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Effects of Ischemic Preconditioning on Apoptotic Cell Death in Ischemia-Reperfused Rat Hearts

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Abstract It was reported that apoptotic cell death was observed in ischemia-reperfused rabbit heart as well as necrotic cell death. Ischemic preconditioning (PC) has a potent anti-myocardial infarction effect on the ischemia-reperfused model. However, the effecs of PC on the apoptosis caused by the ischemia-reperfusion have never been clarified. We performed 30-min ischemia followed by 6-hour reperfusion with and without preceding 3 cycles of 5 minutes ischemia-reperfusion as PC in rats. Risk areas were determined by the injection of dye and infarct areas were defined by triphenyltetrazolium chloride stain. In situ terminal deoxynucleotidyl transferase (TdT) reaction and the electrophoresis of DNA were used for the detection of apoptosis. Hemodynamics showed no significant differences at any stages between control and PC groups. PC reduced infarct size from 25.3 % to 4.3 % of the risk area ($p<.01$). PC reduced the number of TdT positive nuclei in the subendocardium, compared with control group (14.2 ± 1.8 % vs 7.1 ± 0.7 %, $p<.01$). Death of cardiomyocytes caused by ischemia-reperfusion in rats is not only attributed to necrosis but apoptosis, and PC significantly reduced both necrotic and apoptotic cell death especially in the subendocardial area of the ischemic myocardium.

Key words: Apoptosis, Ischemic preconditioning, Myocardial infarction, Ischemia, Reperfusion, Necrosis, Rat

In 1986, Reimer et al reported that a preceding short period of ischemia has a potent anti-myocardial infarction effect on the ischemia-reperfused dog hearts. This phenomenon was termed as ischemic preconditioning (PC).[1] The PC effect has been confirmed in various animals such as dogs[1,2], rats[3], rabbits[4], and pigs[5], and several hypotheses for the underlying mechanisms of PC has been proposed. However, the definite mechanism is still under exploration. Recently, Engler et al reported that apoptotic cell death was observed in ischemia-reperfused rabbit heart as well as necrotic cell death.[6] This report suggests that ischemic cell death is not only caused by necrosis but also by apoptosis. DNA fragmentation is one of the characteristic indicator of apoptosis, and cells exhibiting morphological characteristics related to DNA fragmentation are considered as apoptotic cells.[7-9] Recently, a novel molecular biological-histochemical method of specific staining of new 3'-OH DNA ends generated by DNA fragmentation at a single cell level has been developed, which enables the quantification of the apoptotic cell death. The purpose of the

present study was to clarify how PC influences on the apoptotic cell death in the ischemia-reperfused rat hearts.

Methods

Surgical preparation of Animals

Male Sprague-Dawley rats (380-440 g) were anesthetized with sodium pentobarbital (60 mg/kg intra-peritoneum). The trachea was intubated and ventilation was maintained with a respirator (Model SN-480- 7 Shinano, Japan) using a mixture of 100 % O_2 with a tidal volume of 3.5 ml and a respiratory rate of 50/min. Arterial blood gases were sampled and the PaO_2, $PaCO_2$ and pH were maintained within physiological ranges. Left ventricular pressure was measured by 2F catheter-tip manometer (Model SPC-320 Millar instruments, Inc, USA) inserted via the carotid artery to the left ventricle. The heart was exposed through a left thoracotomy in the third intercostal space and the pericardium was open. A 6-0 silk thread was passed around a left anterior descending coronary artery with a tapered needle. Both ends of the thread were passed through 1-cm length polyethylene tube (outer diameter: 1.5mm). Coronary artery occlusion was made by pulling the thread and reperfusion was obtained by releasing the thread. Myocardial ischemia was confirmed by the visual assessment of regional cyanosis and dyskinesis of the relevant segment of the left ventricle and the increase of left ventricular end-diastolic pressure. Sham-operated animals received identical operative procedures, without coronary artery occlusion.

Experimental protocols

Figure 1 shows protocols used in the present study. Rats were randomly assigned to three groups. In control group (n=7), 30- min coronary occlusion was performed, followed by 360 min reperfusion. In PC group (n=7), three cycles of 5 min coronary occlusion followed by 5 min reperfusion as PC preceded 30 min coronary occlusion followed by 360 min reperfusion. In sham group (n=5), sham -operation was performed. After 360 min reperfusion, the left anterior descending coronary artery was re-occluded and the heart was rapidly excised for the subsequent analysis.

Area at risk and area of necrosis

The heart was cut at the center of the risk area parallel to atrio-ventricular groove. Monastral blue dye (1.5 %, 1 ml) was injected into the ascending aorta to dye the non-risk area. Then the heart was cut into 1 mm thick slices, immersed in 1 % solution of triphenyltetrazolium chloride (TTC) at 37 ℃ for 20 min. Viable myocardium is stained red by TTC, whereas necrotic myocardium is not stained, as previously validated in the rat model.[10] The anatomic area at risk (AAR) was delineated by the absence of the blue dye stain. After immersion in TTC, the heart slices were photographed and the photographic slides were projected and traced, demarcating the areas of the blue dye (perfused tissue), areas without the blue dye (anatomic risk zone), and the areas of infarcted and non-infarcted myocardium. The extent of AAR and area of necrosis (AN) in each slice was quantified by computerized planimetry. The volume of tissue at risk was expressed as a percentage of the left ventricle (AAR/LV), and the volume of necrosis was expressed as a percentage of the volume of the tissue at risk (AN/AAR). The rest of the hearts were fixed with 10% formalin and embedded in paraffin for the subsequent nick end-labeling.

In situ nick end-labeling of apoptotic cells

The method for nick end-labeling of apoptotic cells was adopted from Gavrieli et al.[9] It is based on the preferential binding of terminal deoxynucleotidyl transferase to 3'-OH ends of DNA. Six-micrometer thick sections of hearts were deparaffinized by washing xylene and descending alcohol series (100 %, 95 %, 70 %, and 0 %). The sections were incubated with 20 μg/mL proteinase K for 15 min at room temperature, and endogenous peroxidase was inactivated by treating with 2 % H_2O_2. The sections were incubated with terminal deoxynucleotidyl transferase (TdT; ApopTag, Oncor, USA) for 1 hour at 37 ℃. After end-labeling, the sections were incubated with anti-digoxigenin peroxidase. Aminoethylcarbazole was used

as a chromogen to detect the biotin-labeled nuclei. Then the sections were counterstained with hematoxylin and eosin.

Cell counts of apoptotic cells

We counted the number of cardiomyocyte nuclei exhibiting DNA fragmentation among 300 cardiomyocyte nuclei in subendocardium, midwall and subepicardium respectively in both risk area and non-risk area. The analysis was performed by two independent examiners who were blinded to the information of the individual data.

DNA extraction

Transmural myocardial samples from completely normal and ischemic areas were isolated using the blue dye stain as a guide, frozen in liquid nitrogen, and stored at −80 ℃. A modification of the method of Wyllie and Morris[11] was used to assess the fragmentation of cardiomyocyte DNA. Cardiomyocytes were pelleted by centrifugation at 200 g at 4 ℃ for 5 min, washed once with an ice cold Ca^{++} free buffer, and resuspended in 250 μl of the same buffer. An equal volume of lysis buffer (20 mM EDTA, 0.5% Triton X 100, 5 mM Tris -HCl, pH 8) was added, incubated on ice for 15 min. Intact nuclei were pelleted by centrifugation at 500 g at 4 ℃ for 10 min and the supernatant ultracentrifuged at 27,000 g at 4 ℃ for 20 min. Soluble DNA in the supernatant was precipitated at −20 ℃ for 2 h after the addition of 0.13 M NaCl/70 % ethanol. DNA was pelleted by centrifugation at 4 ℃ for 15 min, dried, then resuspended in 10 mM Tris- HCl/1 mM EDTA pH 7.4. The aqueous phase was extracted twice with chloroform and the DNA precipitated. Aliquots of DNA were loaded onto a 2.0 % agarose gel in TAE (0.04 M TRIS-acetate, 2 mM EDTA, pH 8.0) buffer, and electrophoresed in a flatbed gel apparatus (Advance Inc., Japan) at 30 V. Gels were stained using ethidium bromide at 0.5 μg/ml for 30 min, destained in 1 mM $MgSO_4$ for 30 min, and photographed under transmitted UV light.

Arrhythmias

Arrhythmias such as ventricular fibrillation, ventricular tachycardia and ventricular premature beats were monitored and the effects of ischemic preconditioning on these arrhythmias were studied.

Statistical Analysis

All values are expressed as mean ± SE. Differences in hemodynamic parameters and the percentage of apoptotic cells between the groups were analyzed by a two-way ANOVA. Fisher's least significant difference test was used if significant F values were obtained. Differences between groups in AAR and AN/AAR were analyzed by unpaired t-test. The value less than 0.05 was considered significant.

Results

Hemodynamic Responses

Table 1 summarizes the hemodynamic data. There were no significant differences in either body weight, heart rate, max LV pressure and LV (+) dP/dt and LV (−) dP/dt between control group and PC group throughout the experiments. LVEDP showed significant increase after coronary occlusion ($p<.05$), but there was no difference between the groups.

Myocardial Infarct Size

Fig 2 shows the AAR and AN/AAR. The AAR was not significantly different between the groups (control group, 41.8±1.7 %; PC group, 39.8±1.6 %). AN/AAR was significantly decreased by PC (25.3±1.5 % in control group and 4.3±0.4 % in PC group, $P<.01$).

Cell count of apoptotic cells

Fig 4 shows the percentage of apoptotic cells of 300 nuclei. In the risk area, percentage of TdT positive myocyte nuclei was significantly higher in the subendocardium than in the subepicardium in control group (14.2±1.8 % vs 7.1±0.7 %, $P<.01$). As shown in Fig. 4, ischemic preconditioning significantly reduced the percentage of positive myocyte nuclei in the subendocardium ($p<.01$ vs control group), but not significantly in the mid- and subepicardium (Fig. 5). In the non-risk area of control and PC groups, and in hearts of sham group, positive myocyte nuclei were 0.3±0.5 % observed.

All tissues obtained from the ischemic left

Table 1. Hemodynamics

	Pre-ischemia	30 min ischemia	5 min after reperfusion
HR (bpm)			
Control group	429±10	424±7	420±9
PC group	414±13	410±18	412±19
LVP max (mmHg)			
Control group	122±4	124±5	122±5
PC group	120±5	118±4	118±4
LVEDP (mmHg)			
Control group	4±0.3	9±0.6*	8±0.8*
PC group	4±0.4	8±1.2*	7±1.0*
(+) dP/dt (mmHg/s)			
Control group	5551±302	5847±331	5334±284
PC group	5650±464	5290±404	5034±270
(−) dP/dt (mmHg/s)			
Control group	4259±174	4089±185	3987±188
PC group	4147±243	3756±283	3710±248

HR indicates heart rate; LVP max, maximum left ventricular pressure; LVEDP, left ventricular end diastolic pressure; (+) dP/dt, maximum rate of left ventricular pressure generation; (−) dP/dt, minimum rate of left ventricular pressure generation; PC, ischemic preconditioning
Values are mean ± SE.
* P<.05 compared with pre-ischemia.

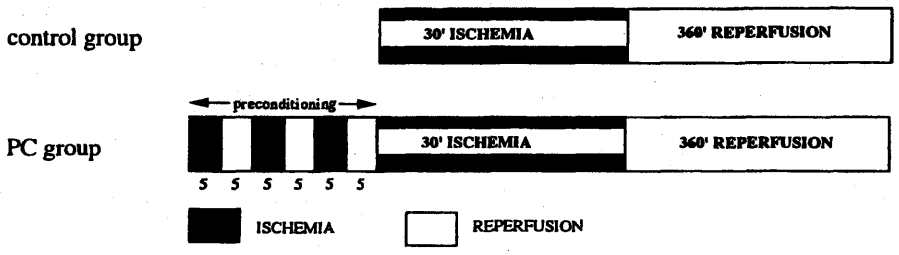

Fig 1. Schematic diagrams of the experimental protocols. Control group and preconditioned (PC) group were subjected to 30 minutes of left coronary artery occlusion followed by 6 hours of reperfusion. In the PC group, three coronary occlusion cycles of 5 min duration, each followed by 5 min of reperfusion was elicited before a subsequent 30 minutes period of ischemia.

ventricle of control group (n=5) and PC group (n=5), demonstrated the presence of the high molecular weight band in addition to a series of smaller DNA fragments with size ranges in multiples of approximately 180- 200 bp units. (Fig 3) The so-called 180 bp "ladder" pattern of DNA fragmentation has been widely reported to provide a biochemical landmark for the process of cellular apoptosis.[12,13,14] The expression of the ladder pattern in control group was more apparent compared with PC group. DNA extracted from the hearts of the sham group rats (n=3) and non-ischemic left ventricle of both control and PC groups showed a single band close to the loading site of the gel, typical of high molecular weight

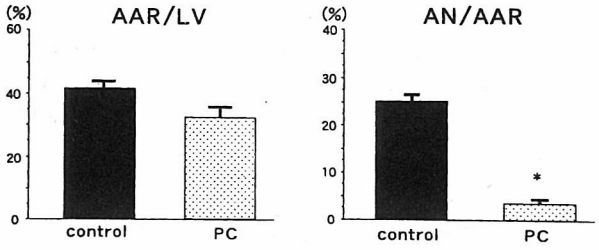

Fig 2. Bar graphs illustrating the effect of ischemic preconditioning on AAR/LV and AN/AAR. There was no significant difference in AAR/LV between control group and PC group. Preconditioning significantly reduced AN/AAR
AAR, volume of area at risk; LV, left ventricular myocardial volume; AN, volume of necrosis; PC, ischemic preconditioning. * P<.01 vs control

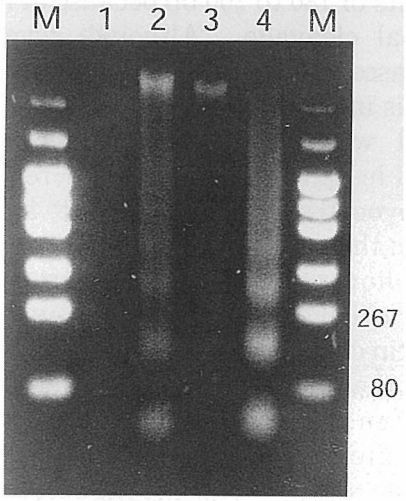

Fig 3. Tissues obtained from the risk area of control group and PC group demonstrated the presence of the high molecular weight band in addition to a series of smaller DNA fragments with size ranges in multiples of approximately 180-200 bp units so-called "ladder pattern". The expression of the ladder pattern of control group showed more apparently compared with that of PC group. Tissues obtained from non-risk area of both control and PC group showed a single band close to the loading site of the gel, typical of high molecular weight nuclear DNA. Ladder pattern was never seen in these tissues. PC, ischemic preconditioning.

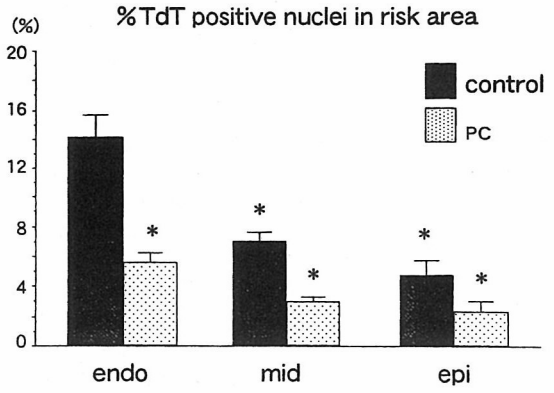

Fig 4. Bar graphs illustrating the effect of ischemic preconditioning on the number of TdT positive nuclei in the risk area. There was a significant trend that the TdT positive myocyte nuclei located more frequently in the endocardium and less toward the epicardium. Preconditioning significantly reduced positive myocyte nuclei in subendocardium. PC, ischemic preconditioning; endo, subendocardial area; mid, mid-myocardial area; epi, subepicardial area, TdT, terminal deoxy nucleotidyl transferase,
* P<.01 vs endo in control group.

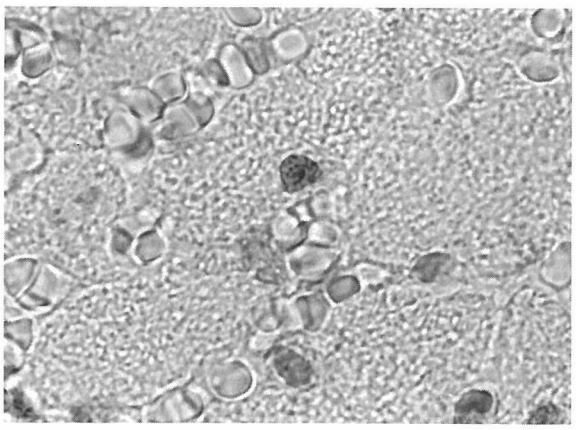

Fig 5. Paraffin sections stained with ApopTag kit and counterstained with hematoxylin and eosin. Subendocardium in the risk area of control group (×400 magnification). The myocyte nuclei stained in red indicate apoptotic cells. The nuclei of infiltrated cells were mostly stained blue with hematoxylin indicates non-apoptotic cells.

nuclear DNA and ladder pattern was never seen in these tissues.

Arrhythmias

During the preconditioning period of ischemia, all rats had VT. During 30 min of ischemia, ventricular tachycardia or ventricular fibrillation developed in all control group rats, but no ventricular tachycardia was observed in PC group. After reperfusion, no significant ventricular fibrillation was observed in both groups.

Discussion

We investigated the expression of apoptosis using TdT reaction for DNA fragmentation in hearts subjected to 30 minutes ischemia and 6 hours reperfusion. Our novel findings are that ischemic preconditioning reduces not only necrosis of the ischemic tissue but DNA fragmentation detected by TdT reaction and the ladder formation, indicative of apoptosis. Previously, it has been noted that ischemia with reperfusion induces apoptosis in the kidney[15,16] and brain[17] in rats. Gottlieb et al[6] reported that ischemia with reperfusion induced apoptosis in rabbit hearts. However, it remains to be clarified how ischemic preconditioning influences on the incidence of apoptosis. In the present study we verified that the apoptosis was induced predominantly in the subendocardial area more frequently than in the subepicardial area. In the preconditioned group the necrotic area significantly reduced from 25.3±6.0% to 4.0±0.1 % and this result was consistent with the previous studies[18]. In this model we verified that apoptosis was significantly attenuated by ischemic preconditioning from 14.2 % to 5.6 % in the subendocardial region ($P<.01$). Several underlying mechanisms of ischemic preconditioning has been proposed, and adenosine A1 receptor stimulation is one of the mechanisms attributed in rabbits[19] and dogs[20] but not in the rat[23]. ATP-sensitive K channels[21,22] are another candidate involved in the mechanism of ischemic preconditioning in dogs but not in the rat. However, protein kinase C (PKC) activation[24,25] plays a crucial role for ischemic preconditioning in rats. Thus, the mechanism for the reduction of apoptosis by ischemic preconditioning might be related to PKC activation, but remains to be clarified. Apoptosis is a physiologic process for disposing senescent or unwanted cells through self-destruction. For example, apoptosis contributes to many fundamental biological processes, including morphogenesis[26,27], remodeling of mature tissues[28], and negative selection of T lymphocytes[29]. Apoptosis differs morphologically from necrosis: i.e. cells exhibit cytoplasmic and nuclear condensation, and then they fragment into apoptotic bodies without membrane disruption. Apoptotic bodies are phagocytosed by neighboring cells or macrophages without inducing an inflammatory response[8]. Nuclear condensation apparently results from release of endonucleases that degrade DNA into large fragments of 50 to 300 kb[30], and then into smaller fragments of 180 to 200 bp because of internucleosomal cleavage. Although several endonucleases were identified and linked to apoptosis in some cell types[31], it has not been clarified which type of endonucleases is involved in apoptosis of ischemia-reperfused cardiomyocytes. We detected apoptotic bodies in the rat heart after 6 hours of reperfusion following 30 min coronary artery occlusion. Gottlieb et al[6] reported the evidence of chromatin condensation and nucleosomal ladders in the rabbit myocytes after 4 hours reperfusion following 30 min coronary occlusion despite the absence of apoptotic bodies. Their data strongly indicate the occurrence of apoptosis and the reason why apoptotic bodies were absent in their study may be attributed to the insufficient reperfusion time, or may be related to the species difference between rats and rabbits. It has already been shown that the ischemia-reperfusion induced apoptosis in the organs other than the heart. For example, in brains, apoptosis appeared within 0.5 hours after reperfusion and peaked at 24-48 hours, and persisted until 4 weeks after reperfusion with TdT reaction assay[17]. In kidney, apoptotic bodies in the epithelial cells were not apparent until 12 hours of reperfusion using hematoxylin and eosin staining.[15,16] In cultured cells, the sequence of apoptosis and phagocytosis is completed within 4 hours[8,32]. In the present study, we did

not follow the time course of the occurrence of apoptosis, however, we demonstrated that 6 hour-reperfusion was sufficient to induce apoptotic bodies in the rat heart. The present study, for the first time, demonstrated the anatomical distribution of apoptotic cells across the wall of the ischemic myocardium and indicated that the preferential occurrence of apoptosis was observed in the subendocardium rather than in the subepicardium. This phenomenon may contribute to the "wave front phenomenon" of the myocardial death after ischemia.[33] There are several pathways to induce apoptosis, however, the mechanism of apoptosis caused by ischemia-reperfusion is still unclear. Apoptotic cells are localized primarily in the inner boundary zones of the evolving infarct. This feature of localization of apoptotic cells suggests that metabolic activity and reoxygenation may provide the conditions for apoptosis to occur since apoptosis requires energy to produce proteins promoting cell death, such as p53[34]. Moreover, inhibitory proteins against apoptosis, such as Bcl-2, might be suppressed in ischemic conditions, which might be one of the important mechanism to induce apoptosis in ischemia-reperfused state[35]. Free radical produced during reperfusion may be another important candidate for inducing apoptosis in ischemia-reperfused state[35]. Other mechanisms such as Fas ligand-antigen system, or cytokines such as TNF-α and interleukin-6 may be involved. The cells expressing Fas antigen initiates apoptotic process by stimulation of Fas ligand. Under hypoxia, cultured rat cardiomyocytes died through apoptosis mediated by Fas antigen[36]. In canine myocardium, induction of interleukin-6 in the ischemia-reperfused myocardium has been demonstrated, and the possibility to cause apoptosis was suggested[37].

Limitations of the present study

The labeling target of the ApopTag kit is the multitude of new 3'-OH ends generated by DNA fragmentation and typically localized in morphologically identifiable nuclei of apoptotic bodies. In contrast, normal or necrotic myocyte nuclei, which have relatively insignificant numbers of DNA 3'-OH ends, do not stain with the ApopTag kit. In the present study, apoptotic bodies were observed, nevertheless, morphologically, apoptotic bodies are similar to polymorphonuclear neutrophils, which are present in the myocardium after reperfusion. However, by using hematoxylin-eosin, and immunohistochemical double stainings with ApopTag kit, apoptotic bodies were indicated brown color. Neutrophils are counterstained blue color by hematoxylin. Thus, neutrophils within the ischemic tissue can be identified by the hematoxylin counterstain and are not confused with apoptotic bodies.

In summary, death of cardiomyocytes caused by ischemia-reperfusion in rats is not only attributed to necrosis but apoptosis, and ischemic preconditioning significantly reduced both necrotic and apoptotic cell death, especially in the subendocardial area of ischemic myocardium. The pathophysiological significance of apoptosis in the development of myocardial dysfunction should be further investigated and the role of ischemic preconditioning should be elucidated.

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