Cardiac myocyte hypertrophy is associated with c-myc protooncogene expression

(catecholamines $/ \alpha$ receptor)

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Communicated by Richard J. Havel, July 9, 1986

The mechanism of hormonally induced cell ABSTRACT hypertrophy is unknown. Stimulation of cardiac myocytes by α_1 -adrenergic agents, phorbol esters, and serum induces an increase in the cell size of nondividing cardiac myocytes in primary culture. Expression of the c-myc gene, known to be increased in growth factor-induced cell division, was studied in this model of cell hypertrophy. The α -adrenergic agonist norepinephrine (0.002–20 μ M) increased levels of c-mycencoded mRNA to 10-fold over control levels. This increase was detectable at 30 min, peaked at 2 hr, and returned to baseline by 6 hr after stimulation. The norepinephrine response was abolished by the α_1 -antagonist terazosin (2 μ M) but was not affected by the β -adrenergic antagonist propranolol (2 μ M) and was only slightly (25%) attenuated by the α_2 -adrenergic antagonist yohimbine (2 μ M). Serum and the phorbol ester tumor promoter phorbol 12-myristate 13-acetate also enhanced c-myc expression in cardiac myocyte cultures. These findings show that the induction of cardiac myocyte hypertrophy is associated with enhanced expression of the c-myc gene and suggest that hormonally induced cell hypertrophy and cell division share common mechanistic pathways.

Tissues grow by two processes: an increase in cell size (hypertrophy) and an increase in cell number (proliferation). Recent studies have correlated growth factor-induced proliferation with increases in the expression of specific genes (1-3). In this respect the protooncogene c-myc has been of interest, since a high level of c-myc expression has been associated with both growth factor stimulation and cell transformation (4-10). Biochemical pathways implicated in cell proliferation might also be involved in cellular hypertrophy. For this reason we studied the expression of the c-myc gene in cardiac myocytes induced to hypertrophy by exposure to α_1 -adrenergic agonists, serum, or phorbol esters.

MATERIALS AND METHODS

Cell Culture and Incubations. Primary cultures of 90% pure cardiac myocytes were prepared from ventricles of day-old rats by gentle trypsinization at room temperature as described (11–13). Cells were maintained for 3 days in serum-free medium 199 supplemented with insulin (10 μ g/ml) and transferrin (10 μ g/ml). Bromodeoxyuridine (0.1 mM) prevented nonmyocardial cell proliferation. After 3 days in culture, cells in 7 ml of medium were treated for 2 hr with 140 μ l of vehicle (100 μ M ascorbic acid in 0.2 μ M HCl) or with agonist suspended in 140 μ l of vehicle.

RNA Preparation. Cells were lysed *in situ* with cold guanidinium thiocyanate. Total RNA was prepared by cen-

trifugation of the guanidinium thiocyanate lysate over a cesium chloride cushion, phenol extraction, and ethanol precipitation (14, 15). RNA was quantitated by measuring absorbance at 260 nm.

Preparation of SP6 c-myc Probe. To prepare a vector that would allow synthesis of a single-stranded probe complementary to rat c-myc encoded-mRNA, the *Bam*HI-Sac I fragment of the rat c-myc gene exon I was subcloned in the plasmid SP65. The vector was linearized with *Bam*HI before use. SP65-directed synthesis of radiolabeled RNA probe utilized [α -³²P]UTP (Amersham) and SP6 RNA polymerase (New England Nuclear) as previously described (16, 17). The product of the reaction was electrophoresed in an 8 M urea/4% polyacrylamide gel, and the full-length probe was eluted from the gel.

Analysis of Rat c-myc Transcripts. Ten micrograms of total cellular RNA was hybridized to an excess $(1 \times 10^6 \text{ cpm})$ of RNA probe complementary to rat c-myc exon 1 in 10 μ l of 75% (vol/vol) formamide/0.1% NaDodSO₄/20 mM Tris HCl, pH 7.0/1 mM EDTA at 52°C for 12-16 hr. RNase T1 (200 units) and RNase A (1.5 μ g) in 150 μ l of 300 mM NaCl/10 mM Tris·HCl, pH 7.5/5 mM EDTA was then added for 1 hr at room temperature, followed by the addition of 10 μ l of 10% NaDodSO₄ and 25 μ l of a 20-mg/ml solution of proteinase K for 20 min at 37°C. The nucleic acids were precipitated by the addition of ethanol to 70% volume and redissolved in formamide loading buffer of 95% formamide/10 mM Tris·HCl/1 mM EDTA with bromphenol blue and xylene cyanol. Samples were then electrophoresed on 8 M urea/4% polyacrylamide gels and visualized by autoradiography. Gel areas corresponding to autoradiographic bands were excised and their ³²P content quantitated by scintillation counting. RNase digestion of the probe RNA (rat c-myc exon 1) hybridized to rat cardiac myocyte RNA yielded protected fragments that migrated as if they were 400 and 310 nucleotides long, the sizes predicted for transcripts using the first or second c-myc promoters (18).

RESULTS

When exposed to α -adrenergic agonists, phorbol esters, or calf serum, cardiac myocytes from neonatal rats in primary culture increase in size and protein content but do not divide or replicate their DNA (11-13). The level of c-myc mRNA was measured in cardiac myocytes after incubation with these hypertrophy-inducing agents (Fig. 1A). A 5- to 10-fold increase in c-myc expression was seen after treatment of cells with phorbol esters, serum, and norepinephrine (Fig. 1A).

Norepinephrine increased c-myc mRNA levels in a concentration-dependent manner (Fig. 1B). Stimulation was detectable with 0.002 μ M norepinephrine and was half maximal at 0.9 μ M norepinephrine. The maximal level of c-myc mRNA stimulated by norepinephrine (2–20 μ M) was 5to 10-fold above the control level (Fig. 1B). Levels of c-myc

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FIG. 1. Norepinephrine, serum, and phorbol esters increase c-myc mRNA in neonatal cardiac myocytes. (A) Autoradiogram of 8 M urea/4% polyacrylamide gel showing stimulation of c-myc mRNA. Cells were treated with vehicle of 100 μ M ascorbic acid in 0.2 μ M hydrochloric acid, 2 μ M norepinephrine (NE), 10% calf serum or 100 nM phorbol 12-myristate 13-acetate (PMA) for 2 hr at 37°C and assayed for c-myc mRNA. Agonist concentrations are final concentrations in culture media. (B) Concentration dependence of norepinephrine-induced c-myc expression. Cells were treated for 2 hr with various concentrations of norepinephrine, and c-myc mRNA levels were determined. Gel areas corresponding to 400-base bands were excised, and radioactivity was determined by scintillation counting. Values shown represent the mean \pm SEM (n = 5) expressed as a percentage of maximal response. Maximal stimulation was 10-fold the basal c-myc expression. Norepinephrine was toxic at 2000 μ M (n = 1).

mRNA increased above control 30 min after the addition of norepinephrine (Fig. 2) and reached a maximum within 1 to 2 hr. By 6 hr after stimulation c-myc mRNA had returned to the control level (Fig. 2). Total RNA as measured by A_{260} was not increased above control levels in cells stimulated for 2 hr with 2 μ M norepinephrine.

The increased expression of c-myc mRNA stimulated by norepinephrine had the specificity of an α_1 -adrenergic response in that it was abolished by the α_1 -antagonist terazosin (2 μ M). The α_2 -antagonist yohimbine (2 μ M) attenuated the response by only 25%, and the β -adrenergic antagonist propranolol (2 μ M) had no effect (Fig. 3). In addition, stimulation with the α -specific agonist methoxamine (2 μ M) increased c-myc mRNA levels 3- to 4-fold above control, whereas isoproterenol had no effect on c-myc mRNA levels (data not shown). The cholinergic agent carbachol had no



FIG. 2. Time course of c-myc mRNA expression after stimulation by norepinephrine. Neonatal cardiac myocyte cultures were treated with vehicle (V) or norepinephrine (NE; 2 μ M final) for the indicated times and assayed for c-myc mRNA. An autoradiogram of an 8 M urea/4% polyacrylamide gel is shown.

effect on c-myc expression and did not induce hypertrophy in cardiac myocytes.

It is unlikely that fibroblast contamination accounts for the c-myc response. Platelet-derived growth factor, an agent known to induce c-myc expression and mitogenesis in fibro-



FIG. 3. α_1 -adrenergic specificity of the stimulation of c-myc expression by norepinephrine. Neonatal cardiac myocyte cultures treated for 2 hr with vehicle (Control, lane a) or 2 μ M norepinephrine (NE, lanes b-e) were assayed for c-myc mRNA as described in Fig. 1. The antagonists propranolol (P, a β -blocker), yohimbine (Y, an α_2 -blocker), and terazosin (T, an α_1 -blocker) were added to the cells at a final concentration of 2 μ M 2 hr before the norepinephrine addition (lanes c, d, and e respectively).

blasts, did not increase c-myc mRNA levels or stimulate hypertrophy in cardiac myocyte cultures. Pure cardiac fibroblast cultures prepared as previously described (11–13) and stimulated with norepinephrine showed no increase in c-myc expression.

DISCUSSION

Serum, phorbol esters, and norepinephrine-agents that induce hypertrophy in cultured cardiac myocytes (11-13)also stimulate expression of the c-myc protooncogene in these cells. Norepinephrine-stimulated c-myc expression, like norepinephrine-induced cellular hypertrophy, is mediated by the α_1 -adrenergic receptor. The mechanism by which the α_1 -adrenergic receptor induces cardiac myocyte hypertrophy and enhances c-myc expression is not known. Stimulation of α_1 -adrenergic receptors results in the accelerated metabolism of membrane phosphoinositides and subsequent production of inositol phosphates and diacylglycerol in both smooth muscle and cardiac myocytes (19-21). We have recently shown that several agonists whose receptors are coupled to phosphoinositide turnover also increase c-myc mRNA levels in fibroblasts, possibly by activation of protein kinase C (16). The α_1 -adrenergic receptor may thus increase c-myc mRNA levels in cardiac myocytes via the phosphoinositide/protein kinase C pathway. In this respect it is noteworthy that phorbol 12-myristate 13-acetate, a direct activator of protein kinase C, also increases c-myc mRNA levels and induces hypertrophy in these cells (Fig. 1A).

Further work will be necessary to determine whether the stimulation of c-myc expression in cardiac myocytes occurs by increasing transcription or by altering message stability. The latter mechanism seems possible in the context of recent data showing that the c-myc gene in fibroblasts may be regulated post-transcriptionally through control of RNA degradation (22-24).

The induction of c-myc expression in cultured cardiac myocytes is rapid and short-lived. However, the hypertrophic response to norepinephrine occurs much later (12-24 hr) and requires the continuous exposure of cells to the catecholamine (11-13). It is likely that the hypertrophic response involves other intracellular pathways in addition to c-myc expression. Thus c-myc expression may be necessary but not sufficient for cellular hypertrophy. This appears to be the case for the mitogenic response associated with short-lived c-myc induction in fibroblasts (16).

Increased expression of the c-myc gene has been associated with cell proliferation in several settings but has not previously been linked to cellular hypertrophy. Our data show that hypertrophy of nondividing neonatal cardiac myocytes in response to α_1 -adrenergic agonists, phorbol ester, or serum is preceded by increased expression of the protooncogene c-myc and suggest a role for the c-myc gene product in processes common to both hypertrophy and cell division. We thank Dr. D. Steffen (Worcester Foundation for Experimental Biology, Worcester, MA) for the *c-myc* clone, Tanny Tsao for her excellent assistance in preparing cells, and Betty L. Cheung for her help and typing of this manuscript. This work was supported by National Institutes of Health National Research Service Award Heart and Vascular Diseases Grant HLO 7192, American Heart Association Grant-in-Aid 831107, National Institutes of Health Grants HL 32898-02, HL 31113, and HL 35561, the American Heart Association California Affiliate, and the Veterans Administration Research Service. P.C.S. is a Clinical Investigator of the Veterans Administration Hospital.

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