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

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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=.002) in 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
rats ~5 minutes after coronary occlusion, but these usually disappeared after 10 minutes of occlusion. After coronary reperfusion, the tie was left loose on the surface of the heart, the chest was closed, and the intratracheal tube and ECG electrodes were removed. The rats were returned to their cages, where they awakened, and they were allowed free access to food and water until they were killed 24 hours later. One milligram of ZVAD-fmk (Enzyme Systems Products) was dissolved in 107 μL of DMSO (Wako Pure Chemicals). 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.2 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 (PE 50; Becton-Dickinson) 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). **Assessment of Infarcted Area and Detection of TUNEL-Positive Cardiomyocytes** After hemodynamics were assessed at 24 hours of coronary reperfusion, 0.5 mL of blood was obtained from the catheter for measurement of blood cells. Then, an intratracheal tube was inserted, and the chest was reopened under artificial ventilation. The coronary artery was again briefly occluded through ligation of the tie that remained at the site of the previous occlusion. Immediately after the ligation, 1% Evans blue solution was infused through the catheter into the beating left ventricular cavity to delineate the ischemic area at risk (underperfused and then reperfused area) of the left ventricle. After administration of an excessive dose of sodium pentobarbital into the left ventricular cavity, the heart was excited and cross-sectioned from the apex to the atrioventricular groove into five specimens of ~2 mm in thickness with the use of a stereoscope. Because there may be some anatomic differences in the left coronary artery of each rat, the three middle slices were prepared for morphometry to determine the ischemic area at risk. These slices were incubated with a 4% TTC solution for 30 minutes at 37°C in a dark room. Then, ischemic but viable (TTC-stained) and infarcted (TTC-unstained) zones within the underperfused and then reperfused area (Evans blue–unstained) and the nonischemic area (Evans blue–stained) were stereoscopically measured using the point-counting method of Weibel31 with an eyepiece equipped with a 25-square grid (Integration No. 1; Zweiss) under 100X magnification, and I/R was calculated. These slices were then fixed in 10% neutral-buffered formalin. Using paraffin sections that were 4 μm thick, TUNEL was performed as described previously32 with minor modifications. Briefly, nuclei of tissue sections were stripped of proteins by incubation with 20 μg/mL proteinase K (Sigma Chemical) for 15 minutes at room temperature. The slides were incubated with 2% H2O2 for 5 minutes to allow inactivation of endogenous peroxidase and then incubated for 60 minutes at 37°C with 0.3 EU/μL TdT (Takara Schuzo Co) and 0.04 mmol/μL biotinylated dUTP (Boehringer-Mannheim Biochemica) in TdT buffer containing 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 (Dojindo) and H2O2. Counterstaining was performed with 2% methyl green. Using this method, each cardiomyocyte could be defined, and TUNEL-positive 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,33 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 K cacodylate, 4 mmol/L MgCl2, and 0.1 mmol/L dithiothreitol); 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 (Fig 1). We also divided the ischemic area at risk into five radial segments, and then these five radial segments were rearranged as (Fig 1) a right lateral border segment adjacent to the interventricular septum; a total of three central segments; and a left lateral border segment adjacent to the left ventricular posterior wall. For each of the segments or layers, I/R and TUNEL-positive cardiomyocytes were calculated, as well as for the entire ischemic area at risk. 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 delinated using Evans blue. The excised heart was sliced immediately as described above. Because TUNEL-positive cardio-
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Figure 1. Myocardial segments and layers for assessment of distribution of the infarcted area and TUNEL-positive cardiomyocytes. The three middle slices of the five left ventricular slices of each heart were analyzed for measurements of the infarcted area and TUNEL-positive cardiomyocytes. The entire ischemic area at risk was first divided into five radial segments and then classified into three segments (right lateral border, central three radial segments, and left lateral border segment). The ischemic area at risk (R) was also divided into three myocardial layers (endocardial, middle, and epicardial layers). The measurements of I/R and TUNEL-positive cardiomyocytes were done in the entire ischemic area at risk, as well as in each ischemic portion.

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.

Results

Hemograms
White blood cell counts just before death revealed no difference between group 1 (9502±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%).

Positive Control for the Rat Model of Reperfusion Injury
The ischemic area at risk was 53.0±2.5% (NS versus group 1 and group 2), and the I/R was 51.0±1.7% (P<.05 versus group 1, NS versus group 2).

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±2.9% 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±5.7%) (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...
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=.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<.05, <.01, respectively) than in the central segment and greater in the endocardial layer (P<.01) but smaller in the epicardial layer (P<.01) than in the middle layer (Fig 4). In group 2, TUNEL-positive cardiomyocytes of all of three segments (P<.01, each) and of endocardial, middle, and epicardial layers (P<.01, <.01, <.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 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. 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=.02) for group 2 than for group 1. The counts of TUNEL-positive cardiomyocytes in group 2 were lower (P=.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. 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<.05, respectively) than the central segment (left). Furthermore, in this group, the I/R was smaller in endocardial and epicardial layers (P<.05, <.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<.05, respectively).

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<.05, <.01, respectively). In this group, TUNEL-positive cardiomyocytes of the endocardial layer or those of the epicardial layer were greater (P<.01) or smaller (P<.01) 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<.05 or <.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 the 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. 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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