Participation of Caspase-3-Like Protease in Necrotic Cell Death of Myocardium During Ischemia–Reperfusion Injury in Rat Hearts

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This experimental study was designed to determine if caspase-3-like protease is activated during a short period of ischemia–reperfusion injury (I-R) that did not induce apoptosis, and whether protease-3-protease inhibitor could prevent myocardial I-R injury, especially necrotic cell death. The subjects were 20 isolated rat hearts; 10 were pretreated for 20 min with 100 μmol/L of the protease-3-protease inhibitor, peptide antagonist Asp-Glu-Val-Asp-CHO (DEVD) (Group D), and compared with the 10 no-pretreated hearts (Group C). The hearts were then subjected to 20, 30, 45, and 60 min of normothermic global ischemia followed by 30 min of reperfusion. Caspase-3-like protease was significantly elevated after 45 min and 60 min in ischemic hearts. Group D had reduced levels of caspase-3-like protease activity after 45 min and 60 min (302±58%, 378±69% of pre-ischemic control, respectively), as compared with Group C (542±74%, 689±85%, respectively) (p<0.05, p<0.05, respectively). Histological analysis also demonstrated a decrease in cellular damage in Group D, as the count ratio of necrotic cells with total cardiomyocytes was 38%, as compared with 78% in the control group (p<0.05). Caspase-3-like protease participated in I-R injury in rat hearts and inhibition of this protease resulted in a reduction of necrotic cell death. (Circ J 2003; 67: 248–252)

Key Words: Apoptosis; Caspase-3-like protease; Ischemia–reperfusion injury; Necrosis

During the previous decade, the mechanisms of apoptosis have been studied extensively and participation of the apoptotic process in ischemia–reperfusion injury (I-R), even in the myocardium, has been suggested. Previous investigators have reported that apoptosis was found in reperfused myocardium after ischemia and that apoptotic cells appeared after at least several hours of reperfusion. The interleukin-1 β-converting enzyme (ICE) family of cysteine proteases, now referred to as caspases, is a group of apoptosis-driving proteases that may also play a role in ischemic injury. Accordingly, the inhibition of caspase activity might protect against I-R injury. Yaoita et al demonstrated in a rat coronary occlusion model that caspase-1 inhibition with the peptide substrate ZVAD-fmk (a relatively nonspecific protease inhibitor) reduced myocardial reperfusion injury by attenuating apoptosis. The caspase-3-like protease inhibitor, peptide antagonist n-acetylglutamate valine aspartate aldehyde (Asp-Glu-Val-Asp-CHO) (DEVD), reduced caspase activity and apoptosis in a rat coronary artery occlusion model.

On the other hand, not only apoptotic cell death but also another type of cell death (necrosis) has been shown to be one of the forms of myocardial I-R injury. Inhibition of ICE family proteases significantly prevented hypoxia-induced apoptosis and necrosis in primary cultured hepatocytes. The participation of caspase-3-like protease in myocardial I-R injury, especially in necrotic cell death, is unknown; however, we consider that it is likely to play an important role. Using isolated rat hearts, this experimental study was designed to determine if caspase-3-like protease is activated during short-time I-R, which does not induce the apoptosis process, and whether the protease-3-protease inhibitor, DEVD, could prevent myocardial I-R injury.

Methods

The experiments were carried out under the supervision of the Animal Research Committee in accordance with the guidelines set by the Japanese Government Animal Protection and Management Law (No. 105).

Animal Model

Adult male Sprague-Dawley rats weighing 200–300 g were anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg body weight). Each heart was excised and the aorta was cannulated promptly (warm ischemia time was within 1 min without preconditioning). Retrograde perfusion was started at a constant pressure of 90 cmH2O using an overflow chamber. The perfusate consisted of (in mmol/L) NaCl 120, KCl 4.5, MgSO4 1.2, KH2PO4 1.2, CaCl2 2.5, NaHCO3 25, and glucose 10, and was equilibrated with 95% O2/5% CO2.

Left ventricular pressure (LVP) was monitored continuously with a thin latex balloon that was inserted into the left ventricle (LV) through the mitral valve and connected to a pressure transducer. The balloon was filled with water to an end-diastolic pressure of 6–10 mmHg and the volume was kept constant throughout the experiment.
Experimental Design
After 20 min of equilibrating perfusion, each heart was subjected to normothermic global no-flow ischemia followed by reperfusion. First, in order to clarify whether caspase-3-like protease was activated during ischemia and reperfusion, 45 min of ischemia was performed. The activity of myocardial caspase-3-like protease after 0, 10, 20, 30, and 40 min of reperfusion was measured in 25 of the hearts. After the duration of reperfusion that activated myocardial caspase-3-like protease was determined from that experiment, the effect of caspase-3-like protease inhibitor DEVD was assessed after 20, 30, 45, and 60 min of ischemia, followed by the defined reperfusion time in another 20 hearts, which were divided into 2 groups of 10: those pretreated for 20 min with 100 μmol/L of DEVD diluted in DMSO (300 μL) (Group D) and those pretreated with the same amount of an inert vehicle (DMSO) (Group C). The dose of DEVD was defined and modified to 100 μmol/L, based on the results of Nicholson et al (2000) and was administrated intravenously.

Measurement of Caspase-3-Like Protease Activity
The activities of caspase-3-like protease were measured according to the method of Shimizu et al (2000). At the indicated time, each heart was chopped up with scissors and homogenized with a Polytron in a buffer containing 50 mmol/L Tris-HCl (pH=7.4), 1 mmol/L EDTA, and 10 mmol/L EGTA. The homogenate was clarified by centrifugation at 5,000 g for 5 min and the supernatant containing 40 μL of protein was incubated with 50 μmol of enzyme substrate Ac-DEVD-MCA (Peptide Institute, Osaka, Japan) at 37°C for 30 min. Concentrations of released 7-amino-4-methylcoumarin were measured using a spectrofluorometer (F-3000; Hitachi, Tokyo, Japan) with excitation set at 380 nm and emission at 460 nm.

Western Blot Analysis
For an immunoblot assay of caspase-3, 400 mg of tissue was homogenized in 800 μL of RIPA buffer (50 mmol/L Tris-HCl (pH=7.2), 150 mmol/L NaCl, 1%). Equivalent amounts of 50–100 μg of protein were separated by 12.5% sodium dodecyl sulfate. After electrophoresis and blotting onto a nitrocellulose membrane (Hybond ECL Amersham, UK), the blots were blocked with phosphate buffered saline (PBS) containing 5% non-fat dry milk and incubated with goat polyclonal anti-human phospho-p38 MAPK (Cell Signaling, Beverly, USA) at a dilution of 1:500 overnight. After washing, the blots were incubated with peroxidase-conjugated goat anti-mouse, or anti-rabbit IgG and ECL reagents (Amersham). All data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using analysis of variance (ANOVA) and a value of p<0.05 was considered statistically significant.

Results
Activation of Caspase-3-Like Protease After Ischemia Followed by Reperfusion
The activity of myocardial caspase-3 like protease was determined after 45 min of ischemia followed by 10, 20, 30, and 40 min of reperfusion in 25 hearts. The activity levels were more than 5-fold higher than the pre-ischemic control after 30 and 40 min of reperfusion (162±35% after 10 min of reperfusion, 342±44% after 20 min, 538±54% after 30 min, and 512±47% after 40 min) (Fig 1). From those results, the optimum reperfusion time was determined to 30 min.

The activity of myocardial caspase-3-like protease was determined after 0, 20, 30, 45, and 60 min of ischemia followed by 30 min of reperfusion. The degree of activity was found to be dependent on the duration of myocardial ischemia. Although activity levels were not significantly different between Group D after 20 and 30 min of ischemia (152±49% after 20 min, 188±55% after 30 min, 302±58% of control after 30 and 40 min of reperfusion (162±35% after 10 min of reperfusion, 342±44% after 20 min, 538±54% after 30 min, and 512±47% after 40 min) (Fig 1). From those results, the optimum reperfusion time was determined to 30 min.

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Histological Examination
Apoptotic and necrotic cell death were determined histologically by the method of Shimizu et al. At the end of the experiment, hearts were stained by perfusion with a medium containing Hoechst 33342 (250 μmol/L) and propidium iodide (PI) (250 μmol/L) for 10 min. These specimens were analyzed under a fluorescence microscope (BX50; Olympus, Tokyo, Japan) with excitation set at 360 nm. Hoechst 33342 stains all nuclei, whereas propidium iodide stains only those nuclei of cells with a disrupted plasma membrane. Whether the nuclei were viable, necrotic or apoptotic was confirmed by electron microscopy and confocal fluorescence microscopy. Under a fluorescence microscope, viable, necrotic and apoptotic nuclei were observed as blue round, pink round or fragmented blue or pink nuclei, respectively. Cell death was quantitated by counting more than 1,000 cells under a non-confocal fluorescence microscope after staining with Hoechst 33342 and PI as described. Cardiomyocytes were carefully distinguished from non-cardiomyocytes.

Detection of TUNEL-Positive Cardiomyocytes
The TUNEL protocol is based on the preferential labeling of terminal deoxynucleotidyl transferase at the 3'-OH ends of DNA. Briefly, the fixed transverse ventricular slices were embedded in paraffin and 4-μm thick sections were deparaffinized by washing in xylene and a descending ethanol series. The sections were subsequently incubated with 20 μg/ml proteinase K for 15 min at room temperature, and endogenous peroxidase was inactivated by a treatment of 3% hydrogen peroxide for 5 min. They were incubated with terminal deoxynucleotidyl transferase (ApopTagR, Oncor, USA) for 1.5 h at 37°C. After the end-labeling, sections were incubated with antidigoxigenin-labeled nuclei and then counterstained by hematoxylin.

Using an eyepiece and a point-counting method, which was performed with a light microscope at a magnification of ×400, we determined the count ratio of necrotic and apoptotic cells with total cardiomyocytes, which had counterstained nuclei. The entire area was searched by means of an orderly shifting of the visual field, using the outer grids of the eyepiece for orientation. TUNEL-positive cardiomyocytes were carefully distinguished from TUNEL-positive non-cardiomyocytes, such as macrophages.
after 45 min, and 378±69% after 60 min) and Group C (168±54% after 20 min, 203±48% after 30 min, 542±74 after 45 min, and 689±85% after 60 min), they were significantly lower in Group D than in Group C after 45 and 60 min of ischemia (p<0.05) (Fig 2). These results were confirmed by western blot analysis. Bands of 17 kD, which indicated the active form of caspase-3-like protease, were expressed in the specimens after 45 and 60 min of ischemia (Fig 3). Accordingly, myocardial caspase-3-like protease was determined to be activated after 45 min of ischemia followed by 30 min of reperfusion.

Cardiac Function Evaluation

In Group D, there was a greater percent recovery of left ventricular developed pressure and coronary flow after 45 min and 60 min of ischemia followed by 30 min of reperfusion (20±11%, 45±13%, respectively), as compared with Group C (20±11%, 45±13%, respectively) (p<0.05) (Fig 4). Further, there was a significantly positive correlation between caspase-3-like protease activity and creatine kinase release (r=0.723, p<0.05). The count ratio of necrotic cells to total cardiomyocytes was 4% after 10 min, 7% after 20 min, 11% after 30 min, 78% after 45 min, and 100% after 60 min of ischemia followed by 30 min of reperfusion. Despite those results, administration of DEVD reduced the number of lethally damaged cells (3.5% after 10 min, 6% after 20 min, 10% after 30 min, 38% after 45 min, and 64% after 60 min). The count ratio of necrotic cells after 45 min of ischemia followed by 30 min of reperfusion was significantly reduced by administration of DEVD (Fig 5, 6). These results show that activation of caspase-3-like protease is a factor in cell injury with necrotic cell death as well as in apoptosis.

In addition, TUNEL-positive cells comprised only 1.5% after 30 min of reperfusion with 45 min of ischemia, although caspase-3-like protease activity had already started to elevate.

Discussion

Not only apoptotic cell death but also necrotic cell death has been shown to be one of the forms of myocardial I-R injury. Recently, ICE family proteases were found to be directly involved in both apoptotic and necrotic cell death induced by hypoxia–reoxygenation, and inhibition of ICE family proteases significantly prevented hypoxia-induced apoptosis and necrosis in primary cultured hepatocytes. Accordingly, the participation of caspase-3-like protease even in myocardial I-R injury, especially in necrotic cell death, is considered to be important.
The results of the present study demonstrate that caspase-3-like protease was activated in the early reperfusion period and that the activation was not necessarily related to morphologically typical apoptotic cell death. Our findings also indicate that caspase-3-like protease inhibition might attenuate I-R injury in association with a reduction in the number of dead cells. It suggests that caspase-3-like protease played a role in the necrosis associated with I-R injuries in the myocardium. Short-time reperfusion following ischemia that did not induce apoptosis, caused necrotic cell death. The inhibition of caspase-3-like protease activity before myocardial I-R injury, lessened the number of dead cells, attenuated cellular damage including a marked reduction of enzyme leakage and lead to recovery of cardiac function. Namely, the reduction in necrotic cells might, in part, contribute to this improvement. Furthermore, cytoplasmic enzyme leakage is a feature of necrotic cell death, and no TUNEL-positive cells could be found. These findings suggest that caspase-3-like protease may participate in necrosis.

Previous investigators have reported that apoptosis occurs in reperfused myocardium after ischemia, particularly after at least several hours of reperfusion. Chen et al used a rat brain model and showed that caspase-3 genes were induced and caspase-3-like protease was activated 4 h after reperfusion1. Yaoita et al reported that a caspase-3 inhibitor reduced the number of TUNEL-positive cells after 24 h of reperfusion in rat hearts3. However, in our results, TUNEL-positive cells could not be found after 30 min of reperfusion with 45 min of ischemia, although caspase-3-like protease activity had already started to elevate, which indicated that short-time I-R did not induce apoptosis.

Eguchi et al reported that intracellular ATP concentrations could indicate the fate of cells in cell death, whether by apoptosis or necrosis? The apoptotic cell death-signaling pathway contains several ATP-dependent steps. Accordingly, ATP-depleting conditions may induce necrotic cell death, even if the apoptosis signaling cascade is activated. On the other hand, I-R may initiate both necrosis and apoptosis.

Clinical Implications

Our data indicate that administration of caspase-3-like protease inhibitor might reduce the amount of cell damage in I-R injuries in human subjects. It goes without saying that consideration must be given to species-to-species differences, the fact that these experiments were performed with normal hearts, and the use of warm ischemia in this study etc.

Many attempts to reduce the extent of myocardial reperfusion injury have been made over the past decades7–15 with most of these efforts directed at lowering the risk posed by certain injurious factors and potentiating various aspects of cardioprotection relating to ischemic duration, oxygen free radicals, pro-inflammatory cytokines, preconditioning, and oxygen tension control. In addition, Minatoguchi et al reported that caspase-dependent DNA fragmentation, which is present in infarcted myocytes, did not have an infarct-size reducing effect16.

Study Limitations

In our experimental setting (45 min of ischemia followed by 30 min of reperfusion), we did not find evidence of apoptosis. Our results seem to indicate that the increased caspase-3-like protease activity seen immediately after ischemia was related to cellular injury, even though that did not include morphological apoptosis.

Using our method, it can be difficult to discriminate myocytes from non-myocytes and therefore to differentiate them, specimens stained with Hoechst 33342 and PI may need to be further stained with monoclonal antibodies against myosin heavy chain and/or α-sarcomeric actin.

Conclusions

Caspase-3-like protease participated in I-R injury in rat hearts and was activated after 45 min of ischemia followed by 30 min of reperfusion. The inhibition of this protease resulted in a reduction of necrotic cell death and an improvement of cardiac function. These results indicate that administration of caspase-3-like protease inhibitor might reduce cell damage in I-R injury via mechanisms that are not dependent on apoptosis.
References