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Induction of Apoptosis of Rat Neonatal Cardiomyocytes by Chemical Ischemia and Reoxygenation: The Role of Phosphatidylserine

Kimiyo Ono, Shingo Ishiguro, Hiroaki Kuroda and Shigetsugu Ohgi

Second Department of Surgery, Faculty of Medicine, Tottori University, Yonago 683-0826, Japan

Ischemia/reperfusion injury plays a crucial role in the induction of the cell death of myocytes. The precise mechanism of the cell death, however, has not been elucidated enough. This study examined the cell death of rat neonatal myocytes induced by chemical ischemia and reoxygenation with an in vitro model, in terms of apoptosis, and the role of phosphatidylserine, which is recognized with annexin V. Chemical ischemia and reoxygenation were conducted on the cultured myocytes obtained from 1- or 2-day-old Wistar rats. The cells were divided into 4 groups exposed to chemical ischemia for 9 h (Group A), 18 h (Group B) and 24 h (Group C) and one group not exposed to chemical ischemia (Control Group). DNA ladder formation on agarose gel electrophoresis was noted in Groups B and C followed by reoxygenation, but not in Group A, as well as all 4 groups without reoxygenation. There were cells positive to terminal deoxynucleotidyl transferase-mediated dUDP-biotin nick end labeling in all 3 groups except for the Control Group; after reoxygenation, the number of cells became larger in Groups B and C than in Group A. Flow cytometry revealed that annexin V-positive cells were 1.15 ± 0.82% in the Control Group, 4.07 ± 3.8% in Group A without reoxygenation and 15.5 ± 6.3% in Group A after 30-min reoxygenation, respectively; the value was significantly higher in the latter than the former two ($P < 0.01$). Although 18-h and 24-h ischemia increased the annexin V-positive cells, reoxygenation did not alter the number of cells in Groups B and C. These results indicate that i) chemical ischemia followed by reoxygenation variably induces apoptosis of rat myocytes, ii) long-term ischemia causes phosphatidylserine translocation on the cell surface membrane, regardless of reoxygenation and iii) mild ischemia necessitates reoxygenation to translocate phosphatidylserine, which might play a crucial role in the initiation of apoptosis of the myocytes.

Key words: annexin V; apoptosis; cardiomyocyte; phosphatidylserine; reoxygenation injury

Recent reports have indicated that apoptosis of myocytes plays an important role in cardiac failure as well as ischemia/reperfusion injury in the myocardium (Olivetti et al, 1994; Kajstura et al., 1996; Dong et al., 1997; Anversa and Kajstura, 1998; Takemura, 1998). Although myocardial infarction was considered to be characterized by necrotic cell death due to the breakdown of cellular energy metabolism, there is some evidence that myocyte loss during the acute stages of myocardial ischemia and reperfusion involves both apoptotic and non-apoptotic cell death (Gottlieb et al., 1994, 1996; Saraste et al., 1997). The role of apoptosis in reperfusion injury has been addressed in rat and rabbit models (Gottlieb et al., 1994; Fliss and Gattinger, 1996) in which reperfusion was shown to accelerate the occurrence of apoptotic cell death. In cardiomyocytes, however, the mechanism of apoptosis has not been elucidated enough (Gottlieb et al., 1996; Misao et al., 1996; Haunstetter and Izumo, 1998; Yaoita et al., 1998; Yue et al., 1998). Therefore, it is important to examine each signal transduction

Abbreviations: FITC, fluorescein isothiocyanate; PS, phosphatidylserine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUDP-biotin nick end labeling

pathway of cell death with reproducible simulated models.

Apoptosis is characterized by chromatin condensation, reduction in cell volume, and cleavage of DNA by endonuclease into oligonucleosomal fragments. The early phase of apoptosis, on the other hand, is accompanied by the translocation of phosphatidylserine (PS) at the surface of the cell (Koopman et al., 1994; Martin et al., 1995; Van den Eijnde, 1997). The expression of PS on the cell surface plays an important role in the recognition and removal of apoptotic cells by macrophages (Verhoven et al., 1995, 1999). A new method for the detection of early phase-apoptotic cells has been used in a variety of organs and tumors (Koopman et al., 1994; Martin et al., 1995; Castedo et al., 1996; van Engeland et al., 1996). Researchers have used a PS-binding protein as a specific probe to detect the redistribution of this phospholipid. Annexins have been found to have a high affinity for aminophospholipids in the presence of Ca^{2+} ions (Bratton et al., 1997; Van den Eijnde et al., 1997). A member of this family, annexin V, preferentially binds PS.

In the present study, we attempted to ascertain whether short-term chemical ischemia followed by reoxygenation of cultured rat neonatal cardiomyocytes induces myocyte apoptosis or not.

Materials and Methods

All animal procedures were conducted in accordance with the Guidelines for Animal Experimentation at the Faculty of Medicine, Tottori University.

Cell cultures and treatments

Hearts were removed from 1- or 2-day-old Wistar rats and the ventricles were separated from the atria and vessels. Neonatal ventricular myocytes were cultured by the modification of a method previously described (Simpson and Savion, 1982; Ichiba et al., 1998). Briefly, myocytes were dissociated with 0.1% collagenase type I (Wako Chemical, Osaka, Japan) at 37˚C. The digestion medium was discarded. The cells were washed twice with Hanks solution and resuspended in medium 199 (M199, Bio-Whittaker, Walkersville, MD), supplemented with 4% fetal bovine serum (JRH biosciences, Lenexa, KS), 100-U/mL penicillin and 100-μg/mL streptomycin (Bio-Whittaker), 10-μg/mL transferrin from bovine plasma (Wako Chemical) and 10-μg/mL bovine pancreas insulin (Wako Chemical). The cell suspension was preplated on a 60-mm diameter plastic dish for 30 min at 37˚C in a 5% CO_2 incubator for isolating fibroblasts. The non-attached cells were sowed onto collagen-coated 60-mm, 35-mm and 24-well culture dishes at a concentration of 2.5×10^4 per 1 mm^2 and incubated in a 5% CO_2 incubator at 37˚C for 48 h prior to use.

Chemical ischemia

The culture medium was replaced with a glucose-free ischemic solution containing 136-mmol/L NaCl, 5.4-mmol/L KCl, 1-mmol/L $CaCl_2$, 0.53-mmol/L $MgCl_2$, 5.5-mmol/L HEPES and 5-mmol/L NaCN to inhibit oxidative phosphorylation adjusted to pH 6.7. We confirmed that the myocytes stopped beating at an early stage. They were then incubated in the same incubator for 9 h (Group A), 18 h (Group B) and 24 h (Group C). The control Group was not exposed to chemical ischemia.

Reoxygenation period

The ischemic solution was removed and myocytes were reoxygenated with M199. They were incubated in the same incubator for 30 min or 6 h.

DNA extraction and electrophoresis

At the end of each incubation period, cardiomyocytes were harvested and washed twice with phosphate-buffered saline. DNA was extracted with a DNA Extractor WB Kit (Wako Chemical, Osaka, Japan). Cells were kept in a 37˚C water bath for 60 min with 1-mg/mL protease. DNA was extracted with sodium

iodide and isopropyl alcohol (Wako Chemical) and then precipitated with ethanol (Wako Chemical). The precipitate was resuspended in 10-mmol buffer of Tris-HCl + 1-mmol EDTA. DNA electrophoresis was carried out with 2% agarose gel for 1.5 h at 50 V. As a size marker, a 200-base pair DNA StepLadder (lot #87203, Promega Corporation, Madison, WI) was used. The DNA was then visualized with ethidium bromide in UV light.

TUNEL assay

Cardiomyocytes were fixed in 90% ethanol for 4 h at room temperature and washed twice with phosphate-buffered saline. Terminal deoxynucleotidyl transferase-mediated dUDP-biotin nick end labeling (TUNEL) staining was performed with a commercially available kit (ApopTag Peroxidase Kit, Intergen Company, Purchase, NY) according to the manufacturer's instructions.

Measurement of PS exposure by annexin V-FITC

Translocation of PS in rat neonatal myocytes was measured with annexin V-fluorescein isothiocyanate (FITC) using an Annexin V-FITC Kit (Immunotech, MC, France). In brief, 5-μL/well annexin V-FITC solution was added to the cells cultured in a 24-well plate with a 500-μL medium and the plate was incubated for 15 min at room temperature. The cells were then harvested and washed twice with M199 containing 2.0-mmol Ca^{2+} ions and resuspended in M199 (10^6 cells/mL). Propidium iodide solution was added 2.55 μL/mL to the samples and the cells were incubated for 15 min on ice. We employed a confocal laser scanning system (INSIGHT PLUS, Meridian Instruments, Okemos, MI) equipped with an Olympus IMT-2 inverted microscope. The fluorescence images were recorded using a CCD camera (Meridian Instruments) and analyzed using INSIGHT-IQ software. For flow cytometric analysis, a FACSort (Becton Dickinson, San Jose, CA) equipped with a single argon ion laser was used. Ten thousand cells per sample were analyzed with the standard Lysis II and Cellfit software (Becton Dickinson). Damaged myocytes exhibit PS on their surface to which FITC-labeled annexin V attaches. Normal cells are annexinV-negative. This method can be used as a specific marker for early cell damage where the cell membrane is still intact.

Statistical analysis

All data are expressed as mean ± SD. The significance of difference was evaluated by Student's *t*-test. Statistical analysis was performed using the software program StatView version 5.0 on a Macintosh computer. Differences were considered significant if *P* was less than 0.05.

Results

The prepared rat neonatal cardiomyocytes contracted spontaneously and synchronously at an average rate of 179 ± 23 beats/min at 37˚C. They were cultured under the chemical ischemia followed by reoxygenation, which provoked the cardiomyocytes to pulsate. For example,

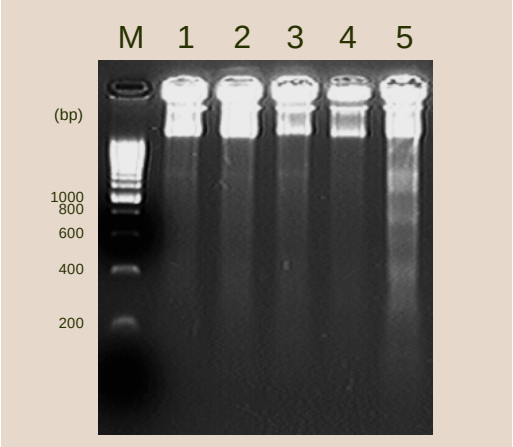

Fig. 1. Internucleosomal DNA cleavage induced by simulated ischemia and/or reoxygenation on 2% agarose gel electrophoresis. A 200-base pair (bp) DNA molecular size marker (Lane M), ischemia-free control (Lane 1), ischemia for 9 h (Lane 2), ischemia for 9 h + reoxygenation for 6 h (Lane 3), ischemia for 18 h (Lane 4) and ischemia for 18 h + reoxygenation for 6 h (Lane 5). Lane 5 shows the ladder pattern.

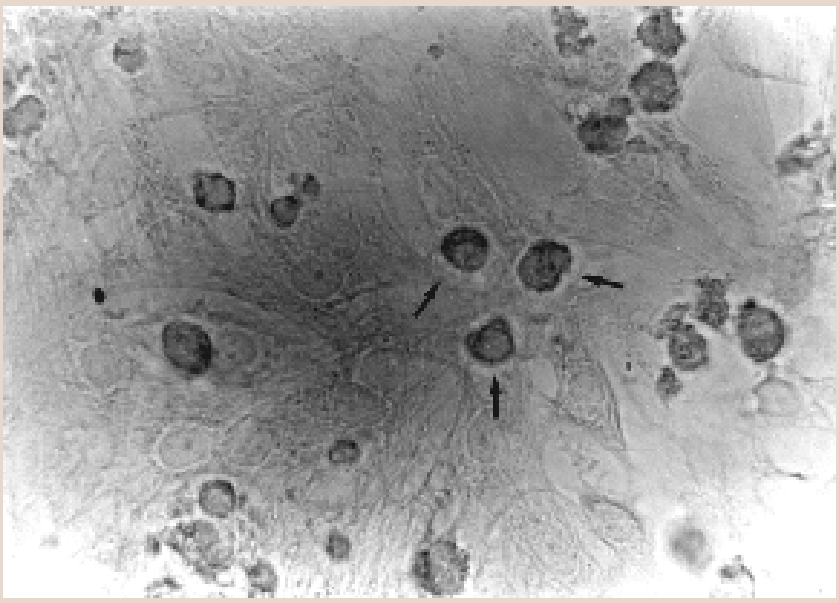

Fig. 2. Light micrograph of the incubated cardiomyocytes with terminal deoxynucleotidyl transferase-mediated dUDP-biotin nick end labeling (TUNEL) staining. The ischemia for 9 h + reoxygenation for 30 min increases TUNEL-positive cells (arrowheads). Original magnification, × 400.

the cardiomyocytes exposed to 9-h chemical ischemia pulsated at an average rate of 176 ± 19 and 226 ± 8.5 beats/min after 30-min and 60-min reoxygenation, respectively.

DNA fragmentation

First, DNA ladder formation was examined on the 2% agarose gel electrophoresis. No DNA ladder was noted in the cardiomyocytes of the 4 groups obtained immediately after chemical ischemia. The cardiomyocytes showed DNA ladders, only when the cells were harvested after 18-h and 24-h chemical ischemia (Groups B and C) followed by 6-h reoxygenation (Fig. 1 lane 5). On the other hand, no DNA ladder was noted in the Control Group without chemical ischemia or in Group A followed by 6-h reoxygenation (Fig.1).

Next, the cardiomyocytes were analyzed by the TUNEL method to confirm apoptotic cell death morphologically. Only a few TUNEL signal-positive cells were noted in all 4 groups, prepared immediately after ischemia without reoxygenation. A good number of TUNEL signal-positive cells were observed in Groups B (Fig. 2) and C, followed by 6-h reoxygenation. The number of TUNEL signal-positive cells was smaller in the groups which were exposed to 0- and 9-h chemical ischemia (Group A), followed by 6-h reoxygenation.

PS exposition after chemical ischemia and reoxygenation

The cardiomyocytes were stained with annexin V-FITC after chemical ischemia with/without reoxygenation. Fluorescence-activated cell sorter analysis showed no signal for FITC or propidium iodide in the myocytes of the Control Group without chemical ischemia and reoxygenation (Fig. 3A). Figure 3B shows autofluorescence of the cardiomyocytes which were exposed to 9-h ischemia and 30-min reoxygenation. On the other hand, annexin V-FITC-positive cardiomyocytes increased in the group exposed to 9-h ischemia and 30-min reoxygenation, and outnumbered the positive cells in the group exposed to 9-h ischemia without reoxygenation (Figs. 3C and D).

Figure 4 shows the percentage of annexin V-positive cells after ischemia with/without re-

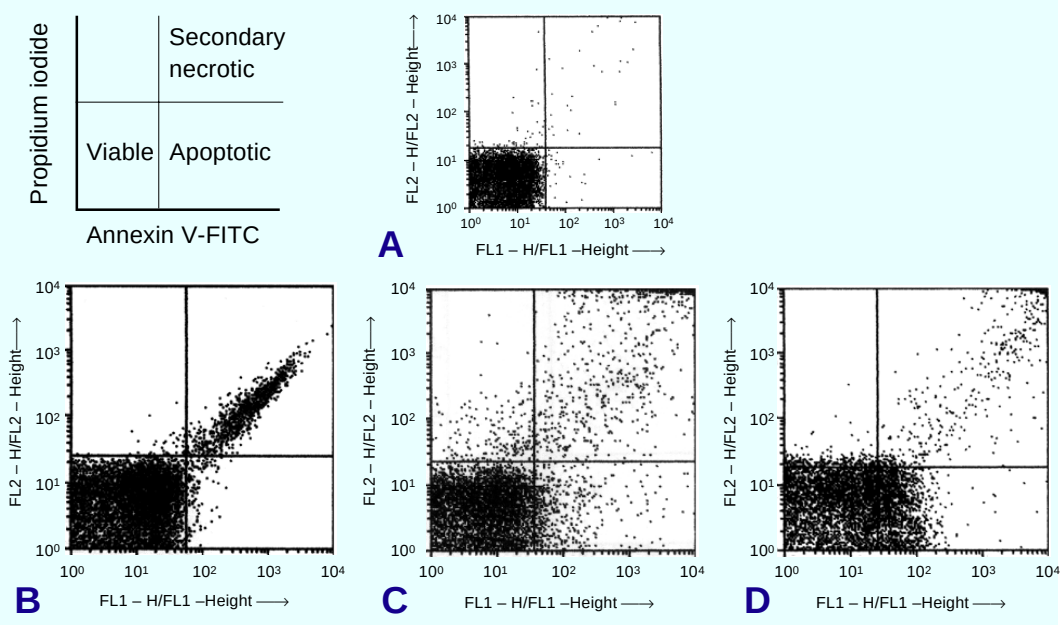

Fig. 3. Schematic presentation of the flowcytometric results obtained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide. **A:** results of control. **B:** ischemia for 9 h, autofluorescence. **C:** ischemia for 9 h. **D:** ischemia for 9 h + reoxygenation for 30 min.

oxygenation. The average percentage of the positive cells was 1.15 ± 0.82 in the Control Group (n = 20), 4.07 ± 3.8 in Group A (n = 8) without reoxygenation, and 15.5 ± 6.3 in Group A (n = 8) with 30-min reoxygenation; the value was significantly higher in the latter than in the former two (P < 0.01). Thus, reoxygenation enhances translocation of annexin V (PS) at the cell surface. Although a number of the annexin V-positive cells significantly increased after 18-h and 24-h ischemia (P < 0.05), reoxygenation did not increase the cells in Groups B and C (Fig. 4).

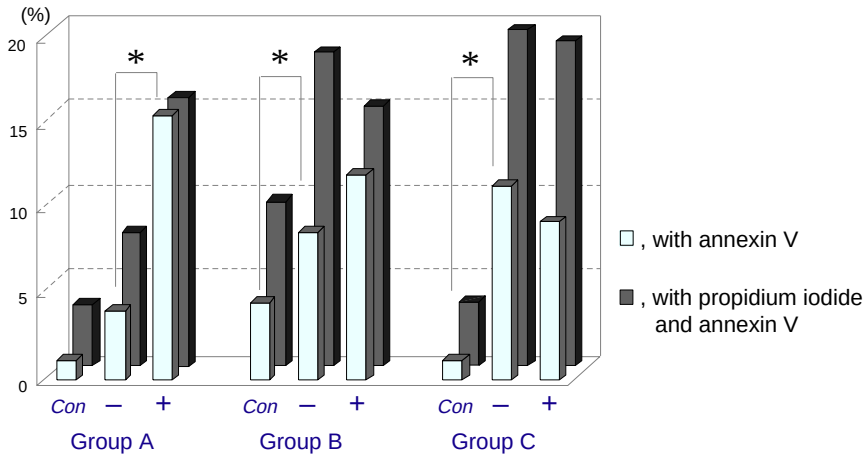

Fig. 4. Effects of chemical ischemia and reoxygenation on the viability of neonatal rat cardiomyocytes. Group A, 9-h ischemia; Group B, 18-h ischemia; Group C, 24-h ischemia. +, with 30-min reoxygenation; –, without reoxygenation; *Con*, control. * P < 0.05.

Discussion

The present study clearly demonstrates that chemical ischemia followed by reoxygenation induces cell death in cultured rat neonatal ventricular myocytes. This model has been well-established and was conducted to elucidate the mechanism of myocardial cell death by ischemia as well as reoxygenation.

Long et al. (1997) used a cultured neonatal rat cardiomyocyte model and showed that exposure of myocytes to hypoxia for 48 h resulted in an intranucleosomal cleavage of genomic DNA characteristic of apoptosis in 60% of the cells. This result is similar to that of Tanaka et al. (1994) who demonstrated that exposure of cultured neonatal cardiomyocytes to more than 12 h of hypoxia induces apoptosis. On the other hand, Karwatowska-Prokopczuk et al. (1998) found that chemical ischemia for 3 h induced myocyte apoptosis in 65% of rat neonatal cardiomyocytes. These results indicate that ischemia alone can induce apoptosis in rat cardiomyocytes. In the present study, it was found that chemical ischemia for 18 and 24 h followed by reoxygenation induces apoptosis, which was confirmed by DNA ladder formation on agarose gel electrophoresis, a hallmark of apoptosis. No DNA ladder was noted in any other group, in which chemical ischemia was loaded for 9 h. On the other hand, the nuclear TUNEL signal has been demonstrated in various levels in the cultured rat myocytes of all 3 groups. Thus, 9-h ischemia followed by reoxygenation did not show any DNA ladder, in spite of the presence of TUNEL signal-positive cells. This discrepancy might be explained by the small number of the TUNEL-positive cells in Group A. Although the standard method for measuring apoptosis is the microscopic detection of morphological changes of the cells, this method is not available for quantitative analysis.

Conceptionally, apoptosis is divided into 3 phases: initiation, propagation and execution. Recent studies have shown that changes in the plasma membrane have been recognized as an attractive target for the analysis of apoptosis of various organs or tumors (Koopman et al., 1994; Martin et al., 1995; van Engeland et al., 1996). We used FITC-labeled annexin V for the detection of PS translocation as an initiation of cardiomyocyte apoptosis (Verhoven et al., 1995; Fadok et al., 1998; Verhoven et al., 1999). This process has been thought to precede DNA fragmentation and loss of membrane integrity. The mechanisms regulating the distribution of transmembrane phospholipids and the implications of transbilayer lipid scrambling and cell death are still under consideration (Castedo et al., 1996; Van den Eijnde et al., 1997; Bevers et al., 1998). The DNA stain propidium iodide was included in the assay mixture to discriminate between the different types of cell death in the present study. Annexin V-positive cells can be divided into 2 populations based on the integrity of the plasma membrane: cells with a damaged plasma membrane and cells with an intact membrane. The former are labeled with propidium iodide and the latter with annexin V.

In response to the addition of cyanide and to the glucose-free conditions during the ischemic phase, the intracellular ATP levels decreased and intracellular Ca^{2+} levels increased in cardiomyocytes. Moreover, reoxygenation markedly increases intracellular Ca^{2+} concentration and causes the hypercontracture of cardiomyocytes within several minutes (Quaife et al., 1991; Siegmund et al., 1994). This might be the main cause of the myocyte injury in the present model because there are few influences from the extracellular environment such as cytokines. Some reports showed that an ATP-dependent transporter called translocase is responsible for the rapid inward movement of phospholipids in normal cells and that the increased levels of intracellular Ca^{2+} of damaged cells accelerates the redistribution of all phospholipids via Ca^{2+} dependent “scramblase” (Bratton et al., 1997; Bevers et al., 1998). Ischemia reduced myocyte ATP levels. When reoxygenation starts, the intracellular ATP level is still low and therefore, an overload of intracellular Ca^{2+} might accelerate the translocation of PS via activation of “scramblase”.

In our study, 9-h ischemia with 30-min reoxygenation induced the translocation of PS. But 18-h ischemia without reoxygenation

potentiated apoptosis or necrosis (secondary necrosis) and the influence of reoxygenation alone was less extensive. Chen et al. (1998) showed that 18-h chemical ischemia triggered apoptosis of isolated neonatal rat cardiomyocytes and they distinguished between a reversible initiation phase and a subsequent irreversible execution phase. Our results suggest that reoxygenation in the early stages of ischemia might result in the additional cell death of potentially viable myocytes. This would explain why apoptotic cardiomyocytes were observed in the transitional areas of the infarcted myocardium of an in vivo experimental model (Saraste et al., 1997). In other words, ischemia for more than 9 h induces myocardial injury and further reoxygenation promotes the damage. The choice of strategies to prevent reperfusion injury might differ with the degree of cell viability.

This study was based on the hypothesis that PS exposure is an early signal of cardiomyocyte apoptosis. However, few reports have investigated whether the transmembrane mobility of phospholipids activates the signal transduction pathways of cell death especially in cardiomyocytes. We did not obtain a DNA ladder at 9-h ischemia with reoxygenation. A variety of stimuli induce apoptosis, acting through several signaling pathways that eventually converge to a sequence of execution steps (Shimizu et al., 1996). This is why results may differ between a DNA ladder and PS exposition. It is necessary to examine each event leading up to myocyte apoptosis with reproducible models.

In summary, chemical ischemia followed by reoxygenation induces apoptosis of neonatal rat cardiomyocytes. The early phase of apoptosis can be initiated by PS, which can then be successfully recognized with annexin V.

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