Cyclin A/cdk2 Activation Is Involved in Hypoxia-Induced Apoptosis in Cardiomyocytes

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Abstract—Cardiomyocytes are terminally differentiated cells characterized as withdrawal cell-cycle machinery, but nonetheless they are known to express cell-cycle regulators. Because many proteins related to the cell cycle induce apoptosis in proliferating cells, we examined the involvement of these proteins in the apoptosis pathway in cardiomyocytes. Primary rat cardiomyocytes were exposed to a severe hypoxic condition to induce apoptosis. The apoptosis rate of cardiomyocytes increased to \( \approx 40\% \) under 24 hours of hypoxia as evaluated by the TUNEL method. The cyclin A protein level assessed by immunoblot analysis accumulated in a time-dependent manner in cardiomyocytes, but there was no increase in nonmyocytes. Hypoxia increased the activity of cyclin A–associated kinase but not the activity of cyclin E–associated kinase, and the apoptosis was inhibited by infection of dominant-negative cdk2 adenovirus, suggesting that cyclin A and its associated kinase play significant roles in the apoptosis of cardiomyocytes. To investigate the cyclin A–mediated apoptosis, we infected cultured cells with cyclin A adenovirus. Apoptosis was induced in 63\% ± 12\% of the infected cardiomyocytes in contrast to only 12\% ± 3\% of the LacZ-infected control cells. In addition, the cells in the hypoxic condition showed an increase in caspase-3 activity and a subsequent decrease in p21\(^{kip1/waf1}\) protein, which is partly cleaved by caspase-3. These findings confirm that cyclin A–associated kinase mediates hypoxia-induced apoptosis in cardiomyocytes, and they also suggest that additional elements of the cell-cycle–dependent machinery participate in this mechanism. (Circ Res. 2001;88:408-414.)

Key Words: apoptosis ■ hypoxia ■ cardiomyocytes ■ cell cycle

In proliferating cells, apoptosis and proliferation are linked by cell-cycle regulators, and apoptotic stimuli affect both cell proliferation and cell death. When cells proliferate, the mitotic cycle progression is tightly regulated by an intricate network of positive and negative signals, and much is known about the molecules involved in cell-cycle control. Progress from one phase of the cell cycle to the next is controlled by the activation or inactivation of several members of a conserved family of serine/threonine protein kinases known as the cyclin-dependent kinases (cdks).1 In recent reports, cdk activity has been shown to be involved in apoptosis. In human endothelial cells, apoptosis induced by deprivation of growth factor is associated with an upregulation of cyclin A–associated cdk2 activity, and inhibition of this activity suppresses apoptosis.2 Inhibitors of cdk in rat cerebellar granule neurons prevent KCl withdrawal–induced apoptosis.3 Other cyclins and cell-cycle regulators, such as c-myc, p53, cdc25, and E2F, have also been mediated in apoptosis events.4

Cardiomyocytes undergo terminal differentiation soon after birth, irreversibly withdrawing from the cell cycle. Previous studies from our laboratory and other groups have determined that cardiomyocytes expressed some of these cell-cycle regulators,5,6 and that cdk activity may be required for the induction of cardiomyocyte hypertrophy. Nonetheless, the exact roles and significance of these regulators in cardiomyocytes are still not precisely understood.

The heart is occasionally exposed to ischemia during episodes of cardiac surgery, angina pectoris, and myocardial infarction. These ischemic episodes cause cellular damage and cell loss, resulting in reduced cardiac function. Part of the cell loss is incurred because of the apoptosis of cardiomyocytes. To prevent the expansion of these apoptotic regions, the underlying mechanisms have to be more thoroughly investigated. We previously demonstrated that hypoxia easily induced apoptosis of cultured cardiomyocytes but had no such effect on nonmyocytes.7 Moreover, in vitro experiments using neonatal rat cardiomyocytes, Long et al8 reported that the apoptosis after hypoxia was partly attributable to the p53-dependent pathway. Several regulatory mechanisms of apoptosis have also been investigated in cardiomyocytes in cellular cytoplasm, such as caspases and the mitogen-activated protein kinase family.9,10 Although ischemia-
induced apoptosis in the heart has been widely recognized and investigated, its underlying mechanisms are not yet understood. To effectively reduce the infarcted size in the treatment of myocardial infarction, it will be important to understand the underlying mechanism of apoptosis in cardiomyocytes.

In the present study, we examined the participation and regulation of cell-cycle molecules in the process of hypoxia-induced apoptosis in neonatal rat cultured cardiomyocytes during their withdrawal from the cell cycle.

Materials and Methods

Cell Cultures and Treatment

Neonatal cardiomyocytes were subjected to Percoll gradient centrifugation and cultured in vitro as described previously.5,7,11

Analysis of DNA Fragmentation and TUNEL Assay

Detection of DNA fragmentation and TUNEL analysis were performed by the methods described previously.12 All experiments were repeated on at least 3 independent occasions with consistent results.

Electron Microscopic Examination

The ultrastructural features of the apoptotic cardiomyocytes exposed to hypoxia were examined by the previously described electron microscopic method.12

Antibodies and Immunoblotting

The following antibodies and reagents were purchased: polyclonal antibodies for rabbit cyclin A (sc-751, Santa Cruz Biotechnology), cyclin E (sc-481), bcl-XL (sc-7195), Bax (sc-493), and cdk2 (sc-163); mouse monoclonal antibodies for p21(crip1, waf1) (sc-6246) and bcl-2 (sc-2003, Santa Cruz Biotechnology) and reviewed previously.13

Immunofluorescent Staining

Immunofluorescent staining of cyclin A with rhodamine-conjugated anti-rabbit IgG antibody (23828, Polysciences, Inc) was performed. Immunofluorescent staining of cyclin A with rhodamine-conjugated anti-rabbit IgG antibody (23828, Polysciences, Inc) was performed. Immunofluorescent imaging were obtained using a ZEISS LSM510 laser scanning confocal microscope.

Adenoviral Constructs and Infection

The recombinant adenoviruses of cyclin A and dominant-negative cdk214 were constructed by the method described previously.5

In Vitro Histone H1 Kinase Assay

The cardiomyocytes were harvested at various time points after being exposed to the hypoxic condition or infected with adenovirus. Whole-cell extract (100 mg) was precleared with protein A/G agarose beads (sc-2003, Santa Cruz Biotechnology) and immunoprecipitated with anti-cdk2, anti-cyclin E, or anti-cyclin A polyclonal antibody overnight at 4°C. After the pellets were washed twice in lysis buffer and twice in kinase buffer, they were incubated in 25 μL of kinase assay solution for 20 minutes at 30°C. The mixtures were boiled for 3 minutes, loaded onto a 10% SDS-polyacrylamide gel, and then exposed to x-ray film (Hyperfilm ECL, Life Science) after electrophoresis.

Results

Hypoxia-Induced Apoptosis in Cardiomyocytes

The apoptosis induced in primary neonatal rat cardiomyocytes in response to hypoxia was investigated. Cultured cardiomyocytes exposed to severe hypoxia for 24 hours exhibited fragmented DNA representing an intranucleosomal DNA cleavage (Figure 1A). Electron microscopic examination confirmed the ultrastructural features of apoptosis. The death of the cardiomyocytes exposed to 24 hours of hypoxia was confirmed to be attributable to apoptosis by an ultrastructure characterized by apoptotic bodies, condensation and margination of chromatin against nuclear membrane, condensation of cytoplasmic organelles, and shrinkage of the cytoplasm. Condensation of chromatin was detected at the periphery of the nucleus, and blebbing and fragmentation of the cytoplasm were observed (Figure 2). Figure 1B shows the
increased end-labeling of DNA in the hypoxia-treated cardiomyocytes as detected by the TUNEL method. There were significant increases in the number of end-labeled cardiomyocytes evaluated after 24 hours of hypoxia, whereas the changes of end-labeled nonmyocytes under hypoxia were minimal. These results demonstrate that hypoxia significantly accelerates apoptosis in cardiomyocytes.

bcl-2 Family Proteins in Cardiomyocytes by Hypoxia

The proteins of the bcl-2 family are critical factors in cell death. Bcl-2 was the first member of the protein family to be identified. Subsequently, several homologous proteins were cloned. Bcl-2 and several other members of this family block apoptosis, whereas others, such as bax, promote cell death, and ultimately the fate of a cell is determined by the ratio between the apoptosis blockers and apoptosis promoters.9,16 To confirm whether bcl-2 family proteins are associated with hypoxia-induced apoptosis, we examined protein levels of several bcl-2 family proteins in cultured rat cardiomyocytes. A decreased bcl-2/bax or bcl-XL/bad ratio is known to increase the probability that a cardiomyocyte will undergo apoptosis.17-19 These protein ratios were not decreased in cardiomyocytes in response to hypoxia (Figure 3). Although the data suggest that the hypoxia-induced apoptosis is unrelated to bcl-2/bax or bcl-XL/bad, there is still a chance that other bcl-2 family members change the expression or the localization.

Changes of Cell-Cycle Regulator Proteins in Hypoxia-Induced Apoptosis in Cardiomyocytes

To determine if the protein levels of cell-cycle regulators change in hypoxia, we used immunoblotting to examine several cell-cycle regulators, ie, c-myc, cdk2, and cyclins. Although cyclin B, cyclin E, c-myc, and cdk2 did not exhibit major changes under the hypoxic condition, the level of cyclin A gradually increased in a time-dependent manner (Figures 4A and 4C). A comparison of cyclin A expression in cardiomyocytes and apoptosis-resistant nonmyocytes is shown in Figure 4B. The level of cyclin A protein slightly decreased in nonmyocytes. To ensure that cardiomyocytes expressed cyclin A, double immunostaining was performed with cyclin A and sarcomeric actin antibodies. Immunofluorescent staining for cyclin A (red) showed that cyclin A was expressed in perinuclear lesions in cardiomyocytes after 48 hours of hypoxia (Figure 4D). The percentage of cardiomyocytes with high cyclin A expression was 72±15% after 48 hours of hypoxia; however, for nonmyocytes the percentage was 10±6% (counted 200 cells, 3 independent experiments). These results suggest that cyclin A expression is related to apoptosis.

Activation of Cyclin A–Associated Kinase Activity in Response to Hypoxia-Induced Apoptosis

To confirm the phosphorylation of cyclin A–associated or cdk2-associated kinase activity during hypoxia, cyclin A–associated, cyclin E–associated, and cdk2-associated complexes were immunoprecipitated by cyclin A, cyclin E, and cdk2 antibody, respectively, and used in an in vitro kinase assay with histone H1 as a substrate. We observed more than a 5-fold increase in the phosphorylation of histone H1 in the complex immunoprecipitated by cyclin A a3-fold increase in the phosphorylation in the complex immunoprecipitated by cdk2. However, cyclin E–associated kinase was slightly decreased (Figures 5A and 5B). Thus, we know that cyclin A–associated kinase activity is indeed activated during hypoxia. Next, to test whether the cyclin A–associated kinase was the effector for apoptosis in this model, cardiomyocytes were exposed to cdk2 inhibitor, butyrolactone-1 (10⁻³ mol/L) (YE-0003, Funakoshi) under a hypoxic condition. When cardiomyocytes were incubated in a hypoxic condition with butyrolactone-1, apoptosis was significantly reduced compared with cardiomyocytes exposed to 24 hours of hypoxia by the TUNEL method (Figure 6E).
Association of Cyclin A/cdk2 and Apoptosis in Cardiomyocytes

To examine the significance of increased cyclin A in response to hypoxia, we determined whether overexpression of cyclin A is sufficient to induce apoptosis in normoxic cardiomyocytes. Cardiomyocytes were infected with a replication-defective adenovirus encoding human cyclin A under the transcriptional control of the \( \beta \)-actin promoter (AxcycA). As a control, the cardiomyocytes were also infected with LacZ adenovirus (AxLacZ). According to the TUNEL method, overexpression of cyclin A induced apoptosis and concomitantly increased cdk2 activity after 72 hours of infection in cultured rat cardiomyocytes but not in control cells infected with AxLacZ (Figures 6A and 6C). In addition, DNA laddering formation was observed in the AxcycA adenovirus–infected cardiomyocytes (Figure 6B), indicating that cyclin A functions as an apoptosis regulator in cardiomyocytes. Furthermore, we compared the respective p53 protein levels in two groups of cells, one overexpressing cyclin A and the other overexpressing LacZ as a control (Figure 6C). There were no significant differences in p53 protein levels between cardiomyocytes and nonmyocytes after 48 hours of hypoxia stimulation. C, Densitometrical analyses of cell-cycle regulator proteins. Values were calculated from 4 independent experiments and were normalized to those at time 0. Results were shown as mean ± SEM. *Significantly different from the 0 time point (P < 0.05). D, Immunofluorescent staining for cyclin A and sarcomeric actin using a laser scanning confocal microscope. Cyclin A protein was highly expressed around nuclei in cardiomyocytes after 48 hours of hypoxia (rhodamine, red) (light blue arrowhead). However, the expression of cyclin A in nonmyocytes was not changed after 48 hours of hypoxia (white arrowhead). Cardiomyocytes were identified using sarcomeric actin antibody (fluorescent, green).

Involvement of Caspase-3 Activation and p21 Cleavage in Hypoxia-Induced Apoptosis

To understand the mechanism underlying the upregulation of cyclin A/cdk2 activity in apoptotic cells, we examined p21^{kip1/waf1}, a cdk2 inhibitor known to play major roles in the regulation of cdk2 activity.21 Western blot analysis with a monoclonal antibody for p21^{kip1/waf1} revealed that the p21^{kip1/waf1} native protein was gradually decreased in a time-dependent fashion during hypoxia. Strikingly, a 14-kDa protein was seen after 6 hours of hypoxia, suggesting that the lower-molecular-weight band corresponds to a truncated p21^{kip1/waf1} molecule (Figure 7A). The amounts of this truncated p21^{kip1/waf1} molecule (14 kDa) present in the apoptotic cells are substantially lower than the amount of the native protein. Because the truncation of p21^{kip1/waf1} by specific
cleavage in apoptotic cells has been ascribed to the activity of caspase 3,2,2,2 we measured caspase 3 activity in cardiomyocytes under a hypoxic condition. The caspase 3 activity level gradually increased, and the maximum activity was induced by 9 hours of hypoxia (Figure 7B).

**Discussion**

Because very little is known about the cell-cycle regulators in the cardiomyocytes that irreversibly withdraw from the cell cycle, we examined whether cell-cycle regulatory proteins play a role in the hypoxia-induced apoptosis of cardiomyocytes. We reported here for the first time that catastrophic cdk2 activity in cultured cardiomyocytes might be the actual effector of hypoxia-induced apoptosis via degradation of p21cip1/waf1 by caspase-3 and upregulation of cyclin A protein. An essential role of cdk activities in the cell death process has been inferred from genetic studies with transiently expressed antisense and dominant-negative cdk mutants.23,24 A recent study reported that the cdks act at an early step in the pathway of KCl withdrawal–induced apoptotic death of cerebellar granule cells, a cell type that shows a differentiation similar to that of cardiomyocytes.3 Induction of apoptosis by several different stimuli is accompanied by upregulation of cyclin A–associated kinase activity, and inhibition of this activity suppresses apoptosis.23,25–30 Levkau et al2 reported that specific cleavage of two cyclin A–associated cdk inhibitors, p21cip1/waf1 and p27kip1, coincided with rapid upregulation of cyclin A cdk2 activity in growth-factor deprivation of cultured human umbilical vein endothelial cells. We have shown increased expression of cyclin A in a hypoxic condition in cardiomyocytes concomitantly with cdk2 activation but not concomitantly with cyclin E cdk2 activation. On the

**Figure 5.** A, Effect of 24 hours of hypoxia on cyclin A–associated, cyclin E–associated, and cdk2–associated kinase activity in cardiomyocytes. Lysates of cultured control cardiomyocytes or cardiomyocytes exposed to 24 hours of hypoxia were immunoprecipitated with anti-cyclin A, anti-cyclin E, or anti-cdk2 antibody. Immune complexes were assayed for histone H1 kinase activity. IP indicates immunoprecipitation. B, Densitometrical analyses of histone H1 kinase activity. Values were calculated from 3 independent experiments.

**Figure 6.** Effects of cyclinA/cdk2 activity on apoptosis in cardiomyocytes. A, Increased apoptosis in normoxic cardiomyocytes infected with cyclin A adenovirus (100 multiplicity of infection [ moi]) (AxcycA). Evaluation of apoptosis rate by TUNEL assay after 72 hours of infection in cardiomyocytes. LacZ-expressed adenovirus was used as control. B, Changes of p53, cyclin A proteins, and cyclin A–associated kinase activity in AxcycA-infected cells after 48 hours. C, DNA fragmentation in adenovirus AxcycA-infected cardiomyocytes. Genomic DNA extracted from cultured cardiomyocytes infected for 72 hours to 20 moi or 100 moi AxcycA. Electrophoresis of genomic DNA reveals a fragmentation pattern characteristic of apoptosis only in AxcycA-infected cells. Representative results characteristic of 2 independent experiments. D, Inhibition of apoptosis in cardiomyocytes by dominant-negative cdk2 adenovirus (dncdk2) (50 moi) under 18 hours and 24 hours of hypoxia. *Significantly different from the 24 hours of hypoxic condition (P < 0.05). E, Inhibition of apoptosis by butyrolactone-1, cdk2 inhibitor, after 24 hours of hypoxia. The number of cardiomyocyte deaths by apoptosis evaluated by TUNEL method was expressed as a percentage. *Significantly different from 24 hours of hypoxia (P < 0.05). F, Activation of the E2F-1 promoter by hypoxia in cardiomyocytes. *Significantly different from normoxia as control (P < 0.05).
analyses of p21cip1/waf1 under hypoxic condition are shown. Both in cardiomyocytes under a hypoxic condition. A, Immunoblot expression by E2F-1 in proliferating cells; therefore, addition, cyclin A induction is known to involve transcriptional regulator, such as E2F, might be responsible. In normoxic cardiomyocytes and that pharmacologically induced inhibition of cdk2 activity suppresses hypoxia-induced apoptosis. Furthermore, to activate E2F-1 promoter by hypoxia, the corresponding downstream changes of cyclin A-associated kinase, such as E2F, might be responsible. In addition, cyclin A induction is known to involve transcriptional activation by E2F-1 in proliferating cells; therefore, our results suggest a role for cyclin A/cdk2 kinase activity in a positive feedback loop regulating the expression of the cyclin A. Taken together, these findings strongly suggest that cyclin A-associated kinase plays a critical role in the hypoxia-induced apoptosis in cardiomyocytes.

The changes in p53 protein levels correlate with the induction of apoptosis in endothelial cells. p53 is a transcriptional regulator of the antiapoptotic gene product bcl-2 and the proapoptotic gene product bax. However, in the immunoblot analysis, exposure of endothelial cells to hypoxia did not alter the levels of the proapoptotic protein bax or the antiapoptotic protein bcl-XL. In cardiomyocytes, p53 is upregulated in hypoxia and mediates apoptosis. The expression of Bcl-2 is increased in ischemic cardiomyocytes, whereas the proapoptotic protein bax remains constant, suggesting the lack of activation of bax under certain conditions. Presently, it is a matter of controversy whether p53 and p53-inducible genes are involved in the modulation of cardiomyocyte apoptosis in hypoxia. In fact, hypoxia did not change our results on the expression ratios of bcl-2 families.

Figure 7. Cleavage of p21cip1/waf1 protein and caspase-3 activity in cardiomyocytes under a hypoxic condition. A, Immunoblot analyses of p21cip1/waf1 under hypoxic condition are shown. Bottom panel is a long exposure of the same blotting shown in the top panel. B, Hypoxia-induced change of caspase-3 activity in cardiomyocytes. The activity was measured by absorbance (405 nm) using a plate reader. Bars = SD of 4 data points.

Although forced expression of wild-type p53 can be sufficient for apoptosis in cardiomyocytes, p53-independent mechanisms have also been noted to induce apoptosis. In MCF-7 cells, human breast-cancer cells, Amellem et al also demonstrated that hypoxia-induced apoptosis occurred independently of the p53 protein level. In the heart, apoptosis was induced by the adenoviral gene transfer of E2F-1 by direct injection into the myocardium of p53 knockout mice. Furthermore, the magnitude of apoptosis in the infarcted myocardium remains unaltered in p53 knockout mice, suggesting the existence of a p53-independent mechanism of apoptosis.

In our study, there were no significant differences in p53 protein levels between the cyclin A overexpression group and control group, indicating that p53 is either uninvolved in cyclin A–mediated apoptosis or acts as an upstream regulator of cyclin A.

The activation of caspases is known as another key regulator of apoptosis. Genetic and biochemical studies indicated that apoptosis is triggered by activation of the members of the caspase protease family. These proteases preferentially cleave protein substrates at certain aspartic acid residues. Caspase-3 activation is known to occur in a hypoxic condition in various cells, including cardiomyocytes. Several groups recently reported that p21cip1/waf1 is truncated to 14 kD by caspase-3 in apoptotic cells, and it was confirmed that this truncation coincides with upregulation of cyclin A–cdk2 activity. In our results, the caspase-3 activation by hypoxia was likely to have selectively cleaved p21, leading to an induction of cdk2 activity before the appearance of apoptosis. Also, given the only partial suppression of apoptosis by dominant-negative cdk2 in the hypoxic condition, we can speculate that another mechanism of apoptosis, such as a p53-dependent mechanism, is at work.

Cell-cycle regulator proteins may play physiologically and pathophysiologically different roles in neonatal myocytes and terminally differentiated adult myocytes; however, we have offered several new insights into the understanding of the cell-cycle regulators and apoptosis in cardiomyocytes. In summary, these findings suggest that cdk2 activation may be instrumental in the occurrence of apoptosis after caspase activation and cyclin A expression in a hypoxic condition, partially through the caspase-mediated cleavage of the cdk inhibitor. These molecules associated with the cell cycle may also contribute to the apoptotic mechanism in the cardiomyocytes.

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