

Low Catecholamine Concentrations Protect Adult Rat Ventricular Myocytes against Apoptosis through cAMP-Dependent Extracellular Signal-Regulated Kinase Activation

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ABSTRACT

Catecholamines have complex effects on cardiac myocyte growth and survival, including the triggering of apoptosis at high concentration. Here, we examined whether at a lower concentration, catecholamine protected adult rat ventricular myocytes from apoptosis *in vitro*. Myocytes were exposed to staurosporine (ST, 10 μ M) for 18 h, with or without epinephrine (0.1 or 10 μ M) or fetal calf serum (10%). Apoptosis was assessed after 48 h of culture in terms of DNA fragmentation (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling method, DNA gel electrophoresis). Epinephrine (0.1 μ M) and serum reduced ST-induced myocyte apoptosis by ~50% ($n = 12$ cultures, $P < .001$), whereas epinephrine and serum alone did not influence the low apoptotic rate in control cultures. In contrast, 10 μ M epinephrine induced marked apoptosis in ST-free conditions. The protective effects of 0.1 μ M

epinephrine and serum were blunted by the tyrosine kinase inhibitor genistein ($n = 12$ cultures, $P < .001$). Extracellular signal-regulated kinase (ERK) activity was stimulated by 0.1 μ M epinephrine but not by 10 μ M epinephrine. Furthermore, the protective effect of epinephrine was mimicked by isoproterenol (1 μ M) and forskolin (1 μ M) but not by phenylephrine (10 μ M) and was blunted by propranolol (10 μ M) but not by prazosin (10 μ M). Finally, isoproterenol and forskolin activated ERK, an effect that was blunted by propranolol. In conclusion, low epinephrine concentrations attenuate ST-induced apoptosis of adult cardiac myocytes *in vitro*, an effect mediated by coupling between the cAMP pathway and ERK activation. This suggests that a minimal adrenergic tone is essential for myocyte survival in conditions of unusual stress.

It was recently reported that the beneficial effects of β -blocking agents on the survival of patients with heart failure (Anonymous, 1994; Packer et al., 1996), a syndrome associated with increased circulating catecholamine concentrations, could be related to the prevention of catecholamine-induced myocyte apoptosis (Communal et al., 1998, 1999; Yue et al., 1998; Iwai-Kanai et al., 1999). However, catecholamines can also promote cardiac myocyte growth (Simpson et al., 1982) and survival (Wu et al., 1997). For instance, atrial natriuretic peptide-induced apoptosis of neonatal rat cardiac myocytes can be prevented by the activation of β -adrenergic receptors and elevation of cAMP levels. This apparent discrepancy among the various studies suggests that catecholamines activate different intracellular signaling

pathways that have distinct effects on cell survival. Depending on their concentration, or on cell stimulation by other peptides or growth factors, catecholamines might thus either activate or protect against programmed cell death.

Other hormones, growth factors, or cytokines that are involved in the onset of cardiac myocyte hypertrophy can either have beneficial effects on cardiac myocyte survival or promote their apoptotic death. This is the case of angiotensin II, the trophic effects of which are well established (Sadoshima et al., 1995) but which was also recently identified as a potent proapoptotic agent (Kajstura et al., 1997). These observations are also in keeping with our current knowledge of apoptosis, a tightly regulated biological process controlled by a subtle balance between a number of cellular signaling pathways promoting or inhibiting activation of the death program. Phosphorylation of various pro- or antiapoptotic factors such as BAD (Wang et al., 1999) and Bcl-2 (Ito et al., 1997) via serine/threonine and tyrosine kinases can either induce or prevent apoptosis, depending on the trigger. The mitogen-activated protein kinase (MAPK) family, which com-

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ABBREVIATIONS: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-related kinase; JNK, c-Jun NH_2 -terminal kinase; ST, staurosporine; PTX, pertussis toxin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; MBP, myelin basic protein; au, arbitrary unit; PK, protein kinase.

prises at least three members, namely, extracellular signal-regulated kinase (ERK), c-Jun NH₂-terminal protein kinase (JNK), and p38-MAPK, also plays a pivotal role in regulating apoptosis and survival, and ERK often contributes to shifting this dynamic balance toward cell survival (Xia et al., 1995). Accordingly, the aim of this study was to determine whether catecholamines can protect adult cardiac myocytes against apoptotic death and, if so, whether this protection involves ERK activation. We used a model of cultured adult rat ventricular myocytes, in which apoptosis can be induced by various stimuli (Andrieu-Abadie et al., 1999; Delpy et al., 1999), including staurosporine (Rücker-Martin et al., 1999), and prevented by agents such as L-carnitine (Andrieu-Abadie et al., 1999). The potential protective effect of catecholamines on staurosporine (ST)-induced apoptosis was compared with that of fetal calf serum, a nonspecific stimulator of myocyte growth pathways.

Materials and Methods

Myocyte Isolation and Culture. Cultures of ventricular myocytes were prepared from adult male Wistar rats (180–200 g) as described in Rücker-Martin et al. (1999). Isolated myocytes were suspended in Dulbecco's modified Eagle's medium (Life Technologies SARL, Cergy Pontoise, France) supplemented with 4% nonessential amino acids (Life Technologies), 1 nM insulin, and antibiotics (100 I.U./ml penicillin and 0.1 µg/ml streptomycin; Life Technologies) and plated in two-well Labtek chambers or in 25-cm² flasks (Polylabo, Strasbourg, France) precoated with 10 µg/ml laminin (Life Technologies). To inhibit nonmuscle cell proliferation, cytosine arabinose (10 µM; Sigma-Aldrich, St. Quentin Fallavier, France) was added throughout the culture period. Myocyte identification was achieved using indirect immunofluorescence and antibodies directed against sarcomeric α -actinin (1/400; Sigma-Aldrich) as described previously (Rücker-Martin et al., 1999).

Cell Treatments. Apoptosis was induced on the day of cell isolation by adding ST (10 µM; Sigma-Aldrich) to the culture medium for 18 h. In some experiments, 10% fetal calf serum (Valbiotech, Paris, France), epinephrine (0.01 to 100 µM; Sigma-Aldrich), or phenylephrine (10 µM; Sigma-Aldrich) dissolved in 100 µM L-ascorbic acid; isoproterenol (1 µM; Sanofi Winthrop Pharmaceuticals, France); or forskolin (1 or 10 µM; Sigma-Aldrich) was added to cultures at the same time as ST. In some cases the protein tyrosine kinase inhibitor genistein (50 µM; Sigma-Aldrich), or the α -adrenoceptor antagonist prazosin (10 µM; Sigma-Aldrich), or the β -adrenoceptor antagonist propranolol (10 µM; Sigma-Aldrich) was added to cultures 1 h before the addition of ST, serum, or epinephrine. In other cases, myocytes were preincubated overnight with the G_i protein inhibitor pertussis toxin (PTX, 1 µg/ml; Sigma-Aldrich) before the addition of test compounds.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Method and DNA Gel Electrophoresis. In situ detection of DNA fragmentation was performed on cultured myocytes by using TUNEL with an in situ cell death detection kit (Roche Diagnostics, Meylan, France) according to the manufacturer's instructions. The labeled myocytes were analyzed by fluorescence microscopy. To quantify apoptotic cells, the percentage of myocytes with DNA nick-end labeling was measured by counting green fluorescent nuclei at 400 \times magnification in 10 randomly chosen fields from 4 to 12 independent cultures (i.e., cultures arising from different rat hearts). The proportion of TUNEL-positive myocytes was expressed as a percentage of the total cells counted. The oligo-nucleosomal-length DNA fragmentation was detected by means of agarose gel electrophoresis as described in Andrieu-Abadie et al. (1999) and Delpy et al. (1999).

Measurement of cAMP Concentration. Myocytes were washed twice with PBS 1 \times and stimulated for 10 min with test compounds in the presence of 0.1 M of the inhibitor of phosphodiesterase 3-isobutyl-1-methylxanthine. Cells were scraped in 250 µl of 0.01 N HCl and frozen in liquid nitrogen until use. Cell extracts were then thawed and sonicated. The lysates were separated by centrifugation (10,000g; 10 min) and cAMP was measured in the supernatant using a radioimmunoassay kit (Biotrak; Amersham Pharmacia Biotech, Piscataway, NJ).

ERK Activity. Myocytes were stimulated with test compounds for 10 min at 37°C, and then rapidly washed in ice-cold PBS and scraped into ice-cold cell lysis buffer [10 mM HEPES, 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 20 mM β -glycerophosphate, 0.1 mM dithiothreitol, 0.1 mM orthovanadate, 0.1 mM PefablocSC (Interchim, Montluçon, France), 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml pepstatin]. Then, cell debris was pelleted at 10,000g at 4°C for 20 min and protein concentrations in the supernatant were determined by Bio-Rad assay. Equal amounts of protein were gently rotated at 4°C with anti-ERK₂ immunoglobulin (Santa Cruz Biotechnology, Le Perray-en-Yvelines, France) for 1 h and then with protein A-agarose (Santa Cruz Biotechnology) for 90 min. The precipitated samples were pelleted, washed, and incubated in kinase buffer containing 20 mM HEPES, pH 7.6, 20 mM MgCl₂, 20 mM β -glycerophosphate, 20 mM *p*-sodium pyrophosphate, 0.1 mM orthovanadate, 2 mM dithiothreitol, 0.01 mM ATP, supplemented with 1 mg/ml myelin basic protein (MBP; Sigma-Aldrich), the kinase substrate, and 1 µCi of [γ -³²P]ATP. The reaction was terminated after 30 min at 30°C by adding protein loading buffer. The samples were heated at 95°C for 5 min, separated by 12% SDS-polyacrylamide gel electrophoresis, electrically transferred to nitrocellulose filters, and visualized by autoradiography. Incorporated [γ -³²P]ATP in the substrate was quantified by radioanalytic scanning of the film (National Institutes of Health software). All assays were performed three to six times.

Statistical Analysis. Data are expressed as means \pm S.E.M. For each set of experiments, differences between the various conditions tested were identified by using one-way ANOVA. When the ANOVA revealed a significant difference, group-to-group comparisons were performed using the *t* test for multiple comparisons. Differences were considered significant when *P* values were below .05.

Results

Serum Protects Adult Ventricular Myocytes against ST-Induced Apoptosis. After 48 h of culture in the absence of ST, myocytes exhibited a low basal percentage of nuclear TUNEL labeling ($8.74 \pm 0.38\%$), which was not modified by the addition of serum ($8.72 \pm 0.39\%$; $n = 12$; NS versus control) (Fig. 1D). In the absence of serum, exposure of myocytes to 10 µM ST for 18 h caused a massive increase in the percentage of labeled nuclei, to $39.25 \pm 0.64\%$ after 48 h of culture ($n = 12$, $P < .001$ versus control). Figure 1 shows a typical example of ST-treated cells after 48 h of culture. The cell showing an intense TUNEL nuclear labeling (Fig. 1C) was identified as an adult ventricular myocyte based upon its rod-shaped morphology (Fig. 1A) and positivity of the immunostaining with antibodies directed against sarcomeric α -actinin (Fig. 1B). It also showed typical shrinkage consistent with an apoptotic process (Andrieu-Abadie et al., 1999; Delpy et al., 1999; Rücker-Martin et al., 1999) contrasting with the normal morphology of surrounding TUNEL-negative myocytes. When ST-treated myocytes were incubated with serum, the percentage of TUNEL-positive nuclei was markedly reduced, at $20.89 \pm 0.62\%$ ($n = 12$, $P < .001$ versus control). Apoptosis in ST-treated cultures was confirmed by the detection of an oligo-nucleosomal-length DNA fragmentation by

means of agarose gel electrophoresis (Fig. 1E). Taken together, these results indicated that ST-induced apoptosis of adult rat ventricular myocytes can be reduced by the addition of serum.

Dual Effects of Epinephrine on Adult Rat Ventricular Myocyte Survival. The effects of epinephrine on myocyte survival were complex (Fig. 2A). In control conditions, 0.01 and 0.1 μM epinephrine failed to induce apoptosis ($9.8 \pm 0.50\%$, $n = 5$ and $8.62 \pm 0.46\%$, $n = 12$; NS versus control). At 1 μM , only $12.74 \pm 0.64\%$ TUNEL-positive myocytes were observed ($n = 5$, $P < .01$ versus control). The percentage rose abruptly to $42.30 \pm 1.42\%$ with 10 μM to plateau at $47.56 \pm 1.37\%$ with 100 μM ($n = 5$, $P < .05$ versus 10 μM).

More interesting was the inhibition of ST-induced apoptosis by epinephrine. In fact, the dose-response curve was J-shaped, with a maximal inhibition being observed with 0.1 μM (23.86 ± 0.76 versus $39.25 \pm 0.64\%$, $n = 12$, $P < .001$). Addition of 10 μM epinephrine to ST showed a cumulative effect (60.12 ± 1.24 versus $39.25 \pm 0.64\%$ or $42.30 \pm 1.42\%$ TUNEL-positive myocytes with ST only and 10 μM epinephrine only, respectively, $n = 5$, $P < .001$ for the two comparisons). This effect increased further with the addition of 100 μM epinephrine to ST. In good agreement with the decreased percentage of TUNEL-positive myocytes, the ST-induced DNA fragmentation appeared to be reduced when cultures were treated with 0.1 μM epinephrine, whereas 10 μM epinephrine was associated with a marked DNA laddering (Fig. 2B). To evaluate whether the opposite effects of high and low

concentrations of epinephrine were tightly related to differences in cellular cAMP concentration, we measured the latter in myocytes exposed to 0.1 and 10 μM epinephrine. Increasing epinephrine concentration from 0.1 to 10 μM raised cAMP concentration from 8.08 ± 0.05 to 10.17 ± 0.14 fmol/ μg of protein ($n = 4$, $P < .01$). Taken together, these results indicated that although 10 μM epinephrine induced adult ventricular myocyte apoptosis, 0.1 μM epinephrine, like serum, was able to modulate the proapoptotic effect that ST exerts on these cells.

Protective Effect of Epinephrine Requires Tyrosine Phosphorylation and ERK Activation. We tested the involvement of tyrosine kinases in the protective effect of 0.1 μM epinephrine by using the tyrosine kinase inhibitor genistein at the concentration of 50 μM that has been previously shown to exhibit specific tyrosine kinase inhibitory effects (Akiyama et al., 1987; Boixel et al., 2000). In control conditions, 50 μM genistein showed no proapoptotic effect on cardiac myocytes (10.03 ± 0.71 versus $8.74 \pm 0.38\%$ TUNEL-positive myocytes, $n = 5$, NS). Myocyte pretreatment with 50 μM genistein totally suppressed the protective effect of epinephrine (41.12 ± 0.85 versus $23.86 \pm 0.76\%$ TUNEL-positive myocytes with and without genistein, respectively, $n = 12$, $P < .001$) or serum (43.24 ± 1.52 versus $20.89 \pm 0.62\%$ TUNEL-positive myocytes with and without genistein, respectively, $n = 6$, $P < .001$) (Fig. 3A). This was confirmed by increased DNA laddering on DNA agarose gels (Fig. 3B), and

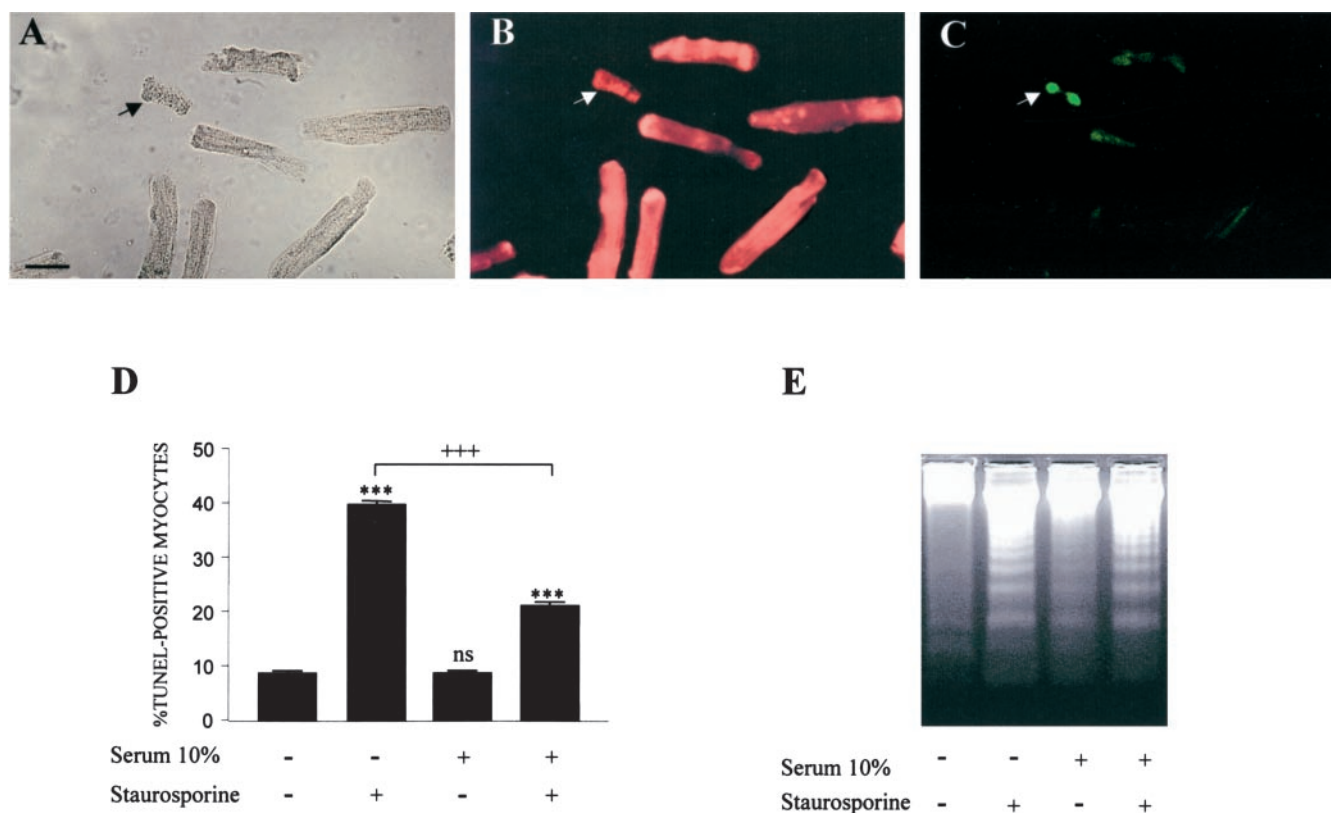


Fig. 1. Serum reduced ST-induced apoptosis of adult rat ventricular myocytes by ~50%. A, most myocytes exhibit the rod shape typical of adult rat ventricular myocytes; the sole apoptotic myocyte indicated by the arrow appears shrunken. B, immunostaining of myocytes with antibodies directed against sarcomeric α -actinin. C, apoptosis of the shrunken myocyte is confirmed by dense TUNEL nuclear labeling. Magnification, 400 \times . Scale bar, 25 μm . D, mean percentage of TUNEL-positive myocytes cultured in the absence or presence of serum, and/or 10 μM ST. *** $P < .001$; NS, not significant versus control. E, serum reduced internucleosomal DNA cleavage in ST-treated myocytes. Ladders shown are representative of two independent experiments.

indicated that epinephrine, like serum, exerted its protective effect through a tyrosine kinase-dependent mechanism.

We examined ERK activation upon its ability to phosphorylate MBP. Figure 4A shows a representative autoradiograph of MBP phosphorylation in which ERK immunoprecipitation was checked by probing the membrane with an anti-ERK antibody. Mean kinase activity is shown as a bar graph in Fig. 4B after densitometric quantification of MBP signals. In control conditions, MBP phosphorylation was weak [$5,067 \pm 2,717$ arbitrary units (au), $n = 6$], suggesting weak ERK activation. Epinephrine ($0.1 \mu\text{M}$), like serum, markedly increased MBP phosphorylation ($60,600 \pm 6,851$ and $72,060 \pm 2,156$ au, respectively, $n = 3$, $P < .001$ versus control). This effect was slightly attenuated in ST-treated cultures ($43,700 \pm 4,809$ and $58,700 \pm 3,153$ au, $n = 3$) although values remained markedly higher than in control

cultures treated with ST ($3,598 \pm 1,431$ au, $n = 6$, $P < .001$ for the two comparisons). In sharp contrast, $10 \mu\text{M}$ epinephrine failed to activate ERK ($5,243 \pm 3,221$ au, $n = 3$, NS versus control) (Fig. 4). Importantly, ERK activation by epinephrine and serum was abolished by genistein ($5,252 \pm 3,165$ and $2,783 \pm 1,880$ au, $n = 3$, $P < .001$). Altogether, these results suggested that the protective effect of epinephrine and serum on ST-induced apoptosis of adult cardiac myocytes could involve tyrosine-kinase-mediated ERK activation.

Protective Effect of Epinephrine Involves the β -Adrenergic Pathway. The α - and β -adrenoceptor antagonists prazosin ($10 \mu\text{M}$) and propranolol ($10 \mu\text{M}$) were used to determine whether the protective effect of epinephrine was mediated by an α - or β -adrenoceptor signaling pathway. As shown in Fig. 5A, propranolol totally suppressed the protec-

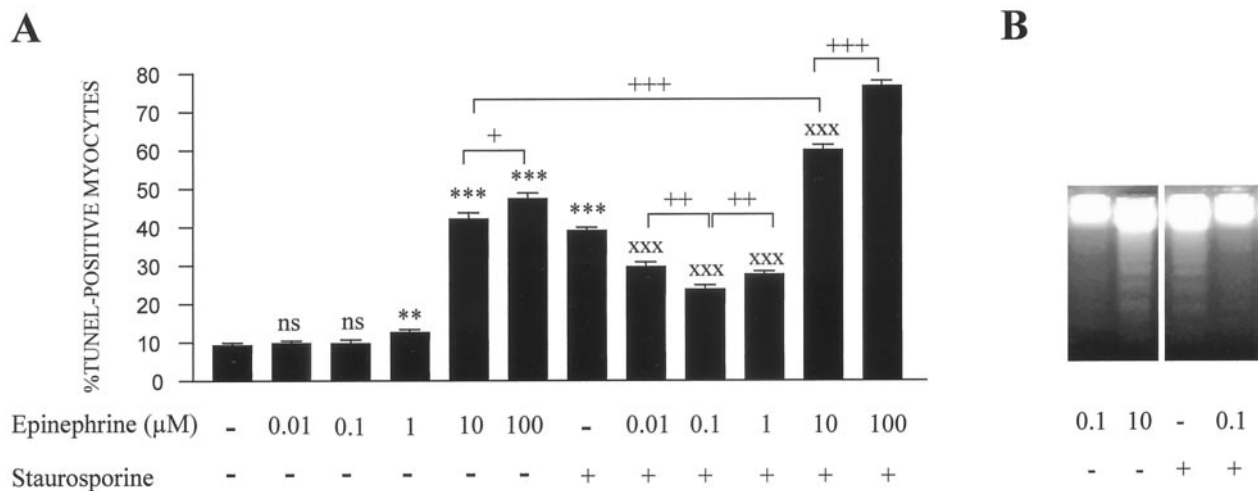


Fig. 2. Concentration-dependent effects of epinephrine upon myocyte survival. A, mean percentage of TUNEL-positive myocytes cultured in the presence or absence of epinephrine (0.01 to $100 \mu\text{M}$) and/or ST. *** $P < .001$; NS, not significant versus control. XXX $P < .001$ versus ST-induced apoptosis. B, $0.1 \mu\text{M}$ epinephrine reduced internucleosomal DNA cleavage from ST-treated myocytes, whereas $10 \mu\text{M}$ epinephrine induced DNA ladders. Ladders shown are representative of two independent experiments.

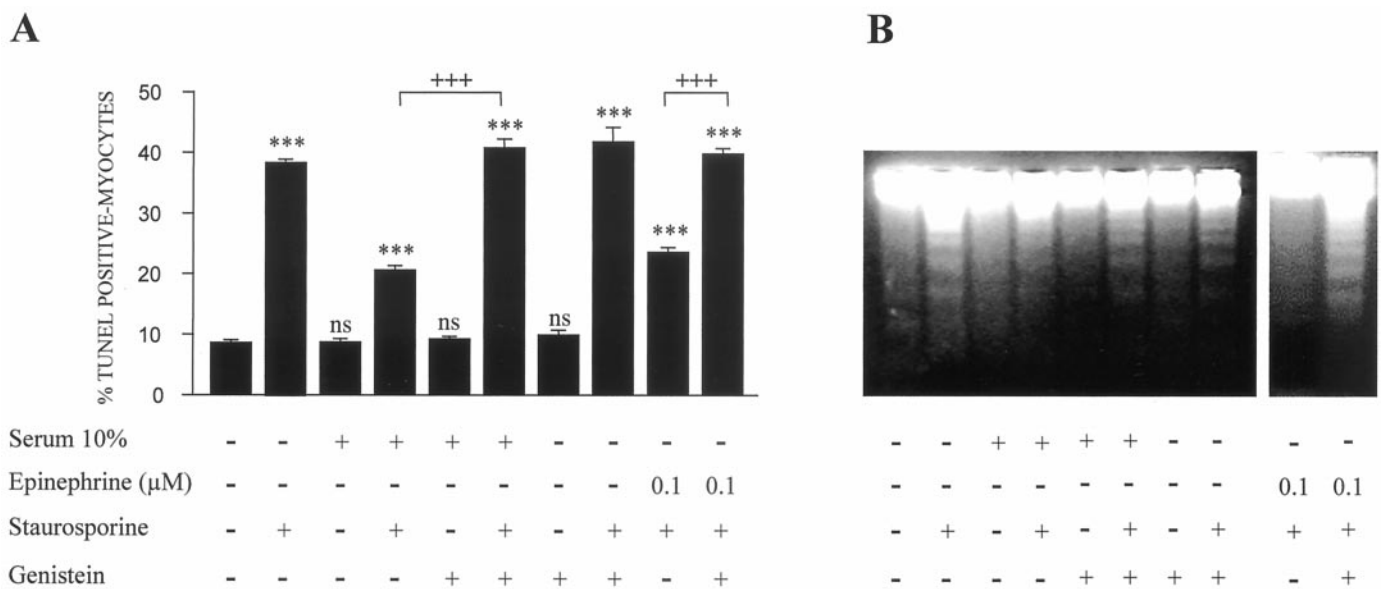


Fig. 3. Tyrosine-kinase inhibition by $50 \mu\text{M}$ genistein blunted the protective effects of $0.1 \mu\text{M}$ epinephrine and serum against ST-induced myocyte apoptosis. A, mean percentage of TUNEL-positive myocytes in various conditions. *** $P < .001$; NS, not significant versus control myocytes. B, internucleosomal DNA cleavage in the various conditions. Genistein blunted the protective effect of $0.1 \mu\text{M}$ epinephrine and serum. Ladders shown are representative of two independent experiments.

tive effect of epinephrine on ST-induced myocyte apoptosis (40.47 ± 0.77 versus $26.37 \pm 0.89\%$ TUNEL-positive myocytes, $n = 8$, $P < .001$), whereas prazosin had no effect (25.39 ± 0.83 versus $26.37 \pm 0.89\%$, $n = 8$, NS), pointing to β -adrenoceptor involvement in this protective effect. Moreover, in contrast to epinephrine, the α -adrenergic agonist phenylephrine ($10 \mu\text{M}$) failed to protect myocytes from ST-induced apoptosis (38.29 ± 0.61 versus $26.37 \pm 0.89\%$ TUNEL-positive myocytes, $n = 4$, $P < .001$) (Fig. 5B).

The β -adrenergic agonist isoproterenol ($1 \mu\text{M}$) mimicked the protective effect of epinephrine (25.89 ± 0.65 versus $26.37 \pm 0.89\%$ TUNEL-positive myocytes, $n = 4$, NS), whereas the addition of phenylephrine to isoproterenol showed no additive effect on isoproterenol protective effect (25.29 ± 1.10 versus $25.60 \pm 0.63\%$ TUNEL-positive myocytes, $n = 5$, NS). Overnight preincubation of myocytes with PTX, the G_i -protein inhibitor, did not blunt the protective effect of $0.1 \mu\text{M}$ epinephrine on ST-induced myocyte apoptosis. Moreover, $1 \mu\text{M}$ forskolin, which directly activates the adenylyl cyclase, also reduced the proportion of TUNEL-positive myocytes in cultures treated with ST (26.18 ± 0.84 versus $40.69 \pm 1.86\%$ TUNEL-positive myocytes, $n = 4$, $P < .004$), thus reproducing the protective effect of epinephrine. In sharp contrast, $10 \mu\text{M}$ forskolin markedly increased the proportion of TUNEL-positive myocytes (46.49 ± 1.25 versus $9.45 \pm 0.48\%$ TUNEL-positive myocytes in the presence of $1 \mu\text{M}$ forskolin, $n = 5$, $P < .001$).

Importantly, the effect of the various compounds on myo-

cyte survival correlated with ERK activation (Fig. 5C). Isoproterenol increased MBP phosphorylation by ERK, whereas culture pretreatment with propranolol ($10 \mu\text{M}$) totally suppressed epinephrine-induced ERK activation. Moreover, $1 \mu\text{M}$ forskolin also increased MBP phosphorylation by ERK (data not shown). Taken together, these results indicated that activation of a cAMP-dependent pathway was involved in epinephrine-induced ERK activation and in the prevention of ST-induced apoptosis of adult myocytes by epinephrine.

Discussion

This study shows for the first time that although $10 \mu\text{M}$ epinephrine as $10 \mu\text{M}$ norepinephrine (Communal et al., 1998, 1999) induces apoptosis of adult rat ventricular myocytes, a low epinephrine concentration ($0.1 \mu\text{M}$) markedly attenuates staurosporine-induced apoptosis of these cells through a pathway involving ERK activation, thereby mimicking the protective effect of serum. This protective effect, independent of G_i , involved coupling between the cAMP signaling pathway and ERK activation, whereas $10 \mu\text{M}$ epinephrine, which triggered cardiac myocyte apoptosis, failed to activate ERK.

ERK is a point of convergence for a number of growth signals (Sadoshima et al., 1995) that modulate the survival of various cell types (Xia et al., 1995), including neonatal cardiac myocytes (Sheng et al., 1997). Our results indicate that ERK is also involved in protecting adult cardiac myocytes

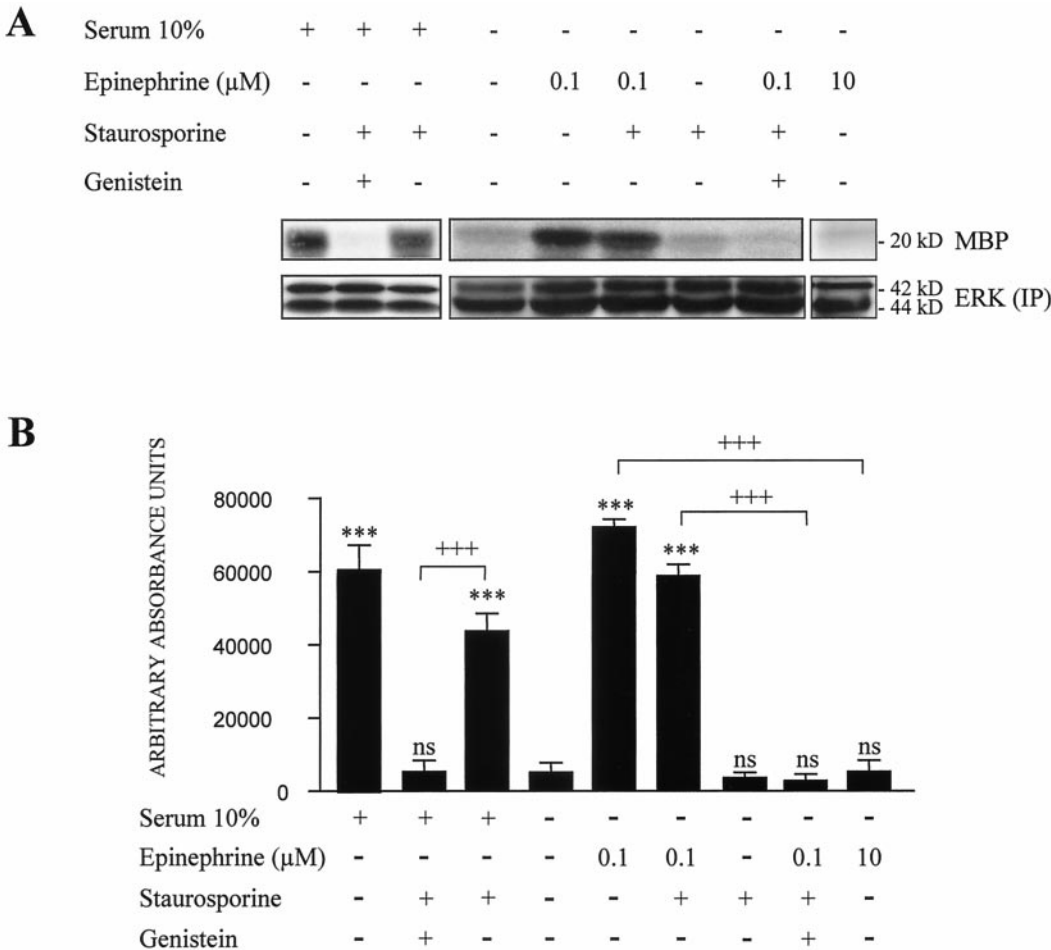


Fig. 4. Tyrosine kinase-dependent pathway underlying the protective effect of epinephrine and serum required ERK activation. A, autoradiograph of MBP phosphorylation representative of three to six independent experiments. The membrane was probed with the anti-ERK2 immunoglobulin as a loading control. B, autoradiographs were scanned using a laser densitometer and the results are expressed as mean \pm S.E.M. of arbitrary absorbance units. *** $P < .001$, ** $P < .01$, * $P < .05$; NS, not significant versus control.

from apoptosis. However, serum privation or myocyte treatment with the tyrosine kinase inhibitor genistein (Akiyama et al., 1987; Boixel et al., 2000) failed to induce apoptosis of the adult myocytes studied here, in contrast to fibroblasts (Kulkarni and McCulloch, 1994), neurons (Ferrari et al., 1993), and neonatal cardiac myocytes (Sheng et al., 1997). This is in keeping with our observation that ERK activation is absent in myocytes grown in the absence of serum or epinephrine. However, when myocytes were committed to die after staurosporine exposure, the protection exerted by serum or epinephrine indicated that the cells became highly sensitive to growth stimuli and ERK activation. This is reminiscent of the situation in cardiotrophin-1 receptor gene knockout mice, which do not develop cardiomyopathy in the absence of exogenous ventricular stress (Hirota et al., 1999). In contrast, massive myocyte apoptosis is observed when pressure overload, a powerful inducer of apoptosis (Teiger et al., 1996), is imposed on the ventricle of these mice (Hirota et al., 1999). The slight inhibition of serum- and epinephrine-induced ERK activation by staurosporine may be because, at 10 μ M, this compound is a nonspecific protein kinase inhibitor, which may interact with the signaling pathways leading to ERK activation. Taken together, these results suggest

that, in normal circumstances, trophic signals such as serum and epinephrine have a limited role in the survival of adult ventricular myocytes, probably because the cells are terminally differentiated, whereas they become powerful survival mechanisms when the cells are submitted to unusual stress.

Another important finding in this study is that the catecholamine epinephrine, at a concentration of 0.1 μ M, protected adult cardiac myocytes against apoptosis induced by the powerful nonspecific apoptosis inducer staurosporine. It is well known that catecholamines stimulate the growth of neonatal (Simpson et al., 1982; Bogoyevitch et al., 1996) and adult (Pinson et al., 1993) cardiac myocytes. Recently, both norepinephrine and isoproterenol were also found to protect brown adipocytes (Lindquist and Rehnmark, 1998), hepatocytes (Zhang et al., 1996), glioma cells (Canova et al., 1997), and neonatal cardiac myocytes (Wu et al., 1997) against apoptotic death. In the latter study, 1 μ M norepinephrine prevented neonatal cardiac myocytes from atrial natriuretic peptide-induced apoptosis through the activation of β -adrenoceptors and an increase in the intracellular cAMP concentration (Wu et al., 1997) but the precise molecular targets of cyclic nucleotide-modulated cell fate decision remained unknown. We found that in adult cardiac myocytes 0.1 μ M

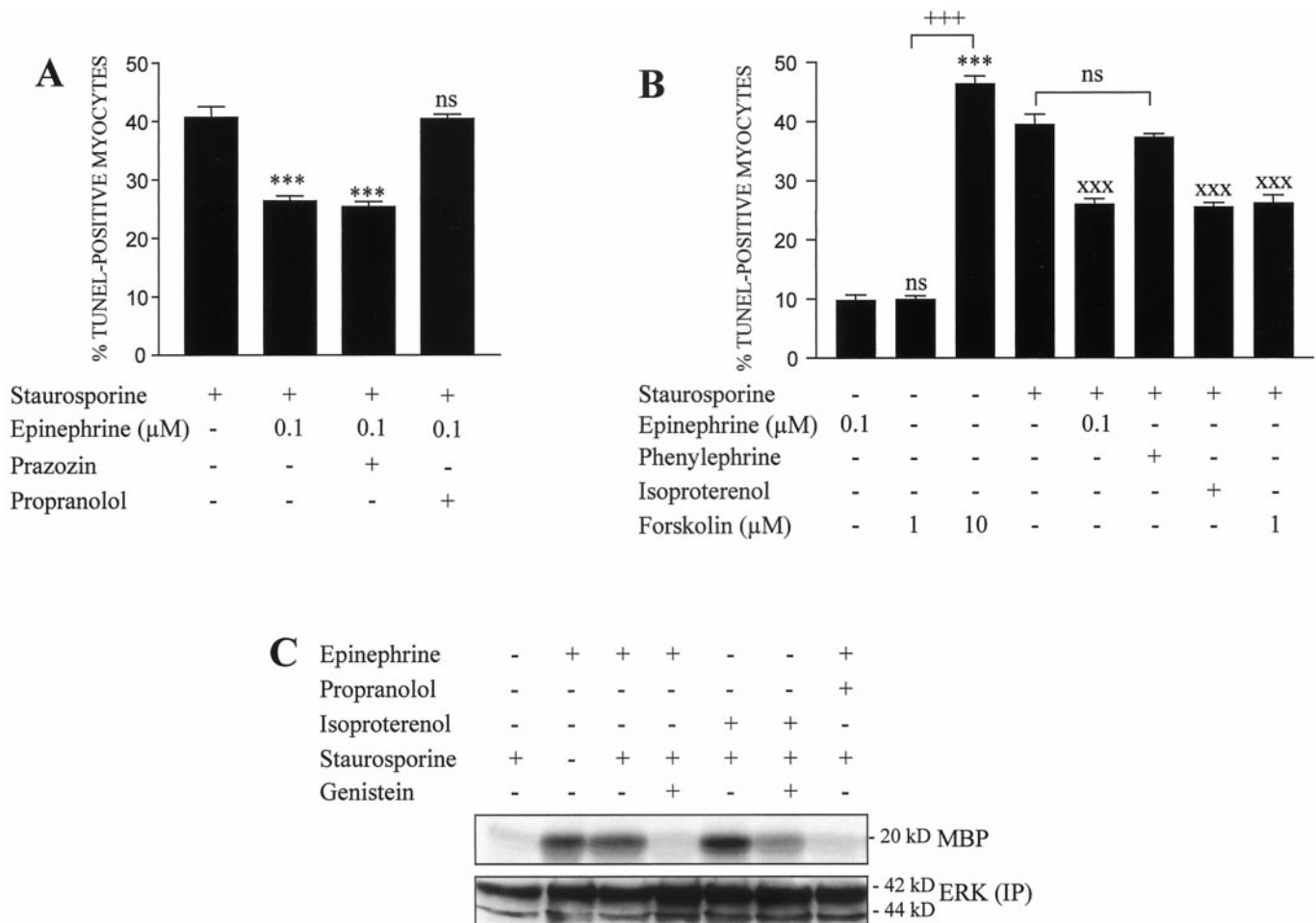


Fig. 5. β -Adrenergic pathway was involved in the protective effect of epinephrine. A, effects of the α - and β -adrenoceptor antagonists prazosin (10 μ M) and propranolol (10 μ M), respectively, on the mean percentage of TUNEL-positive myocytes in the presence of 0.1 μ M epinephrine. B, mean percentage of TUNEL-positive myocytes when epinephrine was replaced by the α - or β -adrenergic agonist phenylephrine (10 μ M) or isoproterenol (1 μ M), respectively, or by the adenylyl cyclase activator forskolin (1 or 10 μ M) in cultures treated with ST. $^{xx}P < .01$, $^{xxx}P < .001$; NS, not significant versus ST-treated control myocytes, $n = 6$. C, autoradiograph of MBP phosphorylation representative of two independent experiments. The membrane was probed with the anti-ERK2 antibody as a loading control.

epinephrine also had an antiapoptotic effect mediated by a cAMP-dependent signaling pathway. Moreover, this cAMP-dependent protective signal appeared to be coupled to ERK activation, a feature generally associated with cell survival. Such coupling has been observed previously in the case of neonatal rat ventricular myocyte growth following α_1 - and β -adrenoceptor stimulation (Yamazaki et al., 1997). In this latter study, α_1 - and β -adrenoceptors were shown to act synergistically through PKC and PKA, respectively, to activate *raf*-1 kinase/ERK cascade-dependent myocyte hypertrophy. More recently, Zou et al. (1999) showed that isoproterenol induces β -adrenoceptor phosphorylation through Gs/cAMP-dependent PKA activation, causing a switch in receptor coupling from G_s to G_i and leading to Src family tyrosine kinase activation and *raf*-1 kinase/ERK cascade-dependent myocyte hypertrophy. Interestingly, Communal et al. (1999) showed that apoptosis of adult rat ventricular myocytes induced by 10 μ M norepinephrine was increased when cells were preincubated with prazosin and PTX. Our results clearly show that G_i proteins are not involved in the protective effect of epinephrine in our experimental conditions.

The potentially deleterious effects of catecholamines are well known. In particular, their ability to induce apoptosis has been observed in PC12 cells (Burke et al., 1997), neuronal cells (Zilkha-Falb et al., 1997), and neonatal (Iwai-Kanai et al., 1999) and adult (Communal et al., 1998) cardiac myocytes. Very high catecholamine concentrations (100 μ M isoproterenol and 10 μ M norepinephrine, respectively) were used in the latter two studies. We also found that high catecholamine concentrations induced adult cardiomyocyte apoptosis. A number of mechanisms could account for the proapoptotic effect of such high catecholamine concentrations, ranging from excessive β -adrenergic pathway stimulation to non- β -adrenoceptor-specific apoptotic stimulation (e.g., accumulation of proapoptotic catecholamine metabolites) (Burke et al., 1997). Regarding the former possibility, Communal et al. (1998) showed that 10 μ M norepinephrine stimulated apoptosis of adult rat ventricular myocytes through a PKA-mediated mechanism that required calcium entry via L-type channels. The proapoptotic effect of excessive β -adrenergic stimulation is also consistent with a recent study indicating that isoproterenol (5 to 20 μ M) promotes apoptosis in concentration-dependent manner in *Gas*-overexpressing myocytes (which do not develop desensitization to catecholamines) but not in wild-type control myocytes (Geng et al., 1999).

In our experiments, a slight although significant increase in cAMP concentration was observed between the antiapoptotic low concentration and the proapoptotic high concentration of epinephrine. Interestingly, 10 μ M epinephrine failed to activate ERK, clearly showing a concentration-dependent effect of catecholamines on protein kinase cascades. Therefore, it is possible a delicate balance in the levels of cAMP determines pro- or antiapoptotic pathways in adult rat ventricular myocytes. Alternatively, high catecholamine concentrations may activate an intracellular pathway preventing ERK activation despite the rise in intracellular cAMP concentration. Together, the studies of Communal et al. (1998, 1999) and ours are consistent with the hypothesis that depending on the concentration used, catecholamines are coupled to distinct signaling pathways resulting in opposite effects on myocyte survival. Similarly, stimulation of α_1 -

adrenoceptors, which are coupled to $G_{\alpha q}$ (Dorn and Brown, 1999), provokes either hypertrophy or apoptosis depending on its degree.

Cardiac β -adrenoceptors are essential regulators of cardiac function. During heart failure, a number of alterations occur in the β -adrenergic signaling pathway, such as 1) increases in plasma catecholamine concentrations; 2) reduction in the myocardial catecholamine concentrations in failing ventricles (Bohm, 1995; Espinasse et al., 1999); 3) decreases in cardiac myocyte β -adrenoceptor density and responsiveness (Bristow et al., 1982; Bohm, 1995) mediated through enhanced β -adrenergic receptor kinase 1 expression (White et al., 2000); and 4) decreases in the cAMP concentration in myocytes from failing ventricles (Bristow et al., 1982; Sethi et al., 1997). A number of β -blocking drugs reduce mortality and morbidity in human heart failure (Anonymous, 1994; Packer et al., 1996), an effect that might conceivably result from protection against catecholamine-induced cardiac myocyte apoptosis (Communal et al., 1998, 1999). However, β -blocking drugs can restore, at least in part, β -adrenoceptor density and function and intracellular cAMP signaling (Bohm et al., 1997), in part probably through their ability to reduce the increased expression of β -adrenergic receptor kinase 1, a reduction that has been suggested recently to preserve normal β -adrenergic receptor-G protein coupling (White et al., 2000). Moreover, in the latter study, such a preservation of the β -adrenergic signaling pathway delays the development of heart failure after myocardial infarction. It is possible that part of this beneficial effect results from the antiapoptotic effect of a preserved β -adrenergic signaling pathway. Taken together, these and our findings suggest that although β -blocking agents protect from adrenergic attacks of heart failure and the resulting myocyte apoptosis (Communal et al., 1998; Yue et al., 1998), they simultaneously restore minimal β -adrenergic and cAMP myocyte tone, thereby favoring myocyte survival.

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