Endothelin-1 as a Protective Factor Against Beta-Adrenergic Agonist-Induced Apoptosis in Cardiac Myocytes

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OBJECTIVES The purpose of this study was to investigate the regulation of beta-adrenergic agonist-induced apoptosis by endothelin-1 (ET-1) in cardiac myocytes.

BACKGROUND Numerous hormonal factors including norepinephrine and ET-1 are activated in patients with heart failure. These factors may be involved in the positive and negative regulation of myocardial cell apoptosis observed in failing hearts. Recently, it has been shown that norepinephrine can induce myocardial cell apoptosis via a beta-adrenergic receptor-dependent pathway.

METHODS Primary cardiac myocytes were prepared from neonatal rats. These cells were stimulated with the beta-adrenergic agonist isoproterenol (ISO) in the presence or absence of ET-1.

RESULTS The administration of $10^{-7}$ mol/liter of ET-1 completely blocked Iso-induced apoptosis. An endothelin type A receptor antagonist, FR139317, negated the inhibitory effect of ET-1 on apoptosis, while the endothelin type B receptor antagonist BQ788 did not show such a negation. Endothelin-1 also inhibited apoptosis induced by a membrane-permeable cAMP analogue (8-Br-cAMP), which bypassed Gi. The effect of ET-1 was neutralized by an MEK-1-specific inhibitor (PD098059), a phosphatidylinositol 3’-kinase inhibitor (wortmannin) and its downstream pp70 S6-kinase inhibitor, rapamycin.

CONCLUSIONS These findings suggest that ET-1 represents a protective factor against myocardial cell apoptosis in heart failure and that this effect is mediated mainly through endothelin type A receptor-dependent pathways involving multiple downstream signalings in cardiac myocytes.

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Apoptosis, or programmed cell death, is an active, gene-directed process in which cells initiate their own death in response to internal or external stimuli. This mode of death serves as an orderly means for multicellular organisms to eliminate unwanted cells without adversely affecting surrounding tissue. Thus, apoptosis is a key mechanism of normal tissue development in the fetus and of cell replacement in certain adult tissues (e.g., the thymus), and is most often encountered in cells that are progressing through the cell cycle (1–4). However, accumulating evidence suggests that terminal differentiated adult cardiac myocytes undergo apoptosis in various animal models of heart failure. These include models of rapid ventricular pacing (5,6) and pressure overload due to aortic constriction (7), and aged spontaneously hypertensive rats (8). Because adult muscle cells have lost their proliferative capacity, the loss of viable cardiac myocytes due to apoptosis results in a further decrease in global cardiac function. As such, identification of the signaling pathways that mediate cardiac myocyte cell death and survival is crucial 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 physiologic stimuli. The control of programmed cell death is dependent on a balance between inhibitors and inducers of apoptosis. Because a number of systemic and local humoral factors are activated in congestive heart failure (9,10), they may possibly play positive and negative roles in the regulation of myocardial cell apoptosis. Norepinephrine is one such factor; the elevation of norepinephrine in plasma closely correlates with the severity and poor prognosis of heart failure (10). It has recently been shown that norepinephrine can induce apoptosis in cultured neonatal cardiac myocytes via a beta-adrenergic pathway (11,12), suggesting that it might be one of the factors involved in myocardial cell apoptosis in heart failure in vivo.

Another factor activated in congestive heart failure is endothelin-1 (ET-1), a 21-residue peptide originally isolated from vascular endothelium (13). The levels of ET-1 in plasma and in ventricular myocardium are markedly increased in human and animal models of heart failure (14–16). Endothelin-1 exerts diverse physiological actions including vasoconstriction and growth-promotion. Moreover, ET-1 is sufficient to induce the myocardial cell hypertrophy associated with the reactivation of the fetal gene program (17,18). These various effects are mediated by two distinct subtypes of G protein-coupled heptahelical receptors, ET_A and ET_B, expressed in a wide variety of tissues (19,20). It was recently reported that ET-1 is a...
synthetic ET-1 and ETB receptor agonist (IRL 1620) were purchased from Peptide Institute (Osaka, Japan). Amino acids, MEM nonessential amino acids, 2 mmol/liter and cultured in media consisting of Hanks’ salts plus penicillin, streptomycin, and 10% (vol/vol) fetal bovine serum (all from Gibco BRL, Gaithersburg, Maryland) at 37°C, 5% CO2.

Cell culture. Primary ventricular cardiac myocytes were prepared as previously described (12,23). Briefly, hearts from one- to two-day-old Sprague-Dawley rats were removed, the ventricles were pooled, and the ventricular cells were dispersed by digestion with pancreatin (Life Technologies, Gaithersburg, Maryland). The cells were preplated for 1 h to enrich the culture with myocytes (90% to 95% of cells after this step). Cells were plated at a high density (1,000 cells/mm2) on 60-mm tissue culture dishes (Primaria, Falcon; Becton Dickinson, Lincoln Park, New Jersey) and cultured in media consisting of Hanks’ salts plus minimal essential medium (MEM) vitamin stock, MEM amino acids, MEM nonessential amino acids, 2 mmol/liter L-glutamine, 0.67 mmol/liter glycine, 0.92 mmol/liter hypoxanthine, 19.6 mmol/liter NaHCO3 (pH 7.1 to 7.2), isoproterenol, 19.6 mmol/liter NaHCO3 (pH 7.1 to 7.2), penicillin, streptomycin, and 10% (vol/vol) fetal bovine serum (all from Gibco BRL, Gaithersburg, Maryland) at 37°C, 5% CO2.

Reagents. Synthetic ET-1 and ETB receptor agonist (IRL 1620) were purchased from Peptide Institute (Osaka, Japan). The ETB receptor antagonist BQ788 was from Calbiochem-Novabiochem (La Jolla, California). The 8-Br-cAMP, wortmannin, and rapamycin were from Sigma Aldrich (Tokyo, Japan). The ETA receptor antagonist FR139317 was provided by Fujisawa Research Laboratory (Osaka, Japan).

Nucleosomal ladder assay. Forty-eight hours after plating, the neonatal rat cardiac myocytes were washed twice with serum-free medium and cultured in serum-free medium in the presence or absence of ET-1 and isoproterenol (Iso) or 8-Br-cAMP for 48 h. The cells were then harvested by scraping into the medium. After centrifugation at 500g for 5 min at 4°C, the cells were lysed in lysis buffer and subjected to a nucleosomal ladder assay using a commercial kit (Takara Biomedicals, Shiga, Japan) according to the manufacturer’s recommendations. The presence of characteristic 180–200-bp multiple oligonucleosomal fragmentation was examined on 1.5% agarose gels stained with SYBR™ Green I (Takara Biomedicals, Shiga, Japan).

In situ labeling of apoptotic cells and their 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, Naperville, Illinois). The in situ TUNEL assay was then performed in accordance with the manufacturer’s protocol for cultured cells (Takara Biomedical) after fixing the cells in 10% neutral buffered formalin for 10 min at room temperature. Individual nuclei were visualized at ×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 indicators were concealed during scoring, and samples from at least three independent experiments were scored per group.

Statistical analysis. Data are presented as mean ± SE. Statistical comparisons were performed using the unpaired two-tailed Student t test or analysis of variance with Scheffe test when appropriate, with a probability value less than 0.05 taken to indicate significance.

RESULTS

Endothelin-1 inhibits beta-adrenergic agonist-induced apoptosis in cardiac myocytes. Neonatal cardiac myocytes retain some ability to undergo mitosis. We investigated whether these cells divide in response to stimulation with ET-1 under our experimental condition. Neonatal cardiac myocytes were treated with saline or ET-1 (10−7 mol/liter) in a serum-free medium for 48 h. These cells were pulsed with 5-bromo-2’-deoxy-uridine (BrdU) between 46 and 48 h after stimulation; then BrdU uptake in these cells was estimated by enzyme-linked immunosorbant assay (Amerham Pharmacia Biotech, Buckinghamshire, UK). The BrdU uptake was very low (almost undetectable) and was not increased by ET-1 stimulation in neonatal cardiac myocytes.

In contrast, in COS7 cells, which can actively proliferate, stimulation with the same concentration of ET-1 increased the uptake of BrdU by 6.7-fold. These results indicate that ET-1 stimulation does not increase DNA synthesis in neonatal cardiac myocytes under our experimental condition.

To determine the effects of ET-1 on beta-adrenergic agonist-induced myocardial cell apoptosis, neonatal rat cardiac myocytes were treated with saline, a beta-adrenergic agonist (10−5 mol/liter of Iso) alone or Iso plus ET-1 (10−7 mol/liter) in the serum-free condition for 48 h. In our experimental conditions in which cells were plated at a high density, serum deprivation alone did not increase the number of TUNEL-positive cells (<10%) (Fig. 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, as no positive cells were found when we omitted the terminal deoxynucleotidyl transferase treatment (Fig. 1C). The cells stimulated with Iso displayed small condensed nuclei, cell shrinkage, and nuclear fragmentation, consistent with the morphologic features of apoptosis (Fig. 1C). Fewer myocardial cells treated with ET-1 in addition to Iso were positive for internucleosomal cleavage by TUNEL staining (Fig. 1D) compared with the cells treated with Iso alone (Fig. 1B).

As shown in Figure 2, lane 4, 10⁻⁷ mol/liter of ET-1 was sufficient to produce a complete blockade of Iso-stimulated increase in TUNEL-positive cells. Figure 3 (lane 2) shows the Iso-induced typical ladder formation of fragmented internucleosomal DNA in agarose gels, a hallmark of apoptosis. As shown in lane 3 of Figure 3, ET-1 completely inhibited the internucleosomal cleavage of genomic DNA in Iso-stimulated cardiac myocytes. These findings are evidence that ET-1 has an antiapoptotic effect on beta-adrenergic agonist-induced apoptosis in cardiac myocytes. The ET₄ receptor-dependent pathway mediates the antiapoptotic effect of ET-1. To characterize the ET-1 receptor subtype mediating the inhibition of apoptosis in cardiac myocytes, we examined whether subtype-specific receptor antagonists can abrogate the protective effect of ET-1. Both FR139317 (24,25) and BQ788 (26) are potent, Araki et al.

Figure 1. ET-1-mediated inhibition of Iso-induced apoptosis in cultured neonatal rat cardiac myocytes, TUNEL-stained myocytes. (A) In the absence of Iso or ET-1; (B and C) in the presence of Iso (10⁻⁵ mol/liter); (D) in the presence of Iso plus ET-1 (10⁻⁷ mol/liter). Terminal deoxytransferase was omitted in C. Arrows show cells with evidence of apoptosis, including chromatin condensation.

Figure 2. Complete inhibition by ET-1 of Iso-induced apoptosis. Cultured cardiac myocytes were treated for 48 h in serum-free media in the presence or absence of Iso (10⁻⁵ mol/liter) and the indicated concentrations of ET-1. TUNEL-positive nuclei were counted and are expressed as the percentage of total nuclei. An average of 400 to 500 nuclei were counted from random fields in each slide. Results are the mean ± SE of three independent experiments.

Figure 3. ET-1-mediated inhibition of genomic DNA fragmentation in cardiac myocytes. Genomic DNA was isolated from myocytes maintained for 48 h in serum-free media in the presence or absence of Iso (10⁻⁵ mol/liter) and ET-1 (10⁻⁷ mol/liter) as indicated and loaded on a 1.5% agarose gel. M, molecular marker.
specific antagonists of ETA and ETB receptors, respectively. It has been shown that $10^{-7}$ mol/liter of FR139317 almost completely (>99%) inhibits the binding of $^{125}$I-ET-1 to the ETA receptor but does not inhibit (<2%) the binding to the ETB receptor (25). As shown in Figure 4, lane 6, $10^{-7}$ mol/liter of FR139317 increased the number of TUNEL-positive myocytes to an extent similar to that observed with Iso stimulation alone. However, the same concentration of FR139317 alone did not increase the number of TUNEL-positive cells (Fig. 4, lane 2), suggesting that this agent specifically blocked the protective effect of ET-1.

In contrast, $10^{-6}$ mol/liter of the ETB antagonist BQ788 (26) only partially negated the inhibitory effect of ET-1 on apoptosis (Fig. 4, lane 7). In addition, the ETB receptor agonist IRL1620 (27,28) did not mimic the inhibitory effect of ET-1 (Fig. 4, lane 8). These results indicate that the apoptotic inhibitory effect of ET-1 is mediated mainly by the ETA receptor.

The ETA receptor is coupled with both Gq and Gi. The ETA receptor-mediated Gi-signaling can inhibit adenylate cyclase activity (29). To investigate whether Gi pathways are required for the inhibition of Iso-induced apoptosis by ET-1, we utilized a cell-permeable cAMP analogue, 8-Br-cAMP ($3 \times 10^{-2}$ mol/liter), which can increase intracellular cAMP levels independent of Gi-coupled adenylate cyclase. Administration of 8-Br-cAMP also increased the number of TUNEL-positive cardiac myocytes to an extent similar to that produced by Iso (Fig. 5, lane 2). Notably, ET-1 almost completely inhibited the 8-Br-cAMP-stimulated increase of apoptosis as well (Fig. 5, lane 3). These findings suggest the Gi-dependent pathways are not indispensable to the apoptotic inhibitory effect by ET-1.

PD098059, wortmannin and rapamycin neutralized the protective effect of ET-1. We searched for intracellular signals essential for the ET-1-induced cell survival by a pharmacological approach. To determine whether the MAP kinase cascade is required for the ET-1 inhibition of apoptosis in cardiac myocytes, we utilized PD098059, a specific MEK inhibitor that selectively inhibits MEK-1 activity (30,31). A previous study confirmed that $10^{-5}$ mol/liter of PD098059 specifically inhibited the ERK1 and ERK2 activity in cardiac myocytes (32). As shown in Figure 6 (lane 7), $10^{-5}$ mol/liter of PD098059 negated the inhibitory effects of ET-1 on myocardial cell
apoptosis. To exclude the possibility of a nonspecific cyto-

toxic effect of PD098059, we tested whether it was capable

of inducing cell death in the serum-free condition. We

found that $10^{-5}$ mol/liter of PD098059 alone did not

induce apoptosis compared with saline stimulation (Fig. 6,

lane 2). This result suggested that PD098059 might block

the downstream signaling pathway by which ET-1 prevents

apoptosis.

To determine whether the effect of ET-1 on the inhibi-
tion of apoptosis involved the phosphatidylinositol 3'-

kinase (PI3) pathway, we used the specific PI3'-kinase

inhibitor wortmannin. Wortmannin has previously been

shown to inhibit PI3'-kinase activity completely at the

concentration of $10^{-7}$ mol/liter (33,34). Cardiac myocytes

were incubated with Iso ($10^{-5}$ mol/liter), ET-1

($10^{-7}$ mol/liter) and wortmannin ($10^{-7}$ mol/liter). This

concentration of wortmannin blocked the inhibition of

apoptosis by ET-1 as demonstrated by increased TUNEL-

positive cells (Fig. 6, lane 8). The 70-kD S6 kinase pp70S6K

is known to play a role in the signaling cascade initiated by

PI3'-kinase (35–37). The inhibitor rapamycin is known to

block the activation of pp70S6K downstream of PI3'-kinase

(38–45). A previous study demonstrated that $10^{-8}$ mol/

liter of rapamycin was sufficient to inhibit the pp70S6K

activity in cardiac myocytes completely (46). This concen-

tration of rapamycin negated the inhibitory effects of ET-1

on myocardial cell apoptosis (Fig. 6, lane 9), while this

concentration of rapamycin alone did not induce apoptosis

compared with saline stimulation (Fig. 6, lane 4). Taken

together, these results provide evidence that PI3'-kinase/

pp70S6K pathways are involved in the antiapoptotic effect of

ET-1 in cultured cardiac myocytes.

**DISCUSSION**

Adult cardiac muscle cells are terminally differentiated and

lose 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 ET-1 antagonized beta-adrenergic agonist-induced

apoptosis in cardiac myocytes and that this inhibition was

mediated mainly through ETA-dependent pathways. The

apoptotic inhibitory effect of ET-1 was neutralized by

PD098059 (an MEK-1-specific inhibitor), wortmannin (a

PI3'-kinase inhibitor), and rapamycin (a pp70 S6-kinase

[pp70S6K] inhibitor), suggesting the involvement of multi-

ple signaling pathways in this inhibition.

**Endothelin-1 as a protective factor against myocardial

cell apoptosis.** Accumulating evidence suggests that myo-
cyte apoptosis occurs in failing hearts (5–8), indicating that

apoptosis contributes to progressive myocardial dysfunction.

However, little is known about the factors that regulate the

program of apoptosis or the molecular and cellular events

that mediate the ensuing cell death. A number of neuro-

hormonal and autocrine substances (including ET-1 and

norepinephrine) are present at high levels in patients with

heart failure (9,10). It was recently shown that beta-

adrenergic stimulation can induce apoptosis in cultured

neonatal rat cardiac myocytes (11,12).

The present study has documented that ET-1 blocked

beta-adrenergic agonist-induced apoptosis. A marked inhi-
bition by ET-1 of apoptosis in rat cardiac myocytes was demonstrated by three lines of evidence: 1) the inhibition of nucleosomal ladder formation of agarose gel electrophoresis; 2) the decrease in the number of TUNEL-positive cells; and 3) the decrease in the number of cells showing nuclear condensation, a morphological feature of apoptosis. We cannot rule out the possibility that TUNEL-positive cells contain a subset of false-positive cells. However, stimulation with Iso markedly increased the number of myocytes showing the typical morphological features of apoptosis, whereas ET-1 decreased the number of such myocytes. Therefore, the number of TUNEL-positive cells correlated well with that of morphologically evidenced apoptotic cells. Although the levels of ET-1 required for the inhibition of apoptosis ($10^{-8} \sim 10^{-7}$ mol/liter) are higher than those normally detected in plasma, local ET-1 levels in the heart markedly increase in the development of heart failure (15,16). Because ET-1 accumulates within cardiac myocytes in failing hearts as shown by immunohistochemistry (15,16), endogenous ET-1 may function as “self-protection” by an autocrine mechanism. Thus, it would be interesting to examine the pathophysiologic role of ET-1 as a protective factor against apoptosis of cardiac myocytes in animal models of heart failure.

**Apoptotic inhibitory effect of ET-1 is mediated by the ETA receptor.** The members of the ET peptide family (ET-1, ET-2 and ET-3) mediate their diverse effects through two distinct subtypes of G protein-coupled heptahelical receptors, termed ETA and ETB (19,20). The ETA receptor is selective for ET-1, and the ETB receptor does not distinguish among these isopeptides. Vascular smooth muscles express mainly ETA receptors mediating contraction, whereas vascular endothelium has ETB receptors that are involved in vasodilation. Cardiac myocytes mainly express ETA receptors and a small amount of ETB receptors. Compatible with the expression pattern in cardiac myocytes, the present study showed that an ETA receptor antagonist (FR139317) but not an ETB receptor antagonist (BQ788) blocked the apoptotic inhibition by ET-1. In addition, an ETB receptor agonist (IRL1620) did not mimic the inhibitory effect of ET-1. These findings are in agreement with the idea that the apoptotic inhibitory effect of ET-1 in cardiac myocytes is mediated mainly by the ETA receptor.

**The MAP kinase-dependent pathways are required for the protective effect of ET-1.** The ETA receptor is coupled to Gi as well as Gq. Signaling through Gi inhibits adenylate cyclase, and thereby decreases the intracellular cAMP contents (29). The present results demonstrated that Gi pathways are not indispensable for the protective effect of ET-1 as ET-1 also inhibited apoptosis induced by the cell-permeable cAMP analogue 8-Br-cAMP, which increases intracellular cAMP levels independent of adenylate cyclase. Although our data do not rule out a possible role of Gi-dependent pathways in the inhibition of apoptosis, the nearly complete blockade of 8-Br-cAMP-mediated apoptosis by ET-1 suggests the important role for more downstream pathways in the cell survival function. The ETA receptor-mediated signaling is functionally linked to phospholipase C to induce phosphoinositide breakdown (47). It is also becoming clear that an ETA receptor pathway crosstalks with Ras and MAP kinase cascades (48–50).

The present findings demonstrated that the treatment of cultured neonatal cardiac myocytes with a MEK-1–specific inhibitor, PD098059, which has been shown specifically to inhibit ERK1 and ERK2 activity in cardiac myocytes (32), negated the apoptotic inhibitory effect of ET-1. These findings demonstrate that MAP kinase–dependent pathways are required for the inhibition of cardiac myocyte apoptosis. Also, MAP kinase pathways have been found to be necessary for the effects of nerve growth factor and insulin growth factor-1 on the promotion of the survival of neuronal cell types (PC12), whereas inhibition of these kinases has been shown to be critical for the induction of apoptosis in these cells (31,33,51). The above studies provide further evidence that MAP kinase–dependent pathways play an important role in promoting the survival of terminally differentiated cell types as well.

**Roles of PI3-kinase/pp70S6K in apoptotic inhibition by ET-1.** Wortmannin, a fungal metabolite, demonstrates a substantial degree of specificity for PI3-kinase compared with a number of other lipid kinases. Experiments with both PI3-kinase inhibitors and mutant growth factor receptors have suggested that pp70S6K is one of the downstream elements of the PI3-kinase signaling pathway (35–37). Rapamycin inhibits the activation of pp70S6K without affecting the activity of tyrosine kinase, MAP kinase, rsk-encoded S6 kinase, or PKC in cardiac myocytes (46). The specific inhibition of the pp70S6K activity by rapamycin is mediated by a high-affinity binding of rapamycin to the cellular receptor protein FRAP, a mammalian homologue of TOR (38–45). Wortmannin and rapamycin exert distinct effects on cell survival among different cell types. Neither wortmannin nor rapamycin had any effect on ET-1–mediated cell survival in fibroblasts (22).

Conversely, in PC12 cells, while wortmannin negated the apoptotic inhibitory effect of growth factors, rapamycin failed to induce apoptosis (33,51), suggesting that PC12 cell survival requires a PI3-kinase signaling pathway that is independent of pp70S6K activation. In contrast to these results in other cells, in cardiac myocytes both rapamycin and wortmannin almost completely inhibited the ET-1 effect of protecting the cells from apoptosis. These findings demonstrate that PI3-kinase/pp70S6K is involved in the protective effect of ET-1 in cardiac myocytes and that the signaling pathways of myocardial cell survival are distinct, in part, from those in other cell types.

**Study limitations.** We utilized pharmacological agents to investigate downstream pathways that mediate apoptotic inhibitory effects of ET-1. Although these agents have been shown to be specific in the concentrations used in this study, we cannot totally rule out the possibility that these agents affected other kinase activities. This is a limitation of
pharmacological studies. Other methodologies, such as the use of dominant negative mutants, are needed to identify signaling pathways that mediate protective effects by ET-1.

In addition, several possibilities should be taken into account when the data of this study are applied to human disease. First, a myocardial development is not complete at birth; differences may exist between neonatal and adult cardiac myocytes. Second, biological properties of the heart may differ between rats and humans. Third, dissociated myocytes in the culture may behave in a different manner with myocytes in the organized heart in vivo. Fourth, stimulation of normal myocytes with Iso or ET-1 may not mimic chronic elevation of these levels in the diseased state. However, a recent study clearly showed that myocardial cell apoptosis results in dilatation of the heart and heart failure (52). Thus, further elucidation of the pathways for apoptosis in cardiac myocytes will enable investigators to design new and effective therapeutic agents for heart failure in humans.

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