Norepinephrine Stimulates Apoptosis in Adult Rat Ventricular Myocytes by Activation of the β-Adrenergic Pathway

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Background—Myocardial sympathetic activity is increased in heart failure. We tested the hypothesis that norepinephrine (NE) stimulates apoptosis in adult rat ventricular myocytes in vitro.

Methods and Results—Myocytes were exposed to NE alone (10 μmol/L), NE+propranolol (2 μmol/L), NE+prazosin (0.1 μmol/L), or isoproterenol (ISO, 10 μmol/L) for 24 hours. NE and ISO decreased the number of viable myocytes by ~35%. This effect was completely blocked by the β-adrenergic antagonist prazosin but was not affected by the α1-adrenergic antagonist prazosin. NE increased DNA laddering on agarose gel electrophoresis and increased the percentage of cells that were stained by terminal deoxynucleotidyl transferase–mediated nick end-labeling from 5.8±1.0% to 21.0±2.3% (P<0.01; n=4). NE likewise increased the percentage of apoptotic cells with hypodiploid DNA content as assessed by flow cytometry from 7.8±0.7% to 16.7±2.2% (P<0.01; n=6), and this effect was abolished by prazosin but not prazosin. ISO and forskolin (10 μmol/L) mimicked the effect of NE, increasing the percentage of apoptotic cells to 14.7±1.9% and 14.4±2.2%, respectively. NE-stimulated apoptosis was abolished by the protein kinase A inhibitor H-89 (20 μmol/L) or the voltage-dependent calcium channel blockers diltiazem and nifedipine.

Conclusions—NE, acting via the β-adrenergic pathway, stimulates apoptosis in adult rat cardiac myocytes in vitro. This effect is mediated by protein kinase A and requires calcium entry via voltage-dependent calcium channels. NE-stimulated apoptosis of cardiac myocytes may contribute to the progression of myocardial failure. (Circulation. 1998;98:1329-1334.)

Key Words: apoptosis ■ norepinephrine ■ receptors, adrenergic, beta ■ myocytes ■ calcium

Increased sympathetic nerve activity to the myocardium is a central feature in patients with heart failure.1,2 It has long been postulated that exposure to high levels of catecholamines might be toxic to cardiac myocytes (for review, see Reference 3). Mann et al4 advanced this thesis by showing that norepinephrine (NE) exerts a direct toxic effect on cardiac myocytes in vitro. They further demonstrated that this effect was mediated by β-adrenergic receptor stimulation and involved increases in cAMP and calcium influx.5

Apoptosis has been demonstrated to occur in the myocardium in a variety of pathological situations.5–7 The number of apoptotic myocytes is increased in myocardium obtained from patients with end-stage heart failure and myocardial infarction8–10 and in myocardium from experimental models of myocardial hypertrophy and failure, including the aortic-banded rat,11 the spontaneously hypertensive rat,12 rats with myocardial infarction,13 and dogs with pacing-induced failure.14 Experiments with cultured cardiac myocytes have demonstrated that apoptosis can be stimulated in vitro by several endogenous peptides that are increased in the hypertrophied or failing myocardium, including tumor necrosis factor-α,15 angiotensin II,16 and atrial natriuretic peptide (ANP).17

The purpose of this study was to determine whether exposure to NE can stimulate apoptosis in cardiac myocytes in vitro, and if so, to delineate the adrenergic receptor and second messenger pathway that mediate this effect. Because the susceptibility to apoptosis may be developmentally linked, we used cardiac myocytes obtained from adult rats. Apoptosis was assessed by DNA laddering, terminal deoxynucleotidyl transferase (TdT)–mediated nick end-labeling (TUNEL assay), and flow cytometric analysis of cellular DNA content.

Methods

Myocyte Isolation and Culture

Calcium-tolerant adult rat ventricular myocytes (ARVMs) were obtained from hearts of male Sprague-Dawley rats (240 to 260 g) as previously described.18 Briefly, animals were anesthetized with sodium pentobarbital (50 mg/kg IP) and heparinized (1000 USP/kg IV), and their hearts were aseptically removed into an ice-cold modified cardioplegic solution (KB solution, in mmol/L: KOH 85, KCl 30, KH2PO4 30, MgSO4 3, EGTA 0.5, HEPES 10, l-glutamic acid 10, KBr 1, NaHCO3 20). Myocytes were isolated by enzymatic digestion with collagenase and dispase, and then further cultured in calcium-free modified Earle’s balanced salt solution (CMEM) containing 1% fetal bovine serum (FBS), 1% nonessential amino acids, 1% glutamine, and 1% penicillin-streptomycin.
acids 50, and taurine 20, at pH 7.4). The hearts were retrograde-perfused on a Langendorff apparatus with Tyrode’s solution (in mmol/L: NaCl 137, KCl 5.4, CaCl₂ 1.2, MgCl₂ 0.5, HEPES 10, and glucose 10, at pH 7.4) for 5 minutes at 37°C. The perfusion solution was switched to a nominally Ca²⁺-free Tyrode’s solution for 6 minutes and then to a nominally Ca²⁺-free Tyrode’s solution containing 0.02% protease (Sigma) and 0.06% collagenase A (Boehringer Mannheim). After 10 to 15 minutes, the enzymatic solution was washed out with KB solution for an additional 5 minutes. After perfusion, cells from the left ventricle were released by shaking the tissue. The cells were filtered through a 150-μm mesh and allowed to settle (40 minutes) in KB solution. The cells were then resuspended in DMEM (Gibco), layered over 60 μg/mL BSA (Sigma) to separate ventricular myocytes from nonmyocytes as described by Ellingsen et al, and allowed to settle for 10 to 15 minutes. Cells were resuspended in ACCT medium consisting of DMEM containing 2 mg/mL BSA, 2 mmol/L L-carnitine, 5 mmol/L creatine, 5 mmol/L taun, 100 IU/mL penicillin, and 100 μg/mL streptomycin.

The ARVMs were then plated in ACCT medium at a density of 100 to 150 cells/mm² on 100-mm or 35-mm plastic culture dishes (Fisher) or 40×22-mm glass coverslips (Fisher) precoated with laminin (1 μg/cm²). Becton-Dickinson. After 1 hour, the dishes were washed with ACCT to remove cells that were not attached. The remaining cells were maintained in ACCT medium for 16 hours before the addition of NE and blocking drugs.

**Cell Treatments**

l-NE (10 μmol/L, Sigma), isoproterenol (ISO, 10 μmol/L, Sigma), and forskolin (FSK, 10 μmol/L, Calbiochem) were added to culture dishes for 24 hours. All dishes were supplemented with ascorbic acid (0.1 mmol/L, Sigma). In some experiments, prazosin (0.1 μmol/L, Sigma), dl-propranolol (2 μmol/L, Sigma), N-[2-(bromocinnamylamino)ethyl]-5-isoquinolinesulfonic acid (H-89, Calbiochem), diltiazem (1 μmol/L, Sigma), or nifedipine (5 μmol/L, Sigma) was added 30 minutes before the addition of l-NE.

**Cell Viability**

Cell viability was assessed by counting the number of adherent cells on 35-mm dishes. Briefly, the nonadherent cells were removed by washing with DMEM, and the adherent cells in 10 randomly chosen fields (1 mm²) were counted at ×20 power with a phase-contrast microscope (Nikon Diaphot) in duplicate dishes at various time points starting at time 0. The percentage of quiescent rod-shaped cells was also scored.

**Agarose Gel DNA Electrophoresis**

Double-stranded DNA breaks were assessed by agarose gel electrophoresis with genomic DNA isolated from ARVMs. Briefly, the cells were washed with DMEM, trypsinized, washed with PBS, and fixed in 4% paraformaldehyde for 30 minutes at 4°C. The cells were then again rinsed with PBS and resuspended in 1 mL of PBS solution containing 0.1% Triton X-100, 50 μg/mL RNase A (GIBCO), and 50 μg/mL of propidium iodide (Sigma). The samples were then kept in the dark at 4°C and analyzed by flow cytometry with excitation at 488 nm and emission measured at 560 to 640 nm (FL2 mode). Apoptotic cells stained with propidium iodide exhibit a reduced DNA content with a peak in the hypodiploid region. The percentage of apoptotic cells was taken as the fraction of cells with a hypodiploid DNA content. As a positive control, ARVMs were treated with DNase I (1 mg/mL, Sigma) for 10 minutes to introduce nicks in the genomic DNA. The coverslips were mounted with glycerol containing antifade agent and visualized under epifluorescence microscopy with FITC filters (excitation, blue; emission, green). The percentage of myocytes with DNA nick-end-labeling was measured by counting cells exhibiting green fluorescent nuclei at ×20 power in 10 randomly chosen fields (1 mm²) in triplicate plates.

**Flow Cytometry Studies**

Flow cytometry studies were performed on a FACScan with Lysis II software (Becton-Dickinson Co). The cells were washed with DMEM, trypsinized, washed with PBS, and fixed in 70% ethanol for 30 minutes at 4°C. The cells were then again rinsed with PBS and resuspended in 1 mL of PBS solution containing 0.1% Triton X-100, 50 μg/mL RNase A (GIBCO), and 50 μg/mL of propidium iodide (Sigma). The samples were then kept in the dark at 4°C and analyzed by flow cytometry with excitation at 488 nm and emission measured at 560 to 640 nm (FL2 mode). Apoptotic cells stained with propidium iodide exhibit a reduced DNA content with a peak in the hypodiploid region. The percentage of apoptotic cells was taken as the fraction of cells with a hypodiploid DNA content. As a positive control, ARVMs were treated with DNase I (1 mg/mL, Sigma) for 10 minutes to introduce nicks into the genomic DNA.

**Statistical Analysis**

All data are expressed as mean±SEM. Comparisons between control and NE-treated cells were performed with a Student’s unpaired t-test. Statistical significance of multiple treatments was determined by ANOVA and a post hoc Tukey’s test. Probability (P) values of <0.05 were considered to be significant.

**Results**

NE Decreases ARVM Viability Through Activation of β-Adrenergic Receptors

In control plates, the number of viable ARVMs decreased by 9% over 24 hours (to 91.0±8.7% of the number present at t₀; n=7). Relative to control plates, the addition of NE further decreased the number of adherent cells by approximately 20% at 12 hours and 35% at 24 hours (to 64.5±6.7% of t₀; P<0.05; n=7) (Figure 1). Exposure to NE for 48 hours caused only a small further decrease in adherent cells (data not shown).
NE-treated dishes, >80% of the remaining adherent cells exhibited a rounded morphology with loss of cross-striations. Cells were pretreated with propranolol (2 μmol/L) or prazosin (0.1 μmol/L) for 30 minutes before addition of NE for 24 hours. The NE-induced decrease in adherent cells was completely blocked by propranolol (89.2 ± 6.7% of t₀; P<0.05; n=4) but was not affected by prazosin (57.01 ± 2.3% of t₀; P=NS; n=3) (Figure 1). Neither prazosin nor propranolol alone affected the number of adherent cells. Addition of the β-adrenergic receptor agonist ISO (10 μmol/L) for 24 hours decreased the number of adherent cells by 40% (to 59.3 ± 7.9% of t₀; P<0.05; n=4), thereby mimicking the effect of NE. Thus, NE exposure for 24 hours decreases the number of viable myocytes via stimulation of β-adrenergic receptors.

**NE Induces Apoptosis in ARVMs**

**DNA Laddering**

Total genomic DNA was isolated from ARVMs treated with NE (10 μmol/L) for 24 hours and labeled with [32P]dCTP with TdT. Control cells exhibited a low level of DNA laddering. Exposure to NE for 24 hours clearly increased the intensity of 180- to 1000-bp DNA fragments in 3 of 3 experiments as assessed by visual inspection (Figure 2). Qualitatively similar results were obtained when low-molecular-weight genomic DNA was isolated, normalized for cell number, and analyzed by ethidium bromide staining of the agarose gel (data not shown).

**TUNEL Staining**

ARVMs were labeled with an in situ TUNEL assay using TdT and fluorescent dUTP. Control cells were mostly rod-shaped (Figure 3A) and exhibited nuclear labeling in 5.8 ± 1.0% of cells. Treatment with NE for 24 hours resulted in a rounded morphology and increased the fraction of TUNEL-positive cells to 21.0 ± 2.3% (P<0.01, n=4) (Figures 3C and 3D). Control ARVMs, treated with DNase I, were used as positive control (Figure 3B).

**Flow Cytometry**

Flow cytometric analysis revealed that 7.8 ± 0.7% of control cells had hypodiploid DNA content (Figure 4A). Treatment with NE (10 μmol/L) for 24 hours increased the percentage of apoptotic cells to 16.7 ± 2.2% (P<0.01; n=6) (Figures 4B and 5A). An increase in the percentage of apoptotic cells was apparent by 6 hours (data not shown) and increased further at 24 hours. Control ARVMs treated with DNase I were used as a positive control (Figure 4C).

**NE-Induced Apoptosis Is Mediated Through β-Adrenergic Receptors**

Cellular DNA content was assessed by flow cytometry in ARVMs pretreated with propranolol (2 μmol/L) or prazosin (0.1 μmol/L) for 30 minutes before addition of NE (10 μmol/L; 24 hours). Propranolol completely blocked the
NE-stimulated increase in the number of apoptotic cells (7.1±1.2%; P<0.05 versus NE alone; n=5) (Figure 5A). The α<sub>1</sub>-adrenergic receptor antagonist prazosin did not reduce the fraction of apoptotic cells (14.0±2.3%; P=NS versus NE alone; n=3). Neither propranolol nor prazosin alone had an effect on the fraction of apoptotic cells. Addition of the β-adrenergic agonist ISO (10 μmol/L) for 24 hours increased the fraction of apoptotic cells to 14.7±1.9% (P<0.01 versus control cells; n=4) (Figure 5B).

**Role of Adenylyl Cyclase, Protein Kinase A, and Calcium Channels**

Consistent with the effects of the adrenergic agonists and antagonists, addition of the direct adenylyl cyclase activator FSK (10 μmol/L) for 24 hours increased the fraction of apoptotic cells to 14.4±2.2% (P<0.01 versus control cells; n=4) (Figure 5B). Pretreatment of ARVMs with H-89 (20 μmol/L), an inhibitor of protein kinase A, caused a modest decrease in the fraction of apoptotic cells in control plates and completely inhibited the effect of NE (NE alone, 10.5±0.8%; NE+H-89, 5.3±0.9%; P<0.05; n=3) (Figure 5C).

β-Adrenergic receptor stimulation increases calcium influx via protein kinase A–mediated phosphorylation of voltage-dependent channels. Pretreatment with the L-type calcium channel blocker diltiazem (1 μmol/L) inhibited the NE-stimulated increase in apoptotic cells (NE alone, 15.5±2.2%; NE+diltiazem, 9.1±1.2%; P<0.05; n=5) (Figure 5D). Similar results were obtained with nifedipine (5 μmol/L; data not shown).

**Discussion**

The major new finding of this study is that exposure to NE for 24 hours stimulates apoptosis in ARVMs in vitro. This effect is mimicked by the β-adrenergic agonist ISO and is blocked by agents that inhibit β-adrenergic receptors, protein kinase A, or voltage-dependent calcium channels. Thus, NE stimulates apoptosis in adult rat cardiac myocytes by activation of the β-adrenergic pathway.

Apoptosis is a genetically orchestrated, energy-dependent mechanism for the programmed removal of cells. Because adult cardiac myocytes are terminally differentiated, it has been thought that they would not be susceptible to apoptosis, which is more often associated with fetal tissue development or rapidly proliferating cells. However, there is now evidence that apoptosis is present in myocardium in a variety of pathological circumstances, including heart failure, pressure overload, and myocardial infarction. The stimuli that modulate apoptosis in myocardium remain to be elucidated. However, it has been shown that angiotensin II, tumor necrosis factor-α, mechanical stretch, hypoxia, and ANP can stimulate apoptosis in cardiac myocytes in vitro. We now demonstrate that NE stimulates apoptosis in ARVMs, as assessed by 3 methods: DNA laddering, in situ TUNEL staining, and flow cytometric analysis of cellular DNA content, the results of which are internally consistent. Thus, NE is another potentially important stimulus for apoptosis in cardiac myocytes.

Catecholamine-induced cardiotoxicity has been appreciated for many years. Several mechanisms have been theorized for the toxic effects of NE, including relative hypoxia, increased sarcolemmal permeability, calcium overload, el-
vation of cAMP, activation of α-adrenergic receptors, activation of β-adrenergic receptors, and the formation of oxidative catecholamine metabolites (reviewed in References 3, 4, and 25). Mann et al demonstrated that exposure to NE was toxic to adult feline cardiac myocytes in vitro via activation of the β-adrenergic pathway. They further provided limited data that indicated a similar effect of NE in ARVMs, albeit at higher concentrations of NE than were required by feline myocytes. Our findings, which confirm these earlier observations, indicate that NE exposure for 24 hours exerts a toxic effect in adult rat cardiac myocytes via activation of the β-adrenergic receptor pathway. The concentration of NE used in our study (10 μmol/L) is similar to that used by Mann et al in rat myocytes and is the concentration required for near-maximal stimulation of contraction in these cells. 

Activation of β-adrenergic receptors in cardiac myocytes increases the cellular cAMP concentration, leading to phosphorylation of L-type calcium channels and an increase in intracellular calcium concentration. We found that direct stimulation of adenyl cyclase with FSK mimicked the effect of NE to cause apoptosis in ARVMs. Conversely, inhibition of protein kinase A activity by H-89 or L-type calcium channels with diltiazem or nifedipine blocked the ability of NE to induce apoptosis. Thus, β-adrenergic stimulation of apoptosis in ARVMs involves protein kinase A and L-type calcium channels. There are multiple mechanisms by which an increase in intracellular calcium might induce apoptosis. These include altering the transcription of a gene(s) involved in the regulation of apoptosis or causing the activation of the β-adrenergic receptor pathway. The concentration of NE used in our study (10 μmol/L) is similar to that used by Mann et al in rat myocytes and is the concentration required for near-maximal stimulation of contraction in these cells. 

Recently, Wu et al demonstrated that β-adrenergic receptor stimulation and 8-Br-cAMP can inhibit apoptosis induced by ANP in neonatal rat cardiac myocytes. In contrast to our findings in ARVMs, ANP-induced apoptosis in neonatal myocytes did not require calcium entry via L-type channels. This differential effect of the β-adrenergic pathway to stimulate or inhibit apoptosis in adult or neonatal cardiac myocytes, respectively, may reflect fundamental differences in the cell biology of these 2 model systems. In particular, neonatal cells retain a limited capacity to proliferate in response to growth stimuli. Neonatal and adult cardiac myocytes also differ with regard to adrenergic physiology and the hypertrophic response to NE. NE stimulates hypertrophy primarily via α-adrenergic receptors in neonatal myocytes, whereas β-adrenergic stimulation is the more potent stimulus in adult myocytes. These observations raise the interesting possibility that the effects of β-adrenergic pathway stimulation on myocyte growth and apoptosis are developmentally regulated (ie, antiapoptotic/hypertrophic in neonatal cells, apoptotic in adult cells).

These experiments must be interpreted with caution, for several reasons. First, the basal rate of apoptosis in this and other studies with cultured cardiac myocytes is relatively high compared with that in intact myocardium, possibly reflecting biological differences that could influence the response to stimuli for apoptosis. Second, although the pharmacological specificity that we observed and the presence of an antioxidant in our experimental system strongly argue that under the conditions of this study, NE stimulates apoptosis by binding to the β-adrenergic receptor, we cannot exclude the possibility that oxidative metabolites of catecholamines contribute to cardiac toxicity in vivo. Finally, although these data suggest that myocyte apoptosis may occur by direct β-adrenergic receptor stimulation, they do not allow us to predict the relative roles of apoptosis versus necrosis in causing catecholamine cardiotoxicity in vivo.

The in vivo relevance of our findings is supported by some but not all recent observations. The infusion of ISO to rats for 24 hours was reported to cause myocardial apoptosis. Likewise, mice overexpressing the Gαo-subunit in the myocardium develop dilated cardiomyopathy. It has been reported that this cardiomyopathy is associated with increased apoptosis and that cells cultured from these mice develop apoptosis in vitro when exposed to ISO. However, it should also be noted that mice overexpressing the β2-adrenergic receptor in the heart do not appear to develop dilated cardiomyopathy. Thus, the in vivo relevance of NE-stimulated myocyte apoptosis remains to be determined.

Activation of the sympathetic nerves to the heart is a common feature of myocardial failure.2,2 Our findings indicate that the stimulation of apoptosis is one mechanism by which overactivity of the cardiac β-adrenergic pathway may lead to the loss of myocytes and thus, to the progression of myocardial dysfunction. Conversely, it appears that treatment with β-adrenergic antagonists can reduce mortality and the progression of disease in patients with heart failure, suggesting that inhibition of this pathway can be of clinical benefit. β-Adrenergic antagonists might improve the biological properties of the heart, at least in part, by protecting cardiac myocytes from NE-stimulated apoptosis.

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References
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