α- and β-Adrenergic Pathways Differentially Regulate Cell Type–Specific Apoptosis in Rat Cardiac Myocytes

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Background—The apoptosis of cardiac myocytes may play a role in the development of heart failure. Norepinephrine is one of the factors activated in heart failure and can induce myocardial cell apoptosis in culture. However, it is unknown if α- and β-adrenergic pathways coordinately or differentially regulate apoptosis and if this apoptotic pathway uses common or cell type–specific apoptotic signals.

Methods and Results—We stimulated cultured neonatal rat cardiac myocytes with an α1-adrenergic agonist (PE, phenylephrine), a β-adrenergic agonist (isoproterenol [Iso]) or a membrane-permeable cAMP analogue (8-Br-cAMP) in serum-free conditions for 48 hours. Iso and 8-Br-cAMP markedly increased the number of TUNEL-positive cells (%TUNEL-positive nuclei >40%) compared with saline stimulation (<10%). DNA fragmentation was also confirmed by ladder formation in agarose gels. Apoptotic myocytes were characterized by cell shrinkage and nuclear condensation, consistent with morphological features of apoptosis. The Iso-induced apoptosis was almost completely inhibited by the protein kinase A–specific inhibitor KT5720. In contrast, PE inhibited 8-Br-cAMP–induced myocardial cell apoptosis. The apoptosis-inhibitory effect by PE was negated by the α1-adrenergic receptor antagonist prazosin and the MEK-1–specific inhibitor PD098059. Interestingly, although 8-Br-cAMP markedly induced apoptosis in cardiac myocytes, it completely blocked serum depletion–induced apoptosis in PC12 cells, a rat pheochromocytoma cell line. Conclusions—These findings indicate that α- and β-adrenergic pathways differentially regulate myocardial cell apoptosis. The results also suggest that a cAMP– protein kinase A pathway is necessary and sufficient for β-adrenergic agonist–induced apoptosis and that this apoptotic pathway is not functional in other cell types, for example, PC12 cells. (Circulation. 1999;100:305-311.)

Key Words: apoptosis ■ myocytes ■ heart failure

Apoptosis, or programmed cell death, is a central feature of normal tissue development in the fetus and of cell replacement in certain adult tissues (eg, thymus).1–5 In contrast to the classic swelling and membrane rupture associated with necrosis, apoptotic cells shrink and maintain their membrane integrity. The hallmarks of apoptosis include cell shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation. Apoptotic cells are phagocytosed by neighboring cells, effectively removing unwanted cells without an inflammatory response. Apoptosis is most often associated with cells that are progressing through the cell cycle. Although adult cardiac myocytes are terminally differentiated and have lost their ability to divide, accumulating evidence suggests that these cells can undergo apoptosis in vivo in various animal models of heart failure including models of rapid ventricular pacing6,7 and pressure overload caused by aortic constriction8 and aged spontaneously hypertensive rats.9 Since the functional aspects of the failing myocardium are impaired in proportion to the reduction in the myocyte fractional area, the apoptosis of myocytes may play a significant role in the deterioration of cardiac function. As such, the identification of the signaling pathways that mediate cardiac myocyte cell death and survival is critical to the ultimate elucidation of the molecular basis of cardiac muscle failure.

Despite an increasing body of evidence concerning myocardial cell apoptosis in vivo, little is known regarding the relevant physiological stimuli. The control of programmed cell death is dependent on a balance between inhibitors and inducers of apoptosis. Since a number of neurohormonal factors are activated in congestive heart failure,10,11 they may play positive and negative roles in regulating myocardial cell apoptosis. Norepinephrine is one such factor, the elevation of which in plasma closely correlates with the severity and poor prognosis of heart failure.11 It was recently shown that norepinephrine can induce apoptosis in cardiac myocytes in vitro.12 Norepinephrine exerts its effect on cardiac myocytes through both α- and β-adrenergic receptor pathways. However, it is not known whether α- and β-adrenergic pathways regulate apoptosis in a coordinated or differential manner.

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Apoptosis in other cell types has been extensively studied. Several reports suggest that a subset of apoptosis inducers is common in cardiac myocytes. For example, reactive oxygen species and tumor necrosis factor-α, both of which are potent apoptosis inducers in most cell types, have also been implicated in myocardial cell apoptosis. It was recently reported that p53, a well-known trigger of apoptosis in a variety of cell types, is sufficient to trigger apoptosis by itself in cardiac myocytes. However, cardiac muscle cells are distinct from other cell types in many biological aspects. Because the signaling pathways leading to apoptosis differ among cell types, it is likely that in addition to the conserved pathways, cardiac myocytes possess their own apoptotic pathways. It is unknown, however, whether norepinephrine-induced apoptosis occurs in a cell type–specific manner or uses common apoptotic signals.

The present study investigated the effects of α- and β-adrenergic stimulation on myocardial cell apoptosis. In this study, we show that a cAMP– protein kinase A (PKA) pathway is necessary and sufficient for β-adrenergic stimulation–induced apoptosis, whereas MEK-1 appears to be involved in an inhibitory effect by α,-adrenergic stimulation. Interestingly, in contrast to cardiac myocytes, cAMP almost completely inhibited apoptosis in PC12 cells, a pheochromocytoma cell line, suggesting that this cAMP-PKA apoptotic pathway might use some cardiac-specific machinery.

Methods

Cell Culture

Primary ventricular cardiac myocytes were prepared as previously described. Briefly, hearts from 1- to 2-day-old Sprague-Dawley rats were removed, the ventricles were pooled, and the ventricular cells were dispersed by digestion with pancreatin (Life Technologies). The cells were preplated for 1 hour to enrich the culture with myocytes (90% to 95% of cells after this step). Cells were plated at a high density (1000 cells/mm²) onto 60-mm tissue culture dishes (Primaria, Falcon; Becton Dickinson & Co) and cultured in media consisting of Hanks’ salts plus minimal essential medium (MEM) vitamin stock, MEM amino acids, MEM nonessential amino acids, 2 mM L-glutamine, 0.67 mM/L glucose, 0.92 mM/L hypoxanthine, 19.6 mM/L NaHCO₃ (pH 7.1 to 7.2), penicillin, streptomycin, and 10% (vol/vol) fetal bovine serum (all from Gibco BRL) at 37°C, 5% CO₂.

PC12 cells, a rat pheochromocytoma cell line, were obtained from a health science research resources bank (JCRB No. 0733) and kept in the RPMI 1640 medium with 10% horse serum and 5% fetal bovine serum.

Nucleosomal Ladder Assay

Forty-eight hours after plating, the neonatal rat cardiac myocytes were washed twice with serum-free media and cultured in serum-free medium in the presence or absence of phenylephrine (PE), isoproterenol (Iso) or 8-B-cAMP for 48 hours. These agents were obtained from Sigma and were of the highest purity available. The cells were then harvested by scraping into the media. After centrifugation at 500g for 5 minutes at 4°C, the cells were lysed in lysis buffer and subjected to a nucleosomal ladder assay with the use of a commercial kit (Takara Biomedicals) according to the manufacturer’s recommendations. The presence of characteristic 180- to 200-bp multiple oligonucleosomal fragmentation was examined on 1.5% agarose gels stained with SYBR Green I (Takara Biomedicals).

In Situ Labeling of Apoptotic Cells and Quantitative Analysis

Terminal deoxynucleotidyl transfer–mediated end-labeling of fragmented nuclei (TUNEL assay) was performed on cardiomyocytes that had been plated on flask-style glass slides (Nalgene Nunc). The in situ TUNEL assay was then performed in accordance with the manufacturer’s protocol for cultured cells (Takara Biomedicals) after fixing the cells in 10% neutral buffered formalin for 10 minutes at room temperature. Individual nuclei were visualized at a magnification of ×400 for quantitative analysis. An average of 400 to 500 nuclei from random fields were analyzed in each slide. The apoptotic index (percentage of apoptotic nuclei) was calculated as (apoptotic nuclei/total nuclei)×100%. Sample indicates were concealed during scoring, and samples from at least 3 independent experiments were scored per group.

Immunocytochemistry

To identify cardiac myocytes, immunocytochemical staining was performed as described with the use of a monoclonal antibody against muscle-specific α-actin (HHF35) at a dilution of 1:100. Signals were detected with the use of an alkaline phosphatase–conjugated Fab fragment of the secondary antibody (a dilution of 1:600, Jackson Immunoresearch Laboratories) and nitroblue tetrazolium dye as the substrate.

Statistical Analysis

Data are presented as mean±SE. Statistical comparisons were performed with the use of unpaired 2-tailed Student’s t tests or ANOVA with Scheffe’s test when appropriate, with a probability value of <0.05 taken to indicate significance.

Results

β-Adrenergic Stimulation Activates an Apoptotic Program in Cardiac Myocytes

For the investigation of the possible contribution of a β-adrenergic pathway to the development of myocardial cell apoptosis, neonatal rat cardiac myocytes were treated with a β-adrenergic agonist (Iso) or saline as a control in the serum-free condition. In our experimental conditions in which cells were plated at a high density, serum deprivation alone did not increase the number of TUNEL-positive nuclei (<10%) (Figure 1A). As shown in Figure 1B, however, the stimulation with Iso markedly increased the number of TUNEL-positive cells (>40%). These positive cells may specifically indicate the presence of internucleosomal DNA fragmentation, since no positive cells were found when we omitted the terminal deoxytransferase treatment (Figure 1C). The cells stimulated with Iso displayed small condensed nuclei, cell shrinkage, and nuclear fragmentation, consistent with the morphological features of apoptosis (Figures 1C and 1D). These cells were derived from cardiac myocytes, as evidenced by the positive immunostaining for HHF35, which reacts with the α-actin of cardiac myocytes but not that of fibroblasts (Figure 1D). Figure 2 and Figure 4 show the Iso-induced typical ladder formation of fragmented internucleosomal DNA in agarose gels, a hallmark of apoptosis. The Iso-induced apoptosis occurred in a dose-dependent manner, as shown in Figure 3. Apoptosis was evident later than 36 hours but not at 24 hours after Iso stimulation (data not shown). The Iso-induced apoptosis was almost completely blocked by a β-adrenergic receptor antagonist, propranolol (10⁻⁴ mol/L) (Figure 2, lane 3 and Figure 5, lane 5), whereas this concentration of propranolol alone did not increase the
number of TUNEL-positive cells (Figure 5, lane 2). To further test the specificity of propranolol, we examined the effect of this agent on 8-Br-cAMP–induced apoptosis. We found that 10^{-4} mol/L propranolol did not affect the 8-Br-cAMP–stimulated increase of TUNEL-positive cells (compare Figure 5, lanes 7 and 8). These findings provide evidence that a β-adrenergic receptor–dependent pathway activates an apoptotic program in cardiac myocytes.

cAMP/PKA Pathway Mediates Iso-Induced Apoptosis

Stimulation of the β-adrenergic receptor activates adenylate cyclase, which increases intracellular cAMP and activates cAMP-dependent PKA. To determine whether the activation of PKA is required for Iso-induced apoptosis, we examined the effect of KT5720, a highly selective inhibitor of PKA.25 As shown in Figure 4, 10^{-6} mol/L of KT5720 was able to completely inhibit the Iso-induced internucleosomal cleavage of genomic DNA. The quantitative analysis by TUNEL staining also revealed that KT5720 (10^{-6} mol/L) almost completely inhibited the Iso-stimulated increase of TUNEL-positive cells (Figure 5, lane 6), whereas the same concentration of this agent alone did not induce apoptosis (Figure 5, lane 3). In accord with these results, the administration of a cell-permeable cAMP analogue, 8-Br-cAMP (30 mmol/L), also induced apoptosis in cardiac myocytes to an extent similar to that shown by Iso (Figure 5, lane 7 and Figure 6A, lane 2). Taken together, these results demonstrate that cAMP-PKA is necessary and sufficient for Iso-induced apoptosis.

α_1-Adrenergic Pathway Inhibits cAMP-Induced Cardiac Apoptosis

To determine the effects of α_1-adrenergic stimulation on myocardial cell apoptosis, neonatal rat cardiac myocytes were treated with an α_1-adrenergic agonist (PE). In contrast to Iso, stimulation with PE did not increase the number of TUNEL-positive cells, even at a high concentration (10^{-4} mol/L) (Figure 3). As shown in Figure 6A, lane 3, PE inhibited the internucleosomal cleavage of genomic DNA in 8-Br-cAMP–

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**Figure 1.** Induction of apoptosis in cultured neonatal rat cardiac myocytes by Iso stimulation. A, In the absence of Iso; B through E, in the presence of Iso. A through C, TUNEL-stained myocytes. Terminal deoxytransferase was omitted in C. D and E, Stained with HHF35 and secondary antibody conjugated with alkaline-phosphatase. Primary antibody was substituted with PBS in E. Arrowheads show cells with evidence of apoptosis, including chromatin condensation.

**Figure 2.** Fragmentation of genomic DNA from Iso-stimulated cardiac myocytes. Genomic DNA was isolated from myocytes maintained for 48 hours in serum-free media in the presence or absence of Iso (10^{-4} mol/L) and propranolol (10^{-4} mol/L) as indicated and loaded on a 1.5% agarose gel. Ladder assays were performed in 3 independent experiments; data presented are representative. M indicates molecular marker.

**Figure 3.** Dose-dependence of Iso-induced apoptosis. Cultured cardiac myocytes were treated for 48 hours with indicated concentrations of Iso or PE. TUNEL-positive nuclei were counted and expressed as percentage of total nuclei. An average of 400 to 500 nuclei were counted from random fields in each slide. Results are mean±SE of 3 independent experiments. *P<0.001 vs saline and Iso 10^{-8} mol/L; P<0.05 vs Iso 10^{-6} mol/L. **P<0.05 vs saline.
stimulated cardiac myocytes. The antiapoptotic effect of PE was further revealed by TUNEL staining (Figure 6B). Fewer myocardial cells treated with PE in addition to 8-Br-cAMP were positive for internucleosomal cleavage by TUNEL staining (Figure 6B, lane 5) compared with the cells treated with 8-Br cAMP alone (Figure 6B, lane 4). These results provide evidence that PE has an antiapoptotic effect in cultured cardiac myocytes. To evaluate whether the inhibition of myocardial apoptosis by PE is mediated through an α₁-adrenergic receptor pathway, we used prazosin, an α₁-adrenergic receptor antagonist. Prazosin negated the PE-mediated inhibition of apoptosis (Figure 6B, lane 6), whereas the same concentration of this agent did not increase the number of TUNEL-positive cells (Figure 6B, lane 2) compared with saline stimulation (Figure 6B, lane 1).

α₁-Adrenergic stimulation has been shown to activate a MAP kinase cascade in cardiac myocytes. To determine whether the activation of MAP kinase is required for the PE inhibition of apoptosis in cardiac myocytes, we used PD098059, a specific MEK inhibitor that selectively inhibits MEK-1 activity.26,27 A previous study confirmed that 10 μM of PD098059 completely inhibits the PE-stimulated activation of ERK1 and ERK2 in cardiac myocytes.28 As shown in Figure 6A, lane 4, and Figure 6B, lane 7, 10 μM of PD098059 negated the inhibitory effects of PE on myocardial cell apoptosis. To exclude the possibility of a nonspecific cytotoxic effect of PD098059, we tested whether it was capable of inducing cell death in the serum-free condition. We found that 10 μM or 50 μM of PD098059 alone did not induce apoptosis (Figure 6B, lane 3) compared with saline stimulation (Figure 6B, lane 1). This result suggested that PD098059 might block the downstream signaling pathway by which PE prevents apoptosis. Taken together, these results provide evidence that PE has an antiapoptotic effect in cultured cardiac myocytes and that a MAP kinase pathway appears to be involved in this process.

cAMP-Mediated Apoptotic Pathway Is Not Functional in PC12 Cells

In cardiac myocytes, as shown in Figure 7A, serum deprivation alone barely induced internucleosomal cleavage of genomic DNA compared with the condition of 10% fetal bovine serum. Cell-permeable 8-Br-cAMP induced marked apoptosis in the serum-free condition (Figure 7A, lane 3). To determine whether cAMP-induced apoptosis is a conserved phenomenon among different cell types or a phenomenon unique to cardiac myocytes, we examined the effect of 8-Br-cAMP on apoptosis in different cell lines. For this purpose, we used PC12 cells, rat pheochromocytoma cells that are dependent on the presence of growth factors such as nerve growth factor and insulin growth factor-1 in the medium and die by apoptosis after serum deprivation.29,30 In these cells, 12 hours after serum deprivation, marked internucleosomal cleavage of genomic DNA was observed. Notably, as shown in Figure 7B, the administration of 30 mmol/L of 8-Br-cAMP completely inhibited the serum deprivation–induced apoptosis in PC12 cells. Antiapoptotic effects of 8-Br-cAMP were also observed in other mitotic cell lines such as mouse fibroblast cells (NIH3T3 cells) (data not shown). These findings indicate that a cAMP pathway is involved in promoting cell survival in these cell lines. Thus 8-Br-cAMP displayed opposite effects on apoptosis in cardiac myocytes and PC12 cells.

Discussion

Adult cardiac muscle cells are terminally differentiated and have lost their proliferative capacity. As a result, the maintenance of cardiac muscle cell survival is critical for the
maintenance of normal cardiac function. The present study demonstrated that a β-adrenergic agonist induced apoptosis in cardiac myocytes and that this apoptosis was mediated through a cAMP-PKA pathway. In contrast, an α1-adrenergic agonist antagonized cAMP-induced apoptosis. Interestingly, cAMP had opposite effects on apoptosis in cardiac myocytes and PC12 cells, suggesting that cAMP involves cardiac-specific signal transduction mechanisms.

Accumulating evidence suggests that myocyte apoptosis occurs in failing hearts,6–9 suggesting that apoptosis contributes to progressive myocardial dysfunction. Nevertheless, little is known about the stimuli that initiate the program of apoptosis or the molecular and cellular events that mediate the ensuing cell death. A number of neurohormonal and autocrine substances, including A-type and B-type natriuretic peptides, endothelin-1, and norepinephrine, are present at high levels in patients with heart failure.10,11 The elevated plasma levels of norepinephrine, an activator of both α- and β-adrenergic pathways, is closely associated with the severity and poor prognosis of heart failure.10,11 With the use of 3 independent criteria, that is, TUNEL staining, DNA ladder formation, and nuclear condensation, the present study has documented that β-adrenergic stimulation can induce apoptosis in cultured neonatal rat cardiac myocytes. In addition, a highly selective inhibitor of PKA, KT5720, completely blocked β-adrenergic–induced cardiac apoptosis, suggesting a PKA-dependent effect. Consistent with these results, the membrane-permeable cAMP analogue (8-Br-cAMP) also induced apoptosis in these cells. These findings demonstrate that a cAMP-PKA pathway mediates β-adrenergic–induced apoptosis. Although small numbers of fibroblasts are present in every culture of neonatal myocytes, the apoptosis that resulted from either β-adrenergic agonist or 8-Br-cAMP was confined to myocytes, as shown by muscle-specific immunostaining with HHF35. Cardiac specificity was also confirmed by the findings that the cellular composition of these cultures
However, the nearly complete blockade of the kinase–independent pathways in the apoptosis inhibition. Our data do not rule out a possible role of MAP pathways are required for the inhibition of cardiac myocyte stimulation inhibited cAMP-induced apoptosis.

These findings demonstrate that MAP kinase–dependent pathways play a particularly important role in promoting the survival of terminally differentiated cell types as well.

Another interesting feature of our results is the opposing effects of cAMP on apoptosis in 2 different cell types. In contrast to the data in primary cardiac myocytes in culture, the administration of 8-Br-cAMP completely inhibited serum deprivation–induced apoptosis in PC12 pheochromocytoma cells. Thus cAMP-mediated apoptosis in cardiac myocytes might involve some cell type–specific signal transduction mechanisms. At present, the precise molecular effectors and targets of cAMP-induced cardiac apoptosis are unclear. Regarding apoptosis in terminally differentiated cells, one of the candidate molecules is the adenovirus E1A-associated cellular protein p300, which appears to play a role in myocardial cell survival.32,33 p300 is a homologue of CBP, a protein that is associated with and coactivates the transcription factor CREB, mediating the induction of cAMP-responsive promoters.34,35 The possible involvement of the p300/CBP family in cAMP-mediated apoptosis in cardiac myocytes requires further investigation.

Several lines of evidence suggest that the activation of the sympathetic nervous system exerts a direct deleterious effect on the heart that is independent of the hemodynamic actions of these endogenous mechanisms. Therapeutic interventions by β-adrenergic receptor blockers favorably alter the natural history of heart failure, and such benefits cannot be explained by the effect of these agents on cardiac contractility and ejection fraction. The present study demonstrated that a β-adrenergic pathway but not the α1-adrenergic pathway induced cell type–specific apoptosis in cardiac myocytes. These findings might indicate a mechanism of the beneficial effects of β-adrenergic receptor blockers in patients with heart failure. It would be of particular interest to test whether β-adrenergic receptor blockers can inhibit myocyte apoptosis in experimental animal models of heart failure. In addition, the elucidation of precise signaling pathways leading to myocardial cell apoptosis may contribute to novel strategies for heart failure therapy in humans.

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