Adrenergic Effects on the Biology of the Adult Mammalian Cardiocyte

Douglas L. Mann, MD; Robert L. Kent, PhD; Bruce Parsons, PhD; and George Cooper IV, MD

Background. To delineate the mechanism(s) of catecholamine-mediated cardiac toxicity, we exposed cultures of adult cardiac muscle cells, or cardiocytes, to a broad range of norepinephrine concentrations.

Methods and Results. Norepinephrine stimulation resulted in a concentration-dependent decrease in cardiocyte viability, as demonstrated by a significant decrease in viable rod-shaped cells and a significant release of creatine kinase from cells in norepinephrine-treated cultures. Norepinephrine-mediated cell toxicity was attenuated significantly by β-adrenoceptor blockade and mimicked by selective stimulation of the β-adrenoceptor, whereas the effects mediated by the α-adrenoceptor were relatively less apparent. When norepinephrine stimulation was examined in terms of cardiocyte anabolic activity, there was a concentration-dependent decrease in the incorporation of [3H]phenylalanine and [3H]uridine into cytoplasmic protein and nuclear RNA, respectively. The decrease in cytoplasmic labeling was largely attenuated by β-adrenoceptor blockade and mimicked by selective stimulation of the β-adrenoceptor, but α-adrenoceptor stimulation resulted in relatively minor decreases in cytoplasmic labeling. The norepinephrine-induced toxic effect appeared to be the result of cyclic AMP-mediated calcium overload of the cell, as suggested by studies in which pharmacological strategies that increased intracellular cyclic AMP led to decreased cell viability, as well as studies that showed that influx of extracellular calcium through the verapamil-sensitive calcium channel was necessary for the induction of cell lethality. Additional time-course studies showed that norepinephrine caused a rapid, fourfold increase in intracellular cyclic AMP, followed by a 3.2-fold increase in intracellular calcium ([Ca2+]i).

Conclusions. These results constitute the initial demonstration at the cellular level that adrenergic stimulation leads to cyclic AMP-mediated calcium overload of the cell, with a resultant decrease in synthetic activity and/or viability. (Circulation 1992;85:790–804)

It is well recognized that the sympathetic nervous system has a major role in the cardiovascular response to stress.1 However, a number of studies have shown that excessive adrenergic stimulation can aggravate or even produce, rather than ameliorate, various manifestations of cardiovascular disease. Catecholamine-mediated myocardial injury has been documented in patients with pheochromocytoma,2 hyperthyroidism,3 myocardial infarction,4,5 and, more recently, congestive heart failure.6 Given the potential importance of catecholamine-mediated cardiotoxic effects in humans, a number of experimental studies have sought to determine the mechanism(s) responsible for catecholamine-induced cardiac injury.7–9 However, although these in vivo studies have yielded important insights into the genesis of catecholamine cardiotoxicity, no definitive mechanism has been identified.

One of the major problems in defining the mechanism(s) of catecholamine cardiotoxicity in vivo is the complex alterations in cardiac loading and blood flow inherent in all such in vivo experimental models. Thus, one cannot fully dissociate any direct toxic effects of the catecholamine from any concurrent cardiac injury that may be produced by mechanical overloading and/or ischemia. A second major prob-
lem in defining these mechanism(s) results from the complexity of cardiac tissue, which comprises cardiac muscle cells, or cardiocytes, admixed with interstitial, neural, and vascular cells. Given that adrenergic stimulation could lead to multiple cell-cell interactions, it becomes very difficult to isolate and precisely define the direct biological effects of catecholamines on the cardiocyte component of the heart.

To provide a more focused approach to defining the proximate mechanism(s) causing catecholamine-induced toxicity, we used a very simple experimental preparation—the isolated cultured adult mammalian cardiocyte—to examine the direct effects of norepinephrine on the biology of this cell. The results of the present study directly support the view that catecholamine cardiotoxicity is the result of cyclic AMP (cAMP)—mediated calcium overload of the cardiocyte.

**Methods**

**Cardiocyte Isolation**

With the exceptions noted below, the methods used to obtain reproducible yields of calcium-tolerant cardiocytes have been described previously. Briefly, adult cats of either sex, weighing 1.4–3.8 kg, were anesthetized with ketamine hydrochloride (50 mg/kg i.m.) and acepromazine maleate (5 mg/kg i.m.) and submitted to rapid cardiectomy under sterile conditions. The aorta was then cannulated, and the coronary arteries were perfused retrograde for 10 minutes with a recirculating modified Krebs-Henseleit buffer (KHB) of the following composition (mm): NaCl 130.0, KCl 4.8, MgSO4 1.2, NaH2PO4 1.2, NaHCO3 4.0, CaCl2 0.5, HEPES 10.0, and glucose 12.5. This was followed by a nonrecirculating buffer of the same composition but without supplemental calcium and concluded with a nonrecirculating calcium-free buffer supplemented with type II collagenase (155 units/ml). Perfusion was terminated when the heart was flaccid. The heart was then removed from the cannula, the ventricles were separated from the atria, and the ventricular cardiocytes were isolated as described previously.

**Cardiocyte Culture**

The characteristics of the cell culture system used herein have been reported in detail. An important feature of this model that bears emphasis is that adult feline cardiocytes remain quiescent (noncontracting) for extended periods of time when maintained in standard serum-free culture conditions. Indeed, the initiation of spontaneous contractions in these cells usually indicates that they have become nonviable. Unless specified otherwise, the culture medium used was medium 199 (Earle’s salts, 0.30 mM dL-phenylalanine, 1.8 mM calcium) supplemented with 10⁻⁶ M insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenium, 5.35 μg/ml linoleic acid, 1.2 μg/ml albumin, 100 units/ml penicillin, and 6.8×10⁻⁵ M streptomycin. At each medium change, 100 μM ascorbic acid was added to retard oxidation of any added catecholamine. To minimize fibroblast contamination, selective adhesion techniques were used; fibroblast contamination is <2% with this method. Cardiocytes were kept in a 37°C humidified incubator equilibrated with 5% CO₂ to achieve a final medium pH of 7.30–7.35.

**Experimental Protocols**

To determine the effects of norepinephrine exposure in vitro, cell cultures were treated continuously for 72 hours with graded concentrations of adrenergic agonists and antagonists. The selected range of adrenergic agonist concentrations was based on previous reports that showed that such levels are attainable at the neural cleft or available as circulating levels under certain pathological conditions. For adrenergic antagonists, the selected concentrations were based on reported values, as well as on studies described below in which we determined the concentration of antagonist necessary to block the norepinephrine-mediated increase in cAMP.

A 2-ml suspension of freshly isolated cardiocytes was plated at a final concentration of 1×10⁶ cells/ml onto laminin-coated (20 μg/ml) coverslips in 35 mm polystyrene petri dishes. Beginning on the first day in culture, the cardiocytes were exposed continuously to concentrations of norepinephrine ranging from 10⁻⁹ to 10⁻⁶ M; each dish was treated with a single concentration of norepinephrine. Control cultures were treated with an equal volume of diluent. Medium changes were performed on the first and second days of culture, at which time freshly prepared adrenergic agents or a diluent was added. After 24 hours, the norepinephrine concentration as measured by high-performance liquid chromatography remained at least 80% of the original value. On the third day of culture, all dishes were labeled either with 40 μCi/ml [³H]phenylalanine or 5 μCi/ml [³H]uridine to determine their incorporation into cellular protein or nuclear RNA, respectively.

In a second series of experiments, we examined the effect of norepinephrine on cardiocytes whose β- and/or α-adrenoceptors were blocked before adrenergic stimulation. For these experiments, the cells were pretreated for 1 hour with the nonselective β-antagonist propranolol (range, 10⁻⁹ to 10⁻⁶ M) or with the nonselective α-antagonist phentolamine (range, 10⁻⁸ to 10⁻⁵ M); for studies using combined β- and α-adrenergic blockade, the cells were pretreated for 1 hour with both 10⁻⁶ M propranolol and 10⁻⁵ M phentolamine. After adrenergic blockade, the cells were treated with 1 μM norepinephrine; the medium was changed on the first and second days in culture, and fresh solutions of adrenergic agonists and antagonists were applied. Each experimental set in this series comprised a negative control group treated with diluent only, a control group treated with the maximum concentration of adrenergic antagonist(s) used, and additional groups that received both 1 μM norepinephrine and incremental concentrations of adrenergic antagonists; each culture dish
was treated with a single concentration of adrenergic agonist and antagonist. After 72 hours of continuous treatment with adrenergic agonists and antagonists, the cells were labeled for 6 hours with 40 μCi/ml \[^{3}H\]phenylalanine.

In a third series of experiments, designed to address the separate effects of β- or α-adrenoceptor stimulation, the cardiocytes were stimulated with incremental concentrations of the nonselective β-adrenergic agonist isoproterenol (range, 10^{-10} to 10^{-7} M) or incremental concentrations of the α1-adrenergic agonist phenylephrine (range, 10^{-8} to 10^{-5} M); control cultures were treated with an equal volume of diluent. Each experimental set always consisted of a control group and an experimental group treated with an adrenergic agonist. The preparation, maintenance, and labeling with \[^{3}H\]phenylalanine were identical to those in the protocols described above.

Cardiocyte Evaluation

Cell viability. For the studies in which cells were exposed continuously to adrenergic agonists and/or antagonists, we examined two viability indexes: the percentage of quiescent rod-shaped cells in 10 randomly chosen 1×1-mm fields enumerated serially over the second and third days in culture and the release of creatine kinase into the medium after 24-hour exposure to 10^{-9} to 10^{-5} M norepinephrine. For the creatine kinase release studies, we also examined norepinephrine-treated (10^{-9} to 10^{-5} M) cells isolated from rat38 and canine39 hearts to determine that the results were not species dependent.

Biochemistry. Cardiocyte RNA and protein synthesis were examined by autoradiography; specifically, we measured the incorporation of \[^{3}H\]phenylalanine into cytoplasmic protein and the incorporation of \[^{3}H\]uridine into nuclear RNA.

Protein labeling. \[^{3}H\]Phenylalanine incorporation into cardiocyte cytoplasmic proteins was measured in three experiments. First, \[^{3}H\]phenylalanine incorporation was examined after 72 hours of continuous exposure to 10^{-9} to 10^{-6} M norepinephrine. Second, the cells were pretreated either with 10^{-9} to 10^{-6} M propranolol or 10^{-8} to 10^{-5} M phentolamine and then exposed to 1 μM norepinephrine for 72 hours. Third, cells were treated for 72 hours with either 10^{-10} to 10^{-7} M isoproterenol or 10^{-8} to 10^{-5} M phenylephrine. After 72 hours of exposure to adrenergic agonists and/or antagonists, the cells were labeled with 40 μCi/ml \[^{3}H\]phenylalanine (110.4 Ci/mmoll) for 6 hours, washed three times with a balanced salt solution containing 10 mM cold phenylalanine, and fixed in 3:1 ethanol:acetic acid before air drying. Autoradiography was performed as we have described30; experimental and control slides were treated identically.

For the above studies, \[^{3}H\]phenylalanine incorporation into cardiocyte cytoplasmic proteins was measured using a previously validated30 computer-assisted microscopic image analysis technique. Briefly, the computer program quantifies both cell surface area and the optical density of silver grains over each cell and then integrates the optical grain density over the entire cytoplasmic area of each cell to provide an integrated grain density per unit area of the cell. We have shown30 that there is a linear correlation (r=0.98) between the autoradiographic grain density and scintillation counting of labeled cardiocyte protein. The distinct advantage of this technique is that it allows only viable rod-shaped cardiocytes to be studied, rather than including nonviable hypercontraction cells in the analysis.

RNA labeling. The method used to quantitate \[^{3}H\]uridine incorporation into nuclear RNA is standard in our laboratory30,40 and was used here after 72 hours of exposure of the cardiocytes to 10^{-9} to 10^{-6} M norepinephrine. At this time, the cells were exposed to 5 μCi/ml \[^{3}H\]uridine (28.5 Ci/mmoll) for 2 hours, washed three times with a balanced salt solution containing 10 mM cold uridine, and fixed in 3:1 ethanol:acetic acid before air drying. Nuclear grain density in excess of that over a background area was enumerated over the nuclei of control and norepinephrine-treated cardiocytes and expressed as the total nuclear grain density per cell. Autoradiography was performed as described before.30

Catecholamine-Mediated Toxicity: In Vitro Mechanisms

During these studies, we noted that exposure to ≥10^{-8} M norepinephrine for 15–20 minutes caused normally quiescent cells to contract spontaneously (see Table 4). This induction of spontaneous beating could be aborted by either removing norepinephrine from the medium or pretreating the cells with propranolol, suggesting that continuous occupancy of the β-receptor was necessary for this result. Further, such spontaneously beating cells would invariably undergo irreversible lethal hypercontracture within hours. Given the striking parallel between these in vitro events and the development of contraction band necrosis in vivo, we made a number of direct and indirect measurements to test the hypothesis18 that β-adrenoceptor stimulation causes cAMP-mediated calcium overload of the cardiocyte and that calcium overload is the proximate cause of catecholamine-mediated cell lethality. For each of the indirect biological studies detailed below, either the induction or complete inhibition of spontaneous cardiocyte beating was used as the end point. To confirm these indirect studies, we directly measured both cAMP and [Ca^{2+}].

cAMP elevation. Three separate studies were done to evaluate the role of cAMP production in inducing spontaneous beating. First, cAMP production was blocked 30 minutes before 1 μM norepinephrine exposure by pretreating the cells with 10^{-4} to 10^{-9} M 2′,5′-dideoxycadenosine, a P-site agonist that inhibits the catalytic activity of adenylate cyclase.41,42 Second, cAMP production was stimulated through a non–β-receptor–mediated pathway; this was accomplished by direct activation of the catalytic subunit of adenylate
cGMP is then catalyzed by cGMP-dependent protein kinase, which phosphorylates specific target proteins, leading to the relaxation of smooth muscle. The effector pathway involves the activation of various intracellular signaling molecules, including cAMP, cyclic nucleotides, and calcium, which are critical for the regulation of cardiac function. This process is facilitated by specific receptors on the surface of cardiocytes, allowing for the selective activation of downstream signaling cascades. The cAMP-dependent mechanism is often associated with the β-adrenergic receptor signaling pathway, which is crucial for the regulation of cardiac contractility and other cardiac functions. The role of cAMP in cardiac physiology is not limited to the regulation of contractility, as it also plays a role in the regulation of gene expression, ion channel activity, and the control of intracellular calcium levels. The increased cAMP concentration leads to the activation of protein kinase A (PKA), which phosphorylates and activates specific target proteins, including the cardiac sodium channel, resulting in the generation of a rapid transient inward current (Ito). This transient inward current can be modulated by β-adrenergic receptor activation, leading to the enhancement of cardiac contractility. The role of cAMP in cardiac function is complex and involves multiple signaling pathways, highlighting the importance of understanding the mechanisms underlying cardiac function and the development of therapeutic strategies for the treatment of cardiac diseases.
exceptions just noted, the conditions for the latter two studies were identical to those described above. To better address the question of whether calcium influx preceded cell injury or whether instead cell injury was the primary event with secondary influx of calcium, the above studies were repeated in individual cells using digital imaging fluorescence microscopy. For the present study, we used a relatively new fluorophore, fluo-3, which has an excitation peak at 503–506 nm and an emission peak at 526 nm. Fluo-3 has two unique advantages over the traditional ultraviolet (UV) excited indicators. First, this fluorophore has a very large (∼40-fold) enhancement in fluorescent intensity upon binding Ca$^{2+}$. Second, fluo-3 has a relatively weaker affinity for calcium, thus permitting measurement of higher levels of [Ca$^{2+}$]. For the studies in individual cells, the preparation of the cardiocytes was exactly the same as that described above for the fura-2 experiments, with the exception that the cells were maintained in culture on laminin-coated petri dishes. On the day of study, the cardiocytes were washed, and the cells were loaded for 60 minutes with 20 μM of fluo-3 acetoxymerthyl ester. To facilitate solubilization of the acetoxymerthyl ester, 0.5–1 μl of 25% (wt/wt) Pluronic F-127 in dry dimethyl sulfoxide was mixed with every 10 nM of AM and the resulting solution thoroughly dispersed in the culture medium. After loading, the cells were washed three times and then equilibrated for an additional 30 minutes to permit complete hydrolysis of any remaining intracellular AM.

Norepinephrine at a final concentration of 1 μM was added to the culture medium, and the resulting change in cardiocyte fluorescence was measured 30 minutes later. Control cells were treated with diluent. Fluorescent brightness was measured using a microspectrofluorometer equipped with a xenon bulb. The filter sets used delivered 490 nm excitation light; emission was measured using a barrier filter that passed light at wavelengths >520 nm. The resulting fluorescence image (×400) was analyzed by digital imaging microscopy, which integrates the intensity of fluorescence brightness over the surface area of an individual cardiocyte and then normalizes this value by the surface area of the cell (μm$^2$); this accounted for any variations in fluorescence brightness that might occur on the basis of cell size alone (expressed as arbitrary units fluorescence brightness/μm$^2$ cell). Nonspecific background fluorescence was eliminated by a background subtraction routine; autofluorescence was not detectable at the low visible light levels used in the present study.

Two additional fluorescence microscopy experiments were done to further define the norepinephrine effect on [Ca$^{2+}$]. First, the role of β-adrenergic receptor activation was assessed by repeating the above experiments after pretreating the cells with 1 μM propranolol for 1 hour. Second, to determine the role of influx of extracellular calcium, the cardiocytes were pretreated with 1 μM verapamil for 1 hour, and the above experiments were repeated. With the exceptions just noted, the conditions for the latter two experiments were identical to those described above.

To be certain that norepinephrine-mediated hypercontracture that followed the induction of spontaneous cardiocyte beating was the result of calcium overloading of the cell and not simply the result of excessive mechanical stimulation, we performed an additional control study in which cell viability was assessed after electrical stimulation at a frequency similar to that of the norepinephrine-induced spontaneous beating. For these experiments, freshly isolated cardiocytes were continuously paced electrically for 30 minutes with a pair of platinum wire electrodes using 0.33-Hz, 100-μA DC pulses of alternating polarity with no voltage offset between pulses; the latter two stimulation conditions were used to minimize electrolysis. The percentage of viable rod-shaped cardiocytes was determined before and 30 minutes after continuous pacing. The extent of creatine kinase release was measured at 24 hours in the cultures that were electrically stimulated, as well as in a group of control cultures that were not electrically stimulated.

To determine whether the norepinephrine-mediated toxic effect was secondary to ATP depletion, we measured intracellular ATP levels in vitro. Preparation and maintenance of the cultures were exactly as described above. On the first day in culture, the cells were stimulated with 1 μM norepinephrine for 0, 15, and 60 minutes and 24 hours; the appropriate control groups for this experiment consisted of parallel cultures treated at identical time points with diluent only. At the end of each indicated time point, the cultures were washed three times with cold KHB buffer and intracellular ATP extracted for 10 minutes with cold 12% trichloroacetic acid. The samples were then ether-extracted five times, and the pH was adjusted to 7.8. Intracellular levels of ATP were measured using the luciferease assay according to the method of Lundin et al. The amount of cellular protein per sample was determined using the bicinechonic acid assay. The level of ATP in the norepinephrine and control cultures was expressed in nanomoles of ATP per milligram of cardiocyte protein.

Drugs

L-Isomers were used except as specifically indicated. Sources of adrenergic agents were norepinephrine, phenylephrine, isoproterenol (Sigma Chemical); propranolol (a gift from Ayerst); and phenotolamine (a gift from CIBA-GEIGY). Other sources were (±)-verapamil, tetrodotoxin, dibutyryl cAMP, forskolin (Sigma), lidocaine (Astra); 2′,5′-dideoxyadenosine (Pharmacia); fura-2 AM (Calbiochem); [γ-32P]-phosphatidic acid and [5-3H]uridine (New England Nuclear); NTB3 emulsion (Kodak); medium 199 with Earle’s salts (GIBCO); and medium supplements (Collaborative Research).
Statistical Analysis

Each value is expressed as mean±SEM. Specific adrenergic agonist and antagonist effects on cell viability were examined by two-way ANOVA, with testing for differences within experimental groups on the second and third days in culture. One-way ANOVA was used to test for overall differences in the extent of [3H]phenylalanine or [3H]uridine incorporation in norepinephrine-treated cells; where appropriate, Dunnett’s multiple comparison was used to test for specific differences between control and experimental groups. A difference was said to be significant at p<0.05.

Results

Cell Viability

An important finding of the present study was that norepinephrine stimulation led to a striking, concentration-dependent decrease in cardiocyte viability. That is, when rod-shaped cardiocytes were exposed to norepinephrine, a large proportion of these normally quiescent cells began to contract spontaneously within 15–30 minutes. When followed over time, virtually all such cells developed irreversible hypercontracture. Figure 1 (upper panel) shows the morphology of a rod-shaped cardiocyte and (lower panel) of a hypercontracted cardiocyte exposed to 1 μM norepinephrine. Figure 2 summarizes the results of the cell morphology studies and shows that norepinephrine treatment caused a major decrease in the percentage of viable rod-shaped cells as a function of increasing agonist concentration. Two-way ANOVA showed that this catecholamine-mediated toxic effect was both concentration and time dependent.

Figure 1. Photomicrographs of cardiocyte morphology before and after norepinephrine exposure. Panel A: Appearance of a typical rod-shaped cardiocyte in culture. Panel B: Appearance of a partially contracted cardiocyte after 30 minutes of exposure to 1 μM norepinephrine. Panel C: Typical appearance of a fully hypercontracted cardiocyte after 60 minutes of exposure to 1 μM norepinephrine.

Figure 2. Bar graph of effect of norepinephrine on cardiocyte viability. For each concentration of norepinephrine shown, the data represent mean±SEM values for a minimum of 16 dishes obtained from a minimum of four primary cardiocyte isolations. Cardiocyte viability, expressed in terms of the percentage of rod-shaped cells in culture, was adversely affected by increasing concentrations of norepinephrine. Analysis of variance indicated that this catecholamine-mediated toxic effect was both concentration and time dependent. Each asterisk (*) indicates a significant difference (p<0.05) from the control value by Dunnett’s test.
(p<0.001); that is, for each norepinephrine concentration used, there was a significant decline in the percentage of rod-shaped cells from the second through the third day in culture.

Two pharmacological approaches were taken in determining the mechanisms for this norepinephrine effect. Figure 3 summarizes the results of the first study, in which we studied the prevention of norepinephrine toxicity by α- and/or β-adrenoceptor blockade. Panel A shows that pretreatment with phentolamine did not significantly attenuate the toxic effect of norepinephrine on cell viability; only with very large (10 μM) concentrations of phentolamine was there a slight protective effect. In contrast, panel B shows that propranolol antagonized the toxic effects of norepinephrine over the full range of concentrations used. Two-way ANOVA showed that this protective effect persisted from the second through the third day in culture. As shown, 1 μM propranolol greatly attenuated but did not fully prevent the toxic effect of norepinephrine. Thus, in an additional experiment the cells were pretreated with both 1 μM propranolol and 10 μM phentolamine before norepinephrine treatment. Figure 3B shows that combined α- and β-adrenoceptor blockade completely prevented the norepinephrine-mediated decrease in cell viability.

Figure 4 summarizes the second pharmacological approach, in which cells were treated with phenylephrine or isoproterenol to study the respective effects of either α- or β-adrenoceptor stimulation on cardiocyte viability. Panel A of Figure 4 shows that phenylephrine treatment resulted in a very small but significant decline in the percentage of rod-shaped cardiocytes;
Dunnett's post-ANOVA showed this to be significant for phenylephrine concentrations $\geq 10^{-7}$ M. Two-way ANOVA further showed that this effect was concentration dependent ($p < 0.001$) but not time dependent; that is, there was no further decline in the percentage of rod-shaped cells from the second through the third day in culture. In contrast to the findings with $\alpha$-adrenoceptor stimulation, panel B of Figure 4 shows that isoproterenol stimulation caused an obvious decrease in the percentage of rod-shaped cells; this effect was very similar to the norepinephrine effect shown in Figure 1. Indeed, two-way ANOVA showed that the isoproterenol effect was both concentration and time dependent ($p < 0.001$).

To confirm that the decline in the number of rod-shaped cells reflected diminished cell viability, we measured the amount of creatine kinase release in cardiocyte cultures treated for 24 hours with $10^{-9}$ to $10^{-5}$ M norepinephrine. Figure 5 shows that there was a stepwise increase in creatine kinase release as a function of increasing norepinephrine concentration. ANOVA indicated that these overall differences in creatine kinase release were statistically significant ($p < 0.001$); post-ANOVA comparison indicated that there were significant differences from control for norepinephrine concentrations $\geq 10^{-7}$ M. Linear regression analysis was also done to determine whether there was a relation between the norepinephrine-mediated decrease in cell viability and the amount of creatine kinase release. This analysis showed that after exposure to $10^{-9}$ to $10^{-5}$ M norepinephrine for 24 hours, there was a significant linear relation ($r = 0.92, p < 0.001$) between the percentage of hypercontracted cardiocytes relative to viable, rod-shaped cells and the percent increase in creatine kinase release.

To be certain that norepinephrine-mediated cardiocyte toxicity was not a strictly species-dependent phenomenon, we repeated these studies using cells isolated from adult rat and canine hearts. Figure 5 shows that norepinephrine treatment led to an overall significant ($p < 0.001$ for both) release of creatine kinase from both rodent and canine cardiocytes, albeit to a lesser extent in the rat. For canine cardiocytes, post-ANOVA testing indicated that there were significant differences from control for norepinephrine concentrations $\geq 10^{-7}$ M, whereas for rodent cardiocytes there were significant differences for norepinephrine concentrations $\geq 10^{-5}$ M.

**Biochemistry**

Adrenergic effects on cardiocyte synthetic activity were assessed in two separate autoradiographic studies of $[^3H]$phenylalanine incorporation into cytoplasmic proteins and $[^3H]$uridine incorporation into nuclear RNA.

**Protein labeling.** A second important finding of the present study was that continuous stimulation with norepinephrine resulted in a concentration-dependent decrease in $[^3H]$phenylalanine incorporation into viable rod-shaped cardiocytes. Figure 6 shows the results of exposing cardiocytes for 72 hours to norepinephrine concentrations ranging from $10^{-9}$ to $10^{-6}$ M. The salient finding is the striking decrease in cytoplasmic labeling with increasing norepinephrine concentrations.
Table 1. Effect of Adrenergic Antagonists on [3H]Phenylalanine Incorporation Into Cardiocyte Cytoplasmic Protein

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<th>Treatment (72 hr)</th>
<th>Change from control (%)</th>
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<tr>
<td>10^{-5} M norepinephrine</td>
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<tr>
<td>10^{-7} M norepinephrine + 10^{-9} M propranolol</td>
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<tr>
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Each value is expressed as the mean±SEM of the percent change from control in the extent of cytoplasmic labeling. For each of the treatments noted above, the data were obtained from a minimum of 100 consecutively studied rod-shaped cardiocytes; nonviable ball-shaped cardiocytes were excluded from the analysis. Norepinephrine treatment (1 μM) led to a significant decline in the extent of cytoplasmic labeling. One-way ANOVA indicated that propranolol exerted a significant concentration-dependent “sparking” of the norepinephrine-mediated decrease in [3H]phenylalanine incorporation; at 10^{-6} M propranolol, the effects of norepinephrine were largely, but not completely, attenuated. There was, however, no significant concentration-dependent “sparking” effect after pretreatment with phentolamine. The combination of 10^{-7} M phentolamine and 10^{-6} M propranolol completely blocked the norepinephrine-mediated effects on cardioocyte protein synthesis. Treatment of cardiocytes with adrenergic antagonists alone did not lead to a significant change in the extent of cytoplasmic labeling compared with control cardiocytes (10^{-6} M propranolol, -6.6±3.6%, p=NS; 10^{-5} M phentolamine, -1.5±3.8%, p=NS; 10^{-5} M phenylephrine + 10^{-6} M propranolol, -4.8±4.9%, p=NS).

†p<0.01 compared with control by Dunnett’s test.

The treatment of cardiocytes with 10^{-5} M norepinephrine was highly significant (p<0.001); Dunnett’s post-ANOVA comparison showed that this catecholamine-mediated effect was significantly different from control for norepinephrine concentrations ≥10^{-8} M.

Table 2 is a summary of the results of autoradiographic studies in which cells were exposed to norepinephrine in the presence of α- and/or β-adrenergic antagonists. The extent of cytoplasmic protein labeling was expressed as a percentage of control. When the cells were pretreated with concentrations of propranolol ranging from 10^{-7} to 10^{-10} M, there was a concentration-dependent amelioration of the norepinephrine-mediated decrease in protein labeling. Nevertheless, the norepinephrine effect was still evident in the cells treated with 1 μM propranolol, albeit to a much lesser extent. In contrast to the findings with β-adrenoceptor blockade, phentolamine did not appreciably block the norepinephrine-mediated decrease in [3H]phenylalanine incorporation. However, the combination of α- and β-adrenoceptor blockade completely blocked the norepinephrine-mediated reduction in cytoplasmic labeling.

To further define the mechanism(s) for catecholamine-mediated effect(s) on protein synthesis, we studied the effects of α- or β-adrenoceptor agonists on cytoplasmic protein labeling. Table 2 shows that 72 hours of continuous exposure to 10^{-10} to 10^{-7} M isoproterenol caused a concentration-dependent decrease in cytoplasmic labeling that was similar to the norepinephrine effect. ANOVA showed that these overall differences were highly significant (p<0.001); Dunnett’s post-ANOVA testing indicated that this effect was significantly different from control for isoproterenol concentrations ≥10^{-8} M. In contrast to the findings with selective β-adrenoceptor stimulation, exposure to phenylephrine caused a small but significant (p<0.05) decrease in [3H]phenylalanine incorporation. Dunnett’s post-ANOVA testing showed that the phenylephrine effect was not significantly different from control until very large concentrations (10^{-5} M) of phenylephrine were used. Blockade of the phenylephrine-treated cells with 10 μM phentolamine prevented the decrease in cytoplasmic labeling.

RNA labeling: Table 3 shows the effects of 72 hours of continuous norepinephrine treatment on cardiocyte nuclear RNA labeling. There was a decrease in nuclear labeling as a function of increasing norepinephrine concentration. ANOVA showed that the overall differences were significant (p<0.001); post-
ANOVA comparison showed that this catecholamine-mediated effect was significantly different ($p<0.01$) from control for norepinephrine concentrations $\geq 10^{-7}$ M. To be certain that these differences in nuclear grains per cell were not due to a greater frequency of multinucleated cells in the control group, we compared the grains per nucleus in both control and norepinephrine-treated cells. For each of the experimental groups, there was again a significant ($p<0.001$) concentration-dependent decrease in the extent of nuclear labeling as a function of increasing norepinephrine concentration.

**Catecholamine-Mediated Toxicity: In Vitro Mechanisms**

**cAMP elevation.** Table 4 summarizes the results of the three studies showing that cAMP elevation induced spontaneous cardiocyte beating that, over time, led to the cell hypercontraction depicted in Figures 1 and 2. Although a range of concentrations was tested for each agent shown in Table 4, for the sake of simplicity only the lowest drug concentration that produced the indicated effect is reported. First, the use of $10^{-5}$ M 2′,5′-dideoxyadenosine to block cAMP production prevented induction of spontaneous beating by 1 $\mu$M norepinephrine. Second, when cAMP production was stimulated through a non-$\beta$-adrenoceptor mechanism by $10^{-6}$ M forskolin, spontaneous cardiocyte beating occurred within 30 minutes. Third, when $10^{-3}$ M dibutyryl cAMP, a cAMP analogue, was added to the culture dishes, the cells began to beat spontaneously within 60 minutes.

Figure 7 shows the results of studies in which the time course of cAMP elevation was measured after stimulation with 1 $\mu$M norepinephrine. There was a sustained fourfold increase in cAMP production after norepinephrine stimulation. Furthermore, pretreatment with 1 $\mu$M propranolol for 60 minutes largely abolished the norepinephrine-induced increase in cAMP.

**Calcium influx.** Table 4 also is a summary of the results of studies of the role of calcium influx in inducing spontaneous cardiocyte beating and hypercontraction. Although a range of concentrations was tested for each agent, only the lowest drug concentration that produced the indicated effect is reported. First, although norepinephrine-induced spontaneous beating was readily induced in cardiocytes in 2.5 mM calcium buffer, this was not seen in norepinephrine-stimulated cells in a calcium-free $10^{-3}$ M EGTA buffer. Furthermore, the absence of norepinephrine-induced spontaneous beating in the EGTA-treated cells was not secondary to damage to cell membranes because the EGTA-treated cells began to contract spontaneously when placed in a 2.5 mM calcium solution and restimulated with 1 $\mu$M norepinephrine. Second, both verapamil (≥1 $\mu$M) and cobalt chloride (≥10 mM) suppressed spontaneous beating completely at concentrations that block the slow calcium channel. Third, neither 10 $\mu$M tetrodotoxin nor...
10 μM lidocaine prevented the induction of norepinephrine-induced spontaneous cardiocyte beating at concentrations that block the fast sodium channel.60–62 Finally, verapamil (≥1 μM) completely suppressed the spontaneous cardiocyte beating induced by the 10−3 M dibutyryl cAMP.

Figure 8 shows the effect of norepinephrine stimulation on [Ca2+]i, expressed as an absolute increase in the 340/380 ratio of fura-2 brightness. The salient finding is that continuous stimulation with 1 μM norepinephrine resulted in a gradual and progressive increase in the 340/380 ratio of fura-2 brightness with time. Two-way ANOVA indicated significant (p<0.001) overall differences between control and norepinephrine-treated cells; post-ANOVA comparison demonstrated significant differences from control beyond 12 minutes. A second important finding was that norepinephrine stimulation did not cause a significant (p>0.05) increase in fura-2 brightness for cardiocytes pretreated with 1 μM propranolol. Finally, to determine whether the norepinephrine-mediated increase in fura-2 brightness was caused by calcium influx rather than calcium release from intracellular stores, these experiments were repeated in an EGTA buffer. As shown, in the absence of extracellular calcium, there was no significant (p>0.05) increase in intracellular fura-2 brightness after norepinephrine stimulation.

Digital imaging fluorescence microscopy confirmed that this increase in fluorescence brightness was the result of norepinephrine-stimulated calcium influx rather than secondary calcium influx from cellular injury. The digital imaging studies, which were confined to single, viable rod-shaped cardiocytes (n=100 cells/group), revealed two important findings. First, stimulation with norepinephrine (1 μM) for 60 minutes led to a significant increase in intracellular fluorescence compared with control values (0.288±0.001 versus 0.10±0.004, respectively; p<0.001). Furthermore, this norepinephrine-induced increase in intracellular fluorescence brightness was significantly attenuated (p>0.30 for both) by pretreating the cells with either 1 μm propranolol (0.10±0.002, n=100 cells) or 1 μm verapamil (0.10±0.004, n=100 cells). An additional important finding of the present study was that norepinephrine caused rhythmic “scintillations” of fluorescence brightness within individual cells that were quite similar in appearance to the rhythmic flashing of the firefly Photuris versicolor on a summer evening. This phenomenon, which is illustrated in Figure 9, was seen in the majority of rod-shaped cardiocytes at various times after norepinephrine stimulation. Furthermore, although not every cell showing rhythmic scintillations developed spontaneous beating, this phenomenon was always observed in cells in which spontaneous beating developed. Although the present study was not designed to elucidate the mechanism(s) for this interesting finding, the rhythmic increase in fluorescence brightness probably represents calcium-triggered calcium release from the sarcoplasmic reticulum.61

Mechanical stimulator. Electrical stimulation of cardiocytes at a frequency of 0.33 Hz resulted in an −2.5% decrease (p=0.82) in the percentage of rod-shaped cardiocytes in a given culture dish (n=8 experiments) over time. Furthermore, compared with control cultures that were not electrically stimulated, there was no significant increase in creatine kinase release (control, 951±22 IU; electrically stimulated, 934±49 IU; p=0.94).
Adenosine triphosphate measurements. Table 5 is a summary of the results of the studies in which we examined the effect of norepinephrine stimulation on intracellular ATP levels. As shown, there was no change in the intracellular levels of ATP in either the control or the norepinephrine-treated cultures. Two-way ANOVA showed that there were no significant differences between groups (p=0.99) or within groups as a function of time of treatment (p=0.97).

Discussion

The present study directly supports the hypothesis that cAMP-mediated calcium overload is the primary mechanism responsible for catecholamine-induced toxicity in cultured adult mammalian cardiocytes. Two distinct lines of evidence support this statement. First, when the effects of adrenergic stimulation were examined in virtually pure populations of cardiocytes using concentrations of norepinephrine that are normally attainable at the neural cleft or available as actual circulating levels in certain pathological conditions, there was both a concentration-dependent decrease in cardiocyte viability, as shown by a striking decrease in viable rod-shaped cells, and a significant release of creatine kinase. The view that these effects were mediated directly by the β-adrenoceptor is supported by the finding that the toxic effects of norepinephrine were attenuated significantly by β-adrenoceptor blockade and mimicked by selective stimulation of the β-adrenoceptor. In contrast, α-adrenoceptor effects were relatively small and evident only when very large concentrations of α-adrenergic agonists and antagonists were used. It is of some interest that the toxic response to norepinephrine was not confined to a single mammalian species; toxicity was elicited in rat, cat, and canine cardiocytes. Of note, toxicity was evident for rat cardiocytes only when very large concentrations of norepinephrine were used, probably reflecting the relatively smaller number of β-adrenoceptors in this species compared with cat, dog, and humans.

Second, continuous adrenergic stimulation led to cAMP-mediated calcium overload of the cardiocyte, as shown indirectly by pharmacological strategies in which increased intracellular cAMP was shown to cause spontaneous beating and cell hypercontracture, as well as by data showing that calcium influx through the verapamil-sensitive calcium channel was necessary for this effect. The role of cAMP-mediated calcium influx supported more directly by the microspectrofluorometric studies, as well as by the time course studies where norepinephrine exposure led first to a rapid fourfold increase in intracellular cAMP and then to a 3.2-fold increase in [Ca^{2+}]. Furthermore, the norepinephrine effects on cAMP and [Ca^{2+}], were significantly attenuated by β-adrenoceptor blockade, as might be expected from the relation between β-adrenoceptor stimulation and activation of the verapamil-sensitive calcium channel. To be certain that cardiocyte hypercontracture was the result of calcium overload of the cell rather than excessive mechanical activity, an additional control experiment was performed in which the cells were electrically stimulated to contract. When the cells were stimulated at a frequency similar to that seen with norepinephrine exposure, there was no significant decrease in the

![Figure 9. Fluorescent “scintillation” in a norepinephrine-stimulated cardiocyte. Left panel: Level of fluorescence brightness in a fluo-3–loaded cardiocyte treated with 1 μM norepinephrine for 30 minutes. Right panel: Same cell during the peak of a fluorescent scintillation. Notice that an increase in fluorescence brightness occurs without a change in cell length, thus obviating depolarization as the mechanism. Also note that the fluorescence intensity for the ball-shaped cell in the left corner of each panel was the same, indicating that the intensity of the light source was unchanged.](http://circ.ahajournals.org/)

**Table 5. ATP Levels in Control and Norepinephrine-Treated Cardiocytes**

<table>
<thead>
<tr>
<th></th>
<th>Control (n=5)</th>
<th>Norepinephrine (1 μM) (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>12.9±1.1</td>
<td>13.6±1.2</td>
</tr>
<tr>
<td>15 Min</td>
<td>12.0±2.1</td>
<td>12.6±1.9</td>
</tr>
<tr>
<td>60 Min</td>
<td>13.1±1.8</td>
<td>12.7±2.2</td>
</tr>
<tr>
<td>24 Hr</td>
<td>13.3±3.3</td>
<td>12.4±2.3</td>
</tr>
</tbody>
</table>

Each value is expressed as mean±SEM of nM ATP/mg cardiocyte protein. Intracellular levels of ATP were measured at the times indicated above using the luciferase assay. Two-way ANOVA indicated that there was no significant difference between groups (p=0.99) or within groups (p=0.97) as a function of time.
number of viable rod-shaped cells or significant increase in creatine kinase release from the paced cells. Finally, to exclude the possibility that calcium influx into the cell was secondary to depletion of high-energy phosphates rather than a direct effect of norepinephrine, we measured intracellular ATP levels in vitro. These results showed that intracellular ATP levels were unchanged when norepinephrine stimulation resulted in cAMP-mediated calcium overload of the cell. Parenthetically, it should be noted that the experimental protocols detailed were designed to minimize the effects of free radicals in culture; therefore, it is not possible to comment on any potential effects that free radicals might have played in the observed catecholamine-mediated toxic effects in our in vitro model.

Another major finding of the present study was that continuous stimulation with norepinephrine caused a concentration-dependent decrease in \(^{[3]H}\)phenylalanine incorporation in cultured adult cardiocytes. Thus, adrenergic stimulation not only resulted in diminished cell viability but also led to a decrease in protein synthetic activity in the remaining viable cardiocytes. The decrease in cytoplasmic labeling was greatly attenuated by \(\beta\)-adrenoceptor blockade and mimicked by selective stimulation of the \(\beta\)-adrenoceptor. In contrast, \(\alpha\)-adrenoceptor stimulation led to a very small but significant decrease in cardiocyte synthetic activity, albeit only with very large concentrations of agonist. Although the exact mechanisms for this decrease in \(^{[3]H}\)phenylalanine incorporation are not known, the concentration-dependent decrease in \(^{[3]H}\)phenylalanine incorporation into nuclear RNA after norepinephrine exposure suggests that the decrease in cardiocyte synthetic activity may be in part mediated at the transcriptional level. Nevertheless, on the basis of the present study, it would be premature to comment on rates of protein or RNA synthesis or degradation, given that size of the phenylalanine and uridine pools were not known.

**Experimental Catecholamine Cardiotoxicity**

In 1959, Rona et al.\(^7\) made the important observation that injection of isoproterenol into rats produces "infarctlike" myocardial necrosis in the absence of significant coronary artery lesions. This observation led to the suggestion that "relative hypoxia" was responsible for the observed cardiac necrosis.\(^7,9,65\)

That is, increased cardiac inotropy and chronotropy after adrenergic stimulation caused a relative imbalance between myocardial oxygen demand and blood flow, such that demand exceeded supply. In support of this view is the observation that isoproterenol decreases coronary blood flow in the early phase of isoproterenol cardiotoxicity.\(^10\) In addition to this relative hypoxia theory, a number of alternative hypotheses have been proposed to explain catecholamine-induced myocardial injury. Rona and coworkers\(^11-14\) also suggested that catecholamine-mediated increases in sarcolemmal permeability contributed to the pathogenesis of myocardial injury. Although the concept of calcium-induced cardiac necrosis had been introduced previously,\(^6,6\) Fleckenstein\(^15\) was the first to propose the radically different concept that calcium overload of the cell was the result of catecholamine-mediated cell injury. According to this theory, the important event in the development of the catecholamine-mediated lesion was a high-energy phosphate deficiency that resulted from excessive activation of calcium-dependent ATPases and calcium-induced impairment of mitochondrial function. Critical support for the calcium overload hypothesis followed in an important study by Bloom and Davis\(^17\) that showed that myocardial calcium increased 10 minutes after injection of isoproterenol into rats, suggesting that calcium influx was the cause rather than the consequence of cardiac injury.\(^16\) Although Bloom and Sweat\(^67\) subsequently showed that increased levels of myocardial calcium were coupled to elevations in myocardial cAMP, the view that cellular calcium overload was consequent to \(\beta\)-adrenoceptor-mediated elevations of cAMP was first suggested by Bhagat et al.\(^18\) This hypothesis was subsequently confirmed in a series of studies by Opie and colleagues,\(^19,20,24\) who demonstrated in Langendorff rat hearts that catecholamine injury was mediated by the \(\beta\)-adrenoceptor. However, the importance of the \(\beta\)-adrenoceptor was not confirmed by an in vivo study in rabbits, which suggested instead that catecholamine cardiotoxicity is largely mediated by the \(\alpha\)-adrenoceptor.\(^21,22\) Finally, others have suggested that oxidative catecholamine metabolites and/or the formation of oxygen or oxygen-derived free radicals causes cardiac damage.\(^25-28\) However, as noted in a recent review,\(^29\) the biological significance of these latter studies is uncertain given the large concentrations of adrenochrome (\(\geq 10^{-4}\) M) needed to cause cardiac injury. Thus, a number of hypotheses have been proposed, but there is no consensus regarding the basic mechanism(s) responsible for catecholamine cardiotoxicity.

**Conclusions**

The toxic cardiac effects of catecholamines have been recognized at least since 1907, when Josue\(^68\) first reported catecholamine-induced cardiac necrosis. Thus, we were not surprised to find that norepinephrine caused toxic effects in isolated adult cardiocytes. This statement notwithstanding, the importance of the present study is twofold. First, this simple experimental study provides straightforward and compelling evidence in support of the viewpoint that cAMP-mediated calcium overload of the cell is a fundamentally important early event in the genesis of catecholamine-induced cardiac injury. Second, the present study constitutes the initial demonstration that so-called "pathological" elevations of norepinephrine lead to a concentration-dependent decrease in cardiocyte RNA and protein synthetic activity, as well as cell viability.

Although direct correlations between short-term effects in isolated cells and long-term effects in the heart in vivo are not appropriate, these data do
provide several potential clinical insights. For example, the prognosis of patients with congestive heart failure is inversely related to the level of circulating plasma norepinephrine. Although this catecholamine response may well be an effect of disease severity, it may also have a deleterious contributory role. Furthermore, our finding that catecholamine-induced cardiac overload occurs predominately via the β-adrenoceptor suggests a mechanism for the salutary clinical effect of β-blockade in dilated cardiomyopathy. Finally, our finding that adrenergic stimulation causes decreased cardiocyte synthetic activity suggests a potential mechanism, albeit speculative, for the progressive pump dysfunction in patients with dilated cardiomyopathy. That is, the norepinephrine-induced decrease in cardiocyte RNA and protein synthesis may compromise the development of fully compensatory hypertrophy in response to some forms of chronic cardiac stress. In summary, catecholamines have a major, and beneficial, role in the short-term cardiovascular response to physiological stress. Unfortunately, during the long-term cardiovascular response to pathological stress, the beneficial role of catecholamines may occur at the price of superimposed cardiac toxicity.

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