings were monitored, and a polyethylene tube was inserted into the left ventricular cavity for perfusion with 0.3 EU/mL DMSO. In group 2 animals (n=8), ZVAD-fmk, at one fourth of a total dose of 3.3 mg/kg body weight, was administered as a bolus into the tail vein four times during the study (first 30 minutes before coronary occlusion and then three times every 6 hours after reperfusion). The same amount of an inert vehicle (DMSO) was administered in the same manner to rats of group 1 (n=8).

To assess whether the amount of DMSO used as a vehicle would have a toxic effect in vivo, one fourth of a total DMSO volume of 353 µL/kg body weight (n=5) or the same volume of saline (n=5) was administered four times to sham-operated rats in the same manner as to groups 1 and 2.

Leukocytes are known to be involved in the formation of myocardial reperfusion injury. As a positive control for this model of coronary reperfusion injury, 4 rats were administered absorbed polymorphonuclear leukocyte PMN antisera at a dose of 3 mL/kg (Inter-Cell Technologies) 36 hours before coronary occlusion. Each was subjected to the 30-minute coronary occlusion and 24-hour reperfusion protocol, and 0.5 mL of blood was taken before occlusion and just before death.

**Hemodynamic Assessment**

Twenty-four hours after coronary reperfusion, rats were anesthetized again through intraperitoneal administration of 30 mg/kg sodium pentobarbital. ECG readings were monitored, and a polyethylene tube equipped with a 25-square grid (Integration No. 1; Zweiss) under light microscopy and visualized with the use of chromogen 3,3’-diaminobenzidine (Dainippon) and H2O2. Counterstaining was performed with 0.3% methyl green. Using this method, each cardiomyocyte could be defined, and TdT-positive or -negative nuclei were stained dark brown or light green, respectively, under light microscopy. When the TUNEL method was performed, positive controls were always included. For DNase treatment in situ, sections were processed with proteinase K, and peroxidase inactivation was carried out as described above. Next, the sections were pretreated with DN buffer (30 mmol/L Tris-HCl, pH 7.2, 140 mmol/L sodium cacodylate, and 1 mmol/L cobalt chloride). The reaction was terminated with buffer containing 300 mmol/L NaCl and 30 mmol/L sodium citrate. The slides were coated with avidin-conjugated peroxidase (Medical and Biological Laboratories) diluted 1:3000 in PBS and visualized with the use of chromogen 3,3’-diaminobenzidine (Dainippon) and H2O2. Counterstaining was performed with 0.3% methyl green. Using this method, each cardiomyocyte could be defined, and TdT-positive or -negative nuclei were stained dark brown or light green, respectively, under light microscopy. When the TUNEL method was performed, positive controls were always included. For DNase treatment in situ, sections were processed with proteinase K, and peroxidase inactivation was carried out as described above. Next, the sections were pretreated with DN buffer (30 mmol/L Tris-HCl, pH 7.2, 140 mmol/L sodium cacodylate, 4 mmol/L MgCl2, and 0.1 mmol/L diethiothreitol); then, DNase I (Sigma) at 100 ng/mL was dissolved in this buffer and used to cover each section. After a 15-minute incubation at room temperature, the slides were washed extensively with double-distilled water, and DNA nick end labeling was carried out.

Using an eyepiece for the point-counting method (Integration No. 1, Zweiss), which was performed under a light microscope at a magnification of 400x, we determined the count ratio of the area of cardiomyocytes with TdT-stained nuclei with that of total cardiomyocytes (TUNEL-positive cardiomyocytes) within the ischemic area at risk. The entire area was searched through an orderly shifting of the visual field using the outer grids of the eyepiece for orientation. TUNEL-positive cardiomyocytes were carefully distinguished from TUNEL-negative noncardiomyocytes, such as macrophages.

To assess the distribution of the infarcted area and TUNEL-positive cardiomyocytes in the left ventricular wall, we subdivided the ischemic area at risk into three transmural stratified layers of equal thickness (epicardial, middle, and endocardial) in each slice mentioned above. Using some of the paraffin sections of groups 1 and 2, hematoxylin and eosin staining was also performed for confirmation of myocardial reperfusion injury, such as myocardial cell coagulation, contraction bands, bleeding, and inflammatory cell infiltration.

**Genomic DNA Extraction and Agarose Gel Electrophoresis**

Rats subjected to the same occlusion and reperfusion protocol as groups 1 and 2, respectively (n=3 each group), had their hearts excised at 24 hours after reperfusion, and underperfused myocardium was delineated using Evans blue. The excised heart was sliced immediately as described above. Because TUNEL-positive cardio-

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**Selected Abbreviations and Acronyms**

- DMSO = dimethylsulfoxide
- ICE = interleukin-1β-converting enzyme
- I/R = infarct size/ischemic area at risk
- (±)-LV dp/dt = peak positive (++) and negative (--) first derivatives of left ventricular pressure
- LVEDP = left ventricular end-diastolic pressure
- LVSP = left ventricular peak systolic pressure
- PMN = polymorphonuclear leukocyte
- TdT = terminal deoxynucleotidyl transferase
- TTC = triphenyltetrazolium chloride
- TUNEL = terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling
- ZVAD-fmk = Z-Val-Ala-Asp(OMe)-CH2F

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**Assessment of Infarcted Area and Detection of TUNEL-Positive Cardiomyocytes**

After hemorrhagic shock, rats were anesthetized again through intraperitoneal administration of 30 mg/kg sodium pentobarbital. ECG readings were monitored, and a polyethylene tube was inserted into the left ventricular cavity via the right carotid artery. LVSP, LVEDP, and (±)-LV dp/dt were measured using a polygraph system (AP601G; Nihon Koden).

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DNA was extracted with phenol and chloroform followed by ethanol precipitation. The pellet was resuspended in TE buffer (10 mmol/L Tris-HCl, pH 8.0, and 1 mmol/L EDTA) and treated with DNase-precipitation. The gel was photographed with the use of a Polaroid camera.

Hemograms

White blood cell counts just before death revealed no difference between group 1 (952 ± 351/μL) and group 2 (9350 ± 435/μL). In anti-PMN-treated rats, white blood cell counts were 956 ± 132/μL (P < .0001 versus group 1 and group 2) before coronary occlusion and 1081 ± 156/μL (P < .0001 versus group 1 and group 2) just before death. In this positive control group, lymphocytes made up most of the white blood cells (>99%).

Statistical Analysis

Data are expressed as mean ± SEM. To compare group 1 (control ischemia/reperfusion) with group 2 or to compare the two groups of sham-operated rats, administration of DMSO or saline resulted in no differences in LVSP/EDP or LV dP/dt value or in the heart rate.

Results

Hemodynamics

Although the LVSP did not differ between groups 1 and 2, the LVEDP of group 2 was lower (P = .04) than that of group 1 (Table). The positive LV dP/dt of group 2 was greater (P = .02) than that of group 1, but the heart rates of the two groups did not differ.

For the sham–operated rats, administration of DMSO or saline resulted in no differences in LVSP/EDP or LV dP/dt value or in the heart rate.

Myocardial Infarct Size and TUNEL-Positive Cardiomyocytes

The ischemic areas at risk of groups 1 and 2 were similar (53.9 ± 2.5% in group 1 and 55.4 ± 3.0% in group 2, NS). In the entire ischemic area at risk, the I/R of group 2 (52.4 ± 4.0%) was significantly (P = .02) smaller than that of group 1 (66.6 ± 3.7%) (Fig 2, left). The I/Rs of left and right lateral border segments or endocardial and epicardial layers were smaller (P < .05, < .05, or P < .05, < .01, respectively) than that of the central segment or that of the middle layer in group 1 (Fig 3). In group 2, the I/Rs of all of three myocardial segments and all of three layers were smaller (P < .05, each) than those of group 1. We confirmed that all nuclei of cardiomyocytes on sections subjected to DNase treatment (as a positive control for the TUNEL negative cardiomyocytes) showed a reduced TUNEL signal compared to the nuclei in sections subjected to DNase treatment in the absence of PMN.

Statistical Analysis

Data are expressed as mean ± SEM. To compare group 1 (control ischemia/reperfusion) with group 2 or to compare the two groups of sham-operated rats, an unpaired t test was performed. For comparisons between the positive anti-PMN control and the other groups, one-way ANOVA followed by Fisher’s posthoc comparison was carried out. For comparisons in I/R and TUNEL-positive cardiomyocytes among different myocardial portions, two-way ANOVA followed by Fisher’s posthoc comparison was carried out. A value of P < .05 was considered statistically significant.
Method) were stained dark brown each time the TUNEL method was performed. The concentration of the TUNEL-positive cardiomyocytes of group 2 (3.1±0.9%) was significantly (P=0.0002) less than that of group 1 (11.1±1.0%) (Fig 2, right). In group 1, TUNEL-positive cardiomyocytes were greater in left and right lateral segments (P<0.05, <0.01, respectively) than in the central segment and greater in the epicardial layer (P<0.01) but smaller in the epicardial layer (P<0.01) than in the middle layer (Fig 4). In group 2, TUNEL-positive cardiomyocytes of all of three segments (P<0.01, each) and of endocardial, middle, and epicardial layers (P<0.01, <0.01, <0.05, respectively) were smaller than those of group 1 (Figs 4 and 5). Therefore, there were no significant differences of TUNEL-positive cardiomyocytes in group 2 among the three myocardial segments or three myocardial layers (Fig 4).

Neither TTC-negative zones nor TUNEL-positive cardiomyocytes were detected in the sham-operated rats administered DMSO or saline.

![Figure 2](image1.png)

**Figure 2.** Infarct size and TUNEL-positive cardiomyocytes in the entire underperfused and then reperfused area. Left, I/R. Right, TUNEL-positive cardiomyocytes in the ischemic area at risk. The column representing the infarcted area was lower (P=0.02) for group 2 than for group 1. The counts of TUNEL-positive cardiomyocytes in group 2 were lower (P=0.0002) than in group 1. Group 1, infarcted rats (n=8) administered vehicle; group 2, infarcted rats (n=8) treated with ZVAD-fmk at a total dose of 3.3 mg/kg.

![Figure 3](image2.png)

**Figure 3.** The I/R in myocardial segments or myocardial layers. In group 1, the I/R was smaller in left and right lateral border segments (P<0.05, respectively) than the central segment (left). Furthermore, in this group, the I/R was smaller in endocardial and epicardial layers (P<0.05, <0.01, respectively) than the middle layer (right). In group 2, the I/R of three myocardial segments (left) and of three myocardial layers (right) was smaller than that of the corresponding segments or layers of group 1 (P<0.05, respectively).

![Figure 4](image3.png)

**Figure 4.** The TUNEL-positive cardiomyocytes in myocardial segments or myocardial layers within the ischemic area at risk. In group 1, TUNEL-positive cardiomyocytes of left and right lateral border segments (left) were greater than that of the central segment (P<0.05, <0.01, respectively). In this group, TUNEL-positive cardiomyocytes of the endocardial layer or those of the epicardial layer were greater (P<0.01) or smaller (P<0.05) than those of the middle layer, respectively (right). In group 2, TUNEL-positive cardiomyocytes were smaller than those of group 1 in all of three myocardial segments and three myocardial layers (P<0.05 or <0.01).

**Agarose Gel Electrophoresis**

DNA laddering indicative of fragmented DNA was clearly demonstrated in myocardial specimens sampled from the lateral border zones and the endocardial side of the ischemic area at risk in group 1 (lane 4) but was attenuated in group 2 (lane 3), as shown in Fig 6. DNA laddering in the core of infarction was attenuated in group 1 (lane 2) and was absent in group 2 (lane 1).

**Discussion**

The present study revealed that administration of ZVAD-fmk reduced both the size of the myocardial infarct, as assessed through TTC staining, and the number of TUNEL-positive cardiomyocytes, with significant hemodynamic improvement in vivo in rats that underwent the 30-minute coronary occlusion and 24-hour reperfusion procedure. TUNEL-positive cardiomyocytes appeared to be apoptotic in this study because well-defined (group 1) or attenuated (group 2) DNA laddering on electrophoresis was consistent with a higher or lower value of TUNEL-positive cardiomyocytes, respectively, in the ischemic area at risk of the two groups. In a preliminary study using frozen sections, we confirmed that none of the TUNEL-positive cardiomyocytes were stained with TTC. Therefore, a reduction in their number appeared to contribute to a reduction in the myocardial infarct size. These results suggested that ZVAD-fmk was effective in reducing myocardial reperfusion injury, which could be at least partially attributed to the attenuation of cardiomyocyte apoptosis.

ZVAD-fmk achieved ∼21% decrease in the I/R and ∼72% decrease in TUNEL-positive cardiomyocytes, as ratios compared with the control ischemia/reperfusion. However, the absolute value for decrease in TTC unstained area (∼14%) appeared somewhat greater than that of TUNEL-positive cardiomyocytes (∼8%) (Fig 2); we must be careful to simply compare the absolute values of TUNEL-positive cardiomyocytes with the I/R because the methodology for quantification...
was not the same between TTC staining (histochemical area measurement on myocardial slices) and the TUNEL method (histological cell counting on paraffin sections). Furthermore, we cannot exclude the possibility that ZVAD-fmk interferes with myocardial necrotic process as well as the apoptotic process. Tsujimoto and colleagues recently revealed that ICE inhibitors retarded necrotic cell death as well as apoptotic cell death in their in vitro system of chemical hypoxia. The authors speculated that there was possible involvement of common mediators in apoptotic and necrotic signal transductions, although their detailed mechanisms remain to be determined. In the present study, we might have observed effects of ZVAD-fmk on these possible but undetermined common mediators. However, our examination in an in vivo system is not suited for approach to signal transductions of these two forms of cell death. The third possibility is the difference in time from initiation of cellular change until elimination between apoptosis and other types of cell death, both forming the infarction. Apoptotic cells are eliminated through phagocytosis in a few minutes in an in vitro condition and in a few hours in an in vivo condition. In contrast, necrotic cardiomyocytes are eliminated much slowly by infiltrating inflammatory cells. Although a turnover of apoptotic cardiomyocytes in vivo has not been clarified so far, it may be speculated that the amount of TUNEL-positive cells quantified at a death stage may not equal the total amount of apoptotic cardiomyocytes that appear during a 24-hour reperfusion period.

To date, nothing is known about the fate of cardiomyocytes that have been exposed to ZVAD-fmk but have not undergone a proapoptotic process, such as initiation of apoptotic signal transduction via TNF receptor. These cardiomyocytes may continue to survive or may undergo an early death because of injury already sustained. Myocardial infarct size was assessed only at 24 hours after reperfusion in the present study. Future studies will be needed to evaluate the viability of cardiomyocytes that escape apoptosis through assessment of infarct extension in the later phase of reperfusion.
References


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