Combination of necroptosis and apoptosis inhibition enhances cardioprotection against myocardial ischemia-reperfusion injury.

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Key Words: necroptosis, necrostatin-1, ischemia-reperfusion, heart

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Abstract

Purpose Necroptosis has been proposed as a mode of cell death which is a caspase-independent programmed necrosis. We investigated whether necroptosis is involved in myocardial ischemia-reperfusion injury in isolated guinea pig hearts, and if so, whether simultaneous inhibition of necroptosis and apoptosis confers an enhanced cardioprotection.

Methods Isolated perfused guinea pig hearts were subjected to 30 min ischemia and 4 h reperfusion (control=CTL, n=8). Necrostatin-1 (necroptosis inhibitor, 10 μM), Z-VAD (apoptosis inhibitor, 0.1μM) and both inhibitors were administered starting 5 min before ischemia and during the initial 30 min of reperfusion (Nec, Z-VAD, Nec+Z-VAD, n=8 each). Contractile recovery was monitored by left ventricular developed (LVDP) and end-diastolic (LVEDP) pressure. Infarct size was determined by triphenyltetrazolium chloride staining. Tissue samples were obtained after 4 hr reperfusion to determine expression of receptor-interacting protein 1 (RIP1) and activated caspase 3 by Western blot analysis.

Results After reperfusion, Nec+Z-VAD had higher LVDP and lower LVEDP compared with CTL. Infarct size was reduced in Nec and Z-VAD compared with CTL. Combination of necroptosis and apoptosis inhibition further reduced infarct size. Expression of activated caspase 3 was not increased in Z-VAD and Nec+Z-VAD compared with Nec and CTL. Expression of RIP1 was preserved in Z-VAD and Nec+Z-VAD compared with CTL, suggesting RIP1-mediated necrosis is involved in myocardial ischemia-reperfusion injury.

Conclusion Necroptosis is involved in myocardial ischemia-reperfusion injury and simultaneous inhibition of necroptosis and apoptosis enhances cardioprotective effect. These findings may provide a novel, additive strategy for cardioprotection in acute myocardial infarction.
Introduction

Acute myocardial infarction remains one of the life threatening complications during the perioperative period. Cardiomyocytes death in ischemia-reperfusion injury has been considered to be an accidental cell death resulting from excessive stress which is mainly caused by necrosis. In contrast to this unexpected death, programmed cell death, apoptosis also has been shown to be involved in myocardial ischemia-reperfusion injury [1-3]. It is a highly regulated mechanism designed to eliminate cells via caspase activation to maintain homeostasis [4]. Stimulation of the death receptors such as Fas and tumor necrosis factor-α (TNF-α) receptors normally leads to the activation of the extrinsic apoptotic pathway. However, it has been demonstrated that death receptor stimulation could still induce cell death with morphological features of necrosis in some cell types when the canonical apoptotic pathway is blocked [5]. This means the existence of regulated necrosis. Recently, as an alternative death pathway, caspase-independent programmed necrosis has been identified by Degterev et al., termed necroptosis [6]. In this pathway, the serine/threonine kinase activity of receptor-interacting protein 1 (RIP1) plays an important role in cell death and its inhibitor, necrostatin-1 has also been identified [7,8]. Necrostatin-1 is a small tryptophan-based molecule that has been shown to reduce infarct volume after middle cerebral artery occlusion following intracerebroventricular injection [6] and block TNF-α-induced necrotic cell death through inhibition of RIP1 kinase activity in Jurkat cells [9]. This drug was also reported to protect the heart against ischemia-reperfusion injury [10]. Furthermore, Wang et al. demonstrated that necrostatin-1 also suppresses apoptosis in mice traumatic brain injury [11]. These findings suggest that necrostatin-1 could represent a potential therapeutic intervention against acute myocardial infarction. As several different
pathways have been reported to be involved in myocardial ischemia-reperfusion injury, a combination therapy may be required for more efficient cardioprotection. Hence, we examined whether necroptosis is involved in myocardial-ischemia reperfusion injury in isolated guinea pig hearts and whether simultaneous inhibition of necroptosis and apoptosis by necrostatin-1 and Z-VAD-fmk (apoptosis inhibitor) confers an enhanced cardioprotection. Elucidating the involvement of necroptosis in myocardial ischemia-reperfusion injury could lead to a novel, additional strategy for cardioprotection in perioperative period.
Materials and Methods

This study was conducted in accordance with the Guidelines for Animals Research at Osaka Dental University, and with the approval of the Animal Experiment Committee of Osaka Dental University, Osaka, Japan. These guidelines conform to those laid out in the Guide for the Care and Use of Laboratory Animals, available from the National Academy of Science. Male Hartley guinea pigs were fed Lab Diet (RC4, Oriental Yeast, Tokyo, Japan) and given water ad libitum. Necrostatin-1 was purchased from Calbiochem (La Jolla, CA, USA) and was used to inhibit necroptosis. Z-VAD-fmk was purchased from PEPTIDE INSTITUTE (Osaka, Japan) and was used to inhibit apoptosis. Necrostatin-1 and Z-VAD were dissolved in dimethyl sulfoxide (DMSO), and were added to Krebs-Henseleit perfusate to give final concentrations of 10 μM and 0.1 μM, respectively and not more than 0.01% DMSO.

Isolated Heart Perfusion and Measurement of Function

Male guinea pigs weighing 550-800g (12-14 weeks old) were given heparin (1000 units intraperitoneally), then anesthetized with pentobarbital (60 mg/kg, intraperitoneally). Hearts were excised and immediately arrested in cold iso-osmotic saline containing 20 mM KCl. The aorta was cannulated and the isolated hearts were perfused at 70 mmHg on a nonrecirculating isovolumic perfused heart apparatus, using a Krebs-Henseleit (KH) perfusate: 118 mM NaCl, 4.0 mM KCl, 2.52 mM CaCl₂, 24.8 mM NaHCO₃, 1.7 mM MgSO₄, 1.2 mM KH₂PO₄, 11.0 mM glucose, 0.5 mM EDTA and 8 units/l insulin. The perfusate was insufflated continuously with 95%O₂/5%CO₂ and was filtered through
cellulose acetate membranes with a pore size of 4.0 μm to remove particulate matter. Hearts were paced at 240 beats/min using platinum-tipped electrodes connected to a Grass Instruments SD-5 stimulus generator (Grass Instruments, Quincy, MA). Left ventricular developed pressure (LVDP; mmHg) was measured using a 2.5 French, high-fidelity micromanometer (Nihon-Kohden, Tokyo, Japan) passed into a compliant latex balloon, inserted into the left ventricle, and recorded on a PowerLab 2/20 Data Recording System (ADInstruments, Hayward, Australia). The balloon was connected to a Y-adapter with one end used to advance the micromanometer and the other used to fill the left ventricular balloon with bubble-free water to an end-diastolic pressure (LVEDP) of 10 mmHg. Coronary flow (CF) was measured by collecting effluent (ml/min). Global ischemia was achieved by clamping the aortic inflow line. During ischemia, hearts were maintained at 37 °C by enclosure in a water-jacketed air chamber. Warmed perfusate kept in the lower part of the chamber saturated the air with humidity and prevented cooling by evaporation. Heart temperature was continuously monitored by a digital thermometer (PTW-100A, Unique Medical, Tokyo, Japan).

Experimental Protocol

Animals were assigned to one of 4 groups (n=8 each; Figure 1). Schematic illustration of the experimental protocol of this study is shown in Figure 1. After a 20 min equilibration, baseline LVDP, LVEDP and CF were recorded. Control hearts were subjected to 30 min of global ischemia followed by 2h (for infarct size study) or 4h (for western blot study) of reperfusion. To investigate the involvement of necroptosis in myocardial ischemia-reperfusion injury, necrostatin-1 (necroptosis inhibitor, 10 μM) was administered starting 5
min before ischemia and during the initial 30 min of reperfusion (Nec). The experiments using Z-VAD (apoptosis inhibitor, 0.1 μM) was done in the same manner. Nec+Z-VAD group was treated with both inhibitors and was subjected to the identical protocol.

Determination of Myocardial Infarct Size

After 2 h reperfusion, the hearts were quickly frozen at −80 °C for 15 min, then sliced into 2 mm thick transverse sections from apex to base (6 slices/heart). After removing the right ventricle and defrosting, each slice was weighed and incubated at 37 °C with 1% triphenyltetrazolium chloride (TTC, Sigma Chemicals) in phosphate buffer (pH 7.4) for 10 min and then fixed in 10% formalin for at least 5 h to distinguish red stained viable tissue from pale unstained necrotic tissue [12]. Each slice was photographed and the necrotic area was determined using Adobe Photoshop® CS (Adobe, San Jose, CA, USA) and multiplied by the weight of the slice, then expressed as a fraction of the left ventricle.

Western Blot Analysis

Separate experiments were performed (n=4 in each group) to examine expression of RIP1, pro-caspase 3 and activated caspase 3. Myocardial tissue samples were collected after 4 h reperfusion, and homogenized in ice-cold homogenizing buffer containing 250 mM sucrose, 20 mM HEPES (pH 7.5), 10 mM KCl, 2 mM EGTA, 2 mM MgCl2, 25 mM NaF, 50 mM β-glycerophosphate, 1 mM Na3VO4, 1mM PMSF, 1% Triton X and protease inhibitor leupeptin (10 μg/ml). The homogenate was centrifuged at 1000g and 4 °C for 5 min. The supernatant was re-centrifuged at 10000g and 4 °C for 15 min. The protein concentration was estimated with a Bradford assay. Equivalent amounts (20 μg) of protein samples were loaded and
separated on a 5-10% SDS-PAGE gradient gel, then electrically transferred overnight to a polyvinylidene difluoride membrane (Millipore Co., Billerica, MA, USA). After blocking with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T), the membranes were incubated for 2 h at 4 °C in TBS-T containing 5% milk and overnight 1:500 dilution of rabbit primary antibody for RIP1, pro-caspase 3 and activated caspase 3 (Santa Cruz Biotechnology, Dallas, TX USA). Membranes were then washed three times with TBS-T for 10 min and subsequently incubated with a 1:1000 dilution of horseradish peroxidase–labeled anti-rabbit immunoglobulin G (NA 934V, GE Healthcare, Buckinghamshire, United Kingdom) in TBS-T containing 5% milk. The same blot was stripped and re-blotted with antibodies to β-actin (Santa Cruz Biotechnology, USA) to confirm equal protein loading. Bound antibody signals were detected with enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL, USA) and visualized using VersaDoc 5000® Imaging System (Bio-Rad, Hercules, CA, USA). Quantitative analysis of the band densities was performed by Quantity One software (Bio-Rad), and the results are presented as the ratio of RIP1, pro-caspase 3 and activated caspase 3 to β-actin, respectively. The average light intensity was multiplied by 100 to facilitate presentation of an x-fold increase.

Statistical Analysis

All data are expressed as mean±SD. Statistical power analysis revealed that a sample size of n=8 would provide sufficient power (0.8) to detect a difference between mean infarct size indices of 15 % (SD=9, α=0.05). A group size of n=4 was used for Western blot to provide a power of 0.8 to detect a difference between means of 20% (SD=10, α=0.05). Hemodynamic data were tested for normal distribution and subsequently analyzed by a two-
factor repeated-measures analysis of variance for time and treatment. If an overall difference between the variables was observed, comparisons were performed as one-way ANOVA followed by Tukey's post-hoc test for inter-group differences and by Dunnett's for intra-group differences with baseline values as the reference time point. Analyses of infarct size and Western blot were performed using one-way ANOVA followed by Student's t-test with Bonferroni's correction for multiple comparisons to avoid type I error. For changes within and between groups a two-tailed p value less than 0.05 was considered significant in advance. (SPSS17 for Windows, SPSS Japan, Tokyo, Japan).
**Results**

Of a total of 56, 4 hearts were not used secondary to intractable ventricular fibrillation after reperfusion (two in CTL and one in Nec and one in Z-VAD). There was no significant difference in body weight among groups.

**Hemodynamics**

Hemodynamic data are shown in Table 1. Baseline LVDP and CF were similar among the 4 groups. Administration of Necrostatin-1 and Z-VAD did not significantly affect LVDP or CF before ischemia. Recovery of LVDP was greater in Nec+Z-VAD compared with other three groups (Nec+Z-VAD: 47 ± 6 vs. CTL: 30 ± 12, Nec: 41 ± 11, Z-VAD: 43 ± 11 mmHg, p< 0.05 at 120 min of reperfusion). LVEDP increased significantly in CTL after ischemia-reperfusion compared with baseline. The increase in LVEDP was significantly less in Nec+Z-VAD compared with CTL during reperfusion period (28 ± 9 vs. 53 ± 20 mmHg, p< 0.05 at 120 min of reperfusion). There was no significant difference in CF among all groups throughout the experiment. This suggests that changes in CF could not account for the improved contractile recovery of Nec+Z-VAD hearts.

**Myocardial Infarct Size**

Myocardial infarct size data are shown in Figure 2. Myocardial infarct size in Nec and Z-VAD groups was significantly reduced by 37% compared with control hearts (Nec: 33 ± 10 %, Z-VAD: 33 ± 5 % vs. CTL: 52 ± 6 %, p<0.05). Combination of necrostatin-1 and Z-VAD further reduced myocardial infarct size in Nec+Z-VAD group (12 ± 2 %).
Western Blot analysis

The expression of RIP1, pro-caspase 3 and activated caspase 3 at 4h after reperfusion is illustrated by a representative Western blot (Figures 3A, 4A). Expression of RIP1 was significantly preserved in Z-VAD and Nec+Z-VAD compared with CTL (Figure 3B). This preservation was not caused by unequal loading of the western blot, as shown by the detection of β-actin. The expression of pro-caspase 3 in Z-VAD and Nec+Z-VAD was better preserved whereas it was decreased in CTL and Nec, suggesting that apoptosis was executed in CTL and Nec, and was blocked in Z-VAD and Nec+Z-VAD. This was confirmed by increased expression of activated caspase 3 in CTL whereas it was attenuated in Z-VAD and Nec+Z-VAD (Figure 4).
Discussion

The present study demonstrated that the caspase-independent programmed necrosis, necroptosis, may be involved in acute myocardial ischemia-reperfusion injury in isolated guinea pig hearts. Further, combination therapy targeting different forms of cell death (necroptosis and apoptosis) confers an enhanced effect in reducing myocardial infarct size. This effect is associated with inhibition of RIP1 by necrostatin-1 and caspase 3 by Z-VAD. As ventricular remodeling due to apoptotic and non-apoptotic programmed cardiomyocytes death is an active process that contributes to cardiac dysfunction long after acute myocardial infarction [13], it is an attractive therapeutic targets. This study could provide an additional therapeutic target to improve left ventricular recovery after myocardial infarction.

Degterev et al. first reported a caspase-independent programmed necrosis as an alternative form of regulated cell death, termed necroptosis. They also identified an inhibitor of necroptosis, necrostatin-1, screening of a chemical library of 15000 compounds and demonstrating that intracerebroventricular injection of necrostatin-1 contributed to delayed mouse ischemic brain injury [6]. They further demonstrated that necrostatin-1 inhibits RIP1 kinase activity, suggesting that RIP1 plays an important role in necroptosis. Although the effect of necrostatin-1 on necroptosis was reported in several cell lines such as Jurkat cells, both in vitro and in vivo data on the use of necrostatin-1 in cardiomyocytes is limited. This is the first study demonstrating the cardioprotective effects of necrostatin-1 in guinea pig hearts. Smith et al. first described that necrostatin-1 protects against myocardial ischemia-reperfusion injury in vitro and in vivo mouse hearts, and cardiomyocytes isolated from rats [10]. They reported that administration of necrostatin-1 at 30 μM, but not 100 μM for 35 min after reperfusion reduced myocardial infarct size approximately 30 % in isolated mouse
hearts. In the present study, administration of necrostatin-1 at 10 μM for 30 min after reperfusion reduced infarct size approximately 30% compared to control, although this dose of necrostatin-1 is relatively low. Z-VAD-fmk is a pan-caspase inhibitor which has been shown to reduce myocardial ischemia-reperfusion injury by attenuating apoptosis [14]. When the cell dies from apoptosis, RIP1 assembles with Fas-associated death domain, caspase 8 and RIP3, then active caspase 8 cleaves RIP1 and induces apoptosis. When caspase 8 is pharmacologically inhibited (Z-VAD-fmk inhibits both caspase 8 and caspase 3), apoptosis cannot be initiated, which results in programmed necrosis as an alternative form of cell death through RIP1 [15]. Necrostatin-1 inhibits this type of cell death by inhibiting RIP1 kinase activity. If apoptosis is not blocked, RIP1 is cleaved by caspase 8, leading to reduced expression of RIP1 after ischemia-reperfusion.

In the present study, the infarct size of Nec group was comparable with that of Z-VAD group, and the combination of necrostatin-1 and Z-VAD-fmk further reduced infarct size. These findings suggest that the further reduction of infarct size in Nec+Z-VAD group was due to inhibition of necroptosis by necrostatin-1, as pro-caspases (3 and 8) are pharmacologically inhibited by Z-VAD. This mechanism was supported by western blot analyses. As shown in Figure 3, expression of RIP1 was significantly reduced in CTL and Nec after 4 h reperfusion, suggesting that RIP1 was cleaved by caspase 8 whereas it remained intact in Z-VAD and Nec+Z-VAD because of suppression of apoptosis. Thus, both apoptosis and necroptosis were likely inhibited in the Nec+Z-VAD group (Figure 4). However, we cannot rule out that there might be another factor affording cardioprotection during combination therapy (Nec+Z-VAD). For example, necrostatin-1 has been shown to exert cardioprotective effects against oxidative stress [16] and to modulate mitochondrial
permeability transition pore [17] which has been shown to be a crucial determinant for cardioprotection [18]. Recently, it has been demonstrated that necrostatin-1 attenuates mitochondrial dysfunction after ischemia by preventing free radical formation [19]. In the present study, the magnitude of reduction of RIP1 expression in Nec was only slightly less than CTL. This suggest that necrostatin-1 itself could also suppress apoptosis, which is consistent with the recent study by Wang et al. Wang and colleagues demonstrated attenuation of apoptosis by necrostatin-1 in traumatic brain injury [11]. Therefore, the finding of the present study demonstrating that treatment with necrostatin-1 alone reduced infarct size does not necessarily mean only reduction of necroptosis by necrostatin-1, but may also be due in part to attenuation of apoptosis by necrostatin-1. One study found that RIP1 may be involved in mediating either apoptosis or necroptosis, depending on the cellular context. The partial protection against TNF-α-induced cell death by Z-VAD-fmk in Jurkat cells is further enhanced by RIP1 inhibition [20].

The following study limitations should be acknowledged. First, although RIP1 is regulated by caspase 8, we examined expression of caspase 3 to assess apoptosis because it is an effector caspase (Z-VAD is a pan-caspase inhibitor). Second, the dose (0.1 μM) of Z-VAD used in the present study was relatively low. However, Mocanu et al. demonstrated that 0.1 μM of Z-VAD effectively reduced infarct size by 36% (similar to the present study) in isolated rat hearts [21]. Of note, if the apoptotic pathway is not completely blocked by this concentration of Z-VAD, we cannot absolutely rule out that the enhanced reduction in infarct size by the combination of Z-VAD and necrostatin-1 could be in part due to suppression of apoptosis by necrostatin-1. Third, we determined infarct size at 2 h after reperfusion whereas myocardial samples for western blot were obtained at 4 h after reperfusion. It has been
shown that a reperfusion period of at least 60 min is required to determine myocardial infarct size by TTC staining [22]. We extended the reperfusion period to 2 h to ensure washout of pyridine nucleotides from necrotic cells, which interact with TTC. Apoptosis was reported to be detectable after approximately 4 h after myocardial ischemia in previous studies [23,24].

Fourth, necrostatin-1 was administered starting 5 min before ischemia and for initial 30 min after reperfusion. Although it has been established that necrostatin-1 exerts a cytoprotective effects by inhibiting RIP1 kinase activity, other effects such as effects on free radical formation and mitochondria were not known. Necrostatin-1 has been shown to prevent free radical formation [19]. As a burst free radical formation occurs during a very early reperfusion period, we targeted this period to test the infarct size limiting effects of necrostatin-1 as well as inhibiting RIP1. However, it remains to be elucidated whether administration of necrostatin-1 throughout the reperfusion period further reduces infarct size. Further studies are needed.

**Conclusions**

This study suggests that a combination of anti-necroptosis and anti-apoptotic therapy is a promising novel strategy for cardioprotection after ischemia-reperfusion injury.
Acknowledgments

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References


Table Legend

Table 1: Data are presented as mean ± SD. LVDP=left ventricular developed pressure; LVEDP=left ventricular end-diastolic pressure; CF=coronary flow; CTL=control; Nec=necrostatin-1; Z-VAD=Z-VAD-fmk. *p<0.05 vs. CTL, n=8 for each group.
Figure Legends

Figure 1: Schematic illustration of the experimental protocol of this study. All hearts were subjected to 30 min global ischemia followed by 120 min reperfusion. Necrostatin-1 (10µM, inhibitor of necroptosis), Z-VAD (0.1µM, inhibitor of apoptosis) and both inhibitors were administered for 5 min before ischemia and 30 min at the onset of reperfusion. Tissue samples were obtained at 4h after reperfusion. n=8 for each group. CTL=control; Nec=necrostatin-1; Z-VAD=Z-VAD-fmk.

Figure 2: Infarct size as a percentage of LV in four groups. Infarct size was significantly reduced in Nec and Z-VAD compared to CTL (33 ± 10%, 33 ± 5% versus 52 ± 6%, p<0.05). Combination of necroptosis and apoptosis inhibition further reduced infarct size (Nec+Z-VAD; 12 ± 2% versus Nec, Z-VAD, p<0.05). Data are presented as mean ± SD. *p<0.05 versus CTL, † p<0.05 versus Nec, Z-VAD (n=8 for each group).

Figure 3: (A) Representative western blot of RIP1 from left ventricular samples acquired at 240 min after reperfusion (n=4 for each group). Expression of RIP1 was significantly preserved in Z-VAD and Nec+Z-VAD compared with CTL. (B) Densitometric evaluation of four experiments as the x-fold increase in average light density (AVI). The results are presented as the ratio of RIP1 to β-actin. The average light intensity was multiplied by 100 to facilitate presentation of an x-fold increase. Data are mean±SD. *p<0.05 vs. CTL, † p<0.05 vs. before ischemia.
Figure 4: (A) Representative western blot of pro-caspase 3 and activated-caspase 3 from left ventricular samples acquired at 4h min after reperfusion (n=4 for each group). Expression of pro-caspase 3 in Z-VAD and Nec+Z-VAD was better preserved whereas it was decreased in CTL. The expression of activated caspase 3 was significantly increased in CTL and was slightly increased in Nec.

(B) Densitometric evaluation of four experiments as the $x$-fold increase in average light density (AVI). The results are presented as the ratio of pro-caspase 3 and activated-caspase 3 to $\beta$-actin. The average light intensity was multiplied by 100 to facilitate presentation of an $x$-fold increase. Data are mean±SD. *p<0.05 vs. CTL, †p<0.05 vs. before ischemia, ‡p<0.05 vs. Nec.
Figure 1

**CTL**
- Baseline
- Ischemia
- Reperfusion

**Nec**
- Baseline
- Necrotatin
- Ischemia
- Necrotatin
- Reperfusion

**Z-VAD**
- Baseline
- Z-VAD
- Ischemia
- Z-VAD
- Reperfusion

**Nec + Z-VAD**
- Baseline
- Necrotatin + Z-VAD
- Ischemia
- Necrotatin + Z-VAD
- Reperfusion

Time points:
- 0: Infarct size measurement
- 30: Tissue samples for Western blot analysis
- 120: Additional time point

Concentrations:
- Necrotatin 10μM
- Z-VAD 0.1μM
- Necrotatin 10μM + Z-VAD 0.1μM
Figure 2
Figure 3

A

RIP1  74kDa
β-actin  43kDa

B

X-fold AVI

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†       †  *  *
Figure 4
Table 1. Hemodynamic Variables

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Data are presented as mean ± SD. LVDP=left ventricular developed pressure; LVEDP=left ventricular end-diastolic pressure; CF=coronary flow; CTL=control; Nec=necrostatin-1; Z-VAD=Z-VAD-fmk. *p<0.05 vs. CTL, n=8 for each group.