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Research Article

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Induction of the Skeletal α -Actin Gene in α_1 -Adrenoceptor-mediated Hypertrophy of Rat Cardiac Myocytes

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Abstract

Myocardial hypertrophy in vivo is associated with reexpression of contractile protein isogenes characteristic of fetal and neonatal development. The molecular signals for hypertrophy and isogene switching are unknown. We studied α (sarcomeric)-actin messenger RNA (mRNA) expression in cultured cardiac myocytes from the neonatal rat. In the cultured cells, as in the adult heart in vivo, expression of cardiac α -actin (cACT) predominated over that of skeletal α -actin (sACT) mRNA, the fetal/neonatal isoform. α_1 -Adrenergic receptor stimulation induced hypertrophy of these cells, increasing total RNA and cytoskeletal actin mRNA by 1.8-fold over control, and total α -actin mRNA by 4.3 fold. This disproportionate increase in total α -actin mRNA was produced by a preferential induction of sACT mRNA, which increased by 10.6-fold over control versus only 2.6-fold for cACT mRNA. The α_1 -adrenoceptor is the first identified molecular mediator of early developmental isogene reexpression in cardiac myocyte hypertrophy.

Introduction

Cardiac hypertrophy in the adult animal is generally an adaptive response to hemodynamic demand and results from enlargement of individual muscle cells, without an increase in muscle cell number (1). Experimental production of hypertrophy by imposition of a hemodynamic load, such as by aortic banding, is accompanied by increased protein synthesis and total RNA content (2-5); a parallel increase in messenger RNAs (mRNAs) for various contractile proteins has also been observed (4, 5). Numerous studies have suggested that the hypertrophic response may include the reappearance, in adult tissue, of contractile protein isoforms characteristic of earlier developmental stages (6-8). In animal models of cardiac hypertrophy, the use of specific recombinant DNA probes has confirmed that the genes for these fetal and neonatal isoforms are reexpressed, including the gene for skeletal α -actin (sACT)¹ (9–11).

The cellular mechanisms that regulate cardiac myocyte growth and early developmental isogene reexpression in re-

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1. Abbreviations used in this paper: cACT, cardiac α -actin; NE, norepinephrine; sACT, skeletal α -actin.

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sponse to hemodynamic demand are unknown. Thyroid hormone is known to regulate the adult-specific isoform of cardiac ventricular myosin (12-16), but recent evidence suggests that thyroid hormone may not have a direct effect on cardiac growth (16). Catecholamines have been proposed as possible signals for myocardial hypertrophy (17-19), but, as with thyroid hormone, it is difficult using in vivo models to distinguish direct cellular effects of catecholamines from indirect effects mediated through hemodynamic or neurohumoral changes. To circumvent this difficulty with in vivo systems, we have been using a cell culture model to identify the molecular events associated with signal-mediated cardiac myocyte growth (20, 21). We have previously shown that α_1 -adrenergic receptor stimulation produces cell enlargement without hyperplasia in primary cultures of neonatal rat ventricular myocytes (20, 21). The present experiments show that α_1 -adrenoceptor stimulation preferentially induces the mRNA for a fetal/neonatal sarcomeric actin isoform, sACT, after its downregulation in culture.

Methods

Cardiac myocyte culture and growth assays

Primary cultures of neonatal rat heart muscle cells were established as previously described (20, 21) and maintained in serum-free medium 199 with transferrin and insulin. Non-muscle cell contamination was reduced by preplating and addition of 0.1 mM bromodeoxyuridine to the medium through day 3 of culture.² Adrenergic agents or their diluent (100 μ M ascorbic acid) were added to the dishes on culture day 4. Norepinephrine (NE) is stable under these conditions for up to 72 h (20, 21); in longer experiments, NE was replenished every 2 d. Cell number was determined by counting under the microscope, and cell size was quantified by measuring surface area, total protein content, and total RNA content (references 20 and 21, and below).

RNA analysis

 $[{}^{14}C]$ Uridine accumulation. Total RNA accumulation was measured by incorporation of $[{}^{14}C]$ uridine into acid-insoluble material. Cultures were incubated with $[{}^{14}C]$ uridine (New England Nuclear, Boston, MA, 500 mCi/mmol), 0.1 μ Ci/ml, 1 or 100 μ M, in the presence of adrenergic agents or their diluent. At designated times cultures were rinsed, extracted with trichloroacetic acid, and dissolved in sodium dodecyl sulfate for liquid scintillation counting.

RNA (Northern) blots. Total cell RNA was extracted (22) from

^{2.} The proportion of contaminating non-muscle cells in these cultures was < 10%, and remained constant under all experimental conditions. Non-muscle cells in this culture system do not grow in response to NE (20, 21), and do not express sarcomeric actin mRNAs in the presence or absence of NE (data not shown).

myocytes 48 h after the addition of adrenergic agents and quantified by ultraviolet (UV) absorbance. For Northern blots, RNA was fractionated by electrophoresis on agarose/formaldehyde gels, blotted (23), and immobilized by UV irradiation (24) on nylon filters (Gene-Screen; New England Nuclear). Blots were hybridized with a nick-translated ³²P-labeled complementary DNA (cDNA) probe derived from chick sACT, p α -actin2 (25). After washing twice in 0.1% sodium dodecyl sulfate, 15 mM NaCl, and 1.5 mM Na citrate at 52°C, blots were exposed to x-ray film. Autoradiographic band intensity was measured by scanning laser densitometry on an Ultroscan (Bromma 2202; LKB Instruments Inc., Bromma, Sweden).

Primer extension. A novel primer extension technique permitted the resolution of cardiac α -actin (cACT) and sACT iso-mRNAs. An 18-base homology in the 5' untranslated region was found by comparing the rat sACT sequence (26) with that of the rat cACT, kindly provided by Dr. U. Nudel, Dept. of Cell Biology, Weizmann Institute of Science, Rehovot, Israel. The oligomer sequence, 5'-CGACCCAC-GATGGATGGG-3', is complementary to a sequence in exon 2, codons 31-37 of both sACT and cACT mRNAs. The oligomer was synthesized by the University of California, San Francisco, Biomolecular Resource Laboratory on a System 1 DNA synthesizer (Beckman Instruments, Inc., Palo Alto, CA) and labeled with [³²P]ATP (see Fig. 2). The labeled primer was hybridized with 5-20 μ g of total RNA for 10 min at 37°C in a solution of 20 mM tricine, 0.1 mM Na EDTA, and 0.3 M NaCl. Reverse transcriptase (8 U/reaction) was added in buffer with a final concentration of 100 mM Tris-Cl, pH 8.3, 12 mM MgCl₂, 10 mM dithiothreitol, 1 mM each of dATP, dCTP, dTTP, and dGTP; and extension was carried out at 42°C for 30 min. Reactions were terminated by the addition of 1 vol of 93% formamide with 90 mM Na EDTA, pH 8.0, and 0.2% each of bromphenol blue and xylene cyanol. Samples were heated uncapped at 100°C for 12 min, immediately loaded onto denaturing 6% acrylamide/urea gels, and fractionated by electrophoresis. Gels were exposed to x-ray film and autoradiographic band intensity was determined by densitometry as above.

Statistics

Results are presented as mean \pm standard error. Mean values were compared by Student's *t* test, or by one-way analysis of variance for more than two groups.

Results

Myocytes exposed to $2 \mu M$ NE for 48 h in serum-free medium showed a 1.5- to 2.0-fold increase in cell size, as measured by cell protein content and surface area, with no change in cell number. Experiments with selective antagonists demonstrated that this was an α_1 -adrenergic response, in agreement with previous results (20, 21). Total cell RNA measured spectophotometrically increased 1.8±0.09-fold (n = 14) over control after treatment with 2 µM NE for 48 h. Trichloroacetic acidinsoluble [¹⁴C]uridine incorporation, a measure of total RNA accumulation, increased by 1.7±0.05-fold (n = 15) over control between 24 and 48 h after exposure to NE. Total RNA thus increased in proportion to the increase in cell protein and surface area. The stimulated increase in total RNA by both assays was inhibited > 80% by the α_1 -adrenergic antagonist terazosin (2 µM, n = 15) but <20% by the β -adrenergic antagonist propranolol (2 µM, n = 15) and by the α_2 -selective antagonist yohimbine (2 µM). Isoproterenol, a nonselective β -adrenergic agonist, was only 15% as effective as an equal concentration of NE (2 µM, n = 4). The NE-stimulated increase in total cell RNA content was therefore an α_1 -adrenergic response.

Total sarcomeric (α -) actin mRNA levels were analyzed in Northern blots of total cell RNA hybridized with a cDNA probe derived from chick skeletal α -actin. This probe contains \sim 500 bases of coding sequence and can cross-hybridize to rat cytoskeletal (β -, γ -) and sarcomeric actin mRNAs (25). In NEtreated myocytes, cytoskeletal actin mRNA content was increased 1.8-fold over control (139±29 vs. 76±14, values in densitometry units), whereas total sarcomeric actin mRNA increased by a mean of 4.3-fold $(1,212\pm84 \text{ vs. } 281\pm26, n=8)$ (Fig. 1, lanes b and e). The stimulated increase in both actin mRNAs was blocked by the addition of 2 μ M terazosin, but not by 2 μ M propranolol (Fig. 1, lanes c and f). Neither actin mRNA was changed by the addition of $2 \mu M$ isoproternol (Fig. 1, lane h). Thus, α_1 -adrenoceptor stimulation regulates myocyte content of both sarcomeric and cytoskeletal actin mRNAs. Cytoskeletal actin mRNA increased in proportion (1.8-fold) to the increases in total cell protein and RNA content, whereas the induction of sarcomeric actin mRNA was disproportionately greater.

Sarcomeric actin has two known isoforms, designated cACT and sACT because of their respective abundance in adult heart and skeletal muscle. An increase in total sarcomeric actin mRNA could therefore be due to increases in the mRNA coding for one or both of these isoforms. Both mRNAs are present in embryonic skeletal and cardiac muscle, although sACT mRNA is down-regulated to very low levels in the adult heart of rats and other species (24–32). sACT and cACT mRNAs cannot be separated on Northern blots because of their close similarity in size. To distinguish them, a primer



- $eta,oldsymbol{\gamma}$ -actin (cytoskeletal) - $oldsymbol{a}$ -actin (sarcomeric)

abc defgh

Figure 1. α_1 -Adrenergic stimulation of sarcomeric actin mRNA. RNA blot analysis of actin mRNA from cultured rat cardiac myocytes treated with: diluent alone (control) (lanes *a*, *d*, and *g*); 2 μ M NE (lanes *b* and *e*); 2 μ M NE + 2 μ M terazosin (lane *c*); 2 μ M NE

+ 2 μ m M propranolol (lane f); and 2 μ M isoproterenol (lane h). The lower band represents sarcomeric (α -) actin mRNA, and the upper band contains mRNAs for cytoskeletal actins (β - and γ - actins). Each lane contains RNA from $\approx 1 \times 10^6$ cells. -cardiac α-actin— — — — —

skeletal *a*-actin - - - - -

C S

a b c d e f



Figure 2. Primer extension method for cACT and sACT iso-mRNAs. (Top) Diagram of primer extension technique. A synthetic 18-base oligomer (heavy line), complementary to a sequence in exon 2, codons 31-37 of both sACT and cACT mRNAs, is labeled with ³²P and hybridized to RNA (thin line). The primer is extended to the 5' end of the mRNA by reverse transcriptase (wavy line). Because sACT and cACT mRNAs differ in length at their 5' ends, two characteristic extension products are generated (186 bases for sACT and 195 bases for cACT) which can be resolved on sequencing gels. (Bottom) Autoradiogram of a 6% sequencing gel of primer extension products generated by yeast transfer RNA (lane a), and total RNAs derived from rat tissues as follows: adult brain (lane b), adult skeletal muscle (lane d), adult heart (lane e), and neonatal cardiac myocytes after 7 d in primary culture (lane f). Lane c contains labeled DNA restriction fragments as size markers. In adult skeletal muscle (lane d) only a 186-base extension product is detected, corresponding to sACT mRNA. A single, larger extension product of 195 bases is seen in RNA from adult heart (lane e). The longer (cACT) band also predominates in RNA from cultured neonatal cardiac myocytes (lane f). (See also Figs. 3 and 5.) No extension products are obtained from brain RNA, which contains abundant cytoskeletal actin mRNA, or from yeast transfer RNA (lanes b and a, respectively). C, cACT, S, sACT.

201-190-180extension technique was developed that takes advantage of a small difference in length between the 5' untranslated regions of the two mRNAs (see Fig. 2). As expected, the 195-base cACT mRNA runoff product was the only isoform detected in adult heart RNA, and only the 186-base sACT mRNA was seen in adult skeletal muscle (Fig. 2, bottom, lanes e and d, respectively).

In control cultured myocytes, cACT mRNA was predominant (Fig. 2, lane f; Fig. 3, lane b; Fig. 5 A, lanes b-e; and Fig. 5 B, lanes a, c, and e). In seven experiments, sACT was $18\pm5\%$ of the total sarcomeric actin mRNA in control cells. By comparison, sACT was $\approx 50\%$ of total sarcomeric actin in neonatal rat ventricle, the source of the cultured cells (Fig. 3, lane a; Fig. 5 A, lane a). These data indicate that sACT mRNA is down-regulated when neonatal cardiac myocytes are placed in culture. Exposure to 2 μ M NE for 48 h increased myocyte content of sACT mRNA nearly 11-fold over control $(10.6 \pm 1.0$ -fold, n = 7); the increase in cACT mRNA was much less striking $(2.6\pm1.0$ -fold) (Figs. 3 and 5). As a consequence, NE-treated cultured myocytes had approximately equivalent levels of sACT and cACT mRNAs (sACT \approx 50% of total), similar to the relative levels present in neonatal ventricle (compare Fig. 3, lane a; Fig. 5 A, lane a). The disproportionate NE-stimulated increase in sarcomeric actin mRNA, as compared with cytoskeletal actin mRNA, was therefore largely due to a preferential increase in the sACT iso-mRNA level.

In three experiments, the increase in sACT mRNA induced by NE was strongly inhibited by the α_1 -antagonist, terazosin, whereas the β -antagonist, propranolol, had significantly less effect (Fig. 3, lanes d and e). Similarly, treatment with the β -agonist isoproterenol did not increase total sarcomeric actin mRNA (Fig. 1, lane h) or sACT mRNA levels (Fig. 3, lane f; two experiments). Thus, up-regulation of sACT mRNA is mediated through the α_1 -adrenergic receptor. The receptor specificity of the NE-stimulated increase in cACT mRNA was similar to that for sACT, with no induction by isoproterenol (Fig. 3, lane f) and no inhibition of the NE response by propranolol



a b c d e f

Figure 3. α_1 -Adrenergic induction of sACT iso-mRNA expression. Autoradiogram of primer extension analysis of total RNA from 1-dold-rat ventricle (lane a) and from separate groups of equal numbers of cultured myocytes (lanes b-f): control (CON) (lane b); NE (lane c); NE + terazosin (TZN) (lane d); NE + propranolol (PROP) (lane e); and isoproterenol (ISO) (lane f) (all 2 μ M). In seven experiments, the mean induction of sACT mRNA by NE (lane c) was ~ 11-fold, an effect that is potently inhibited by terazosin (lane d) but not by propranolol (lane e) (see Results).

(Fig. 3, lane e). However, the inhibitory effect of terazosin on cACT mRNA (Fig. 3, lane d) was not statistically significant (P > 0.05), possibly because of the low level of induction of this mRNA by NE and the limited number of observations.

Up-regulation of sACT mRNA by NE was dose dependent, with a maximum at 2 μ M and an estimated EC₅₀ of 0.4 μ M (Fig. 4). This concentration is within the range estimated for an active sympathetic nerve-smooth muscle synapse (33). Interestingly, at 20 μ M NE, the highest concentration used, there was a decrease in the per cell amount of sACT mRNA (Fig. 4). The explanation for this decrease is unclear; however, since total RNA per cell was unchanged between 2 and 20 μ M, the reduction of sACT at the higher NE dose does not reflect a nonspecific toxic effect.

Up-regulation of sACT mRNA was apparent by 12 h after exposure to NE, was maximal by 24 h, and persisted for up to 6 d (Fig. 5, A and B; three experiments). Over the same time interval, control cells maintained very low levels of sACT mRNA. The induction of sACT mRNA by NE was thus rapid and sustained.

Discussion

The central finding in this report is that α_1 -adrenergic stimulated myocardial cell hypertrophy includes a specific up-regulation of the sACT iso-mRNA. The sACT isogene is expressed in fetal and neonatal myocardium and is down-regulated to very low levels in adult ventricle (27-32), as well as in the cultured neonatal myocytes used in this study. Under the culture conditions used, the neonatal myocytes assume an adultlike phenotype with respect to α -actin gene expression. During hypertrophy in culture, α_1 -adrenergic stimulation switches the pattern of actin gene expression in favor of the developmentally immature isogene. The α_1 -induced reexpression of sACT mRNA during hypertrophy in culture is reminiscent of the hemodynamic load-induced reexpression of sACT during myocardial hypertrophy in vivo (11). These results provide evidence linking activation of a specific cell surface receptor with up-regulation of an early developmental isogene.

The intracellular events that couple α_1 -adrenoceptor activation to cardiac gene expression remain to be defined. Of potential relevance is the finding that stimulation of the α_1 -



Figure 4. Dose dependence of the NE-stimulated increase in sACT mRNA and total RNA. Plotted against the indicated final concentration of NE are the percent maximum per cell increases in total RNA (dashed line, three experiments) and sACT mRNA (solid line, two experiments). Each control myocyte contained ~ 10 pg total RNA.



a b c d e f

Figure 5. Time course of sACT down-regulation in control myocytes and up-regulation by 2 μ M NE. Both panels show autoradiograms of primer extension assays as in Figs. 2 and 3. (A) Neonatal rat ventricle (lane a); control myocytes at 12, 24, 48, and 72 h after the addition of diluent on culture day 4 (lanes b-f); and NE-treated cells at 12, 24, and 72 h after stimulation (lanes f-h). (B) Paired control (lanes a, c, and e) and NE-treated cells (lanes b, d, and f) on culture day 2 (lanes a-b), day 4 (lanes c-d), and day 6 (lanes e-f). Each lane contains RNA from an equal number of cells.

adrenoceptor increases phosphoinositide turnover in this and other systems (34–38). Activation of protein kinase C by endogeneously generated diacylglycerol may be an early step in gene regulation by α_1 -adrenergic agonists (36–38).

A new perspective on the α_1 -adrenoceptor has recently emerged. In addition to its well-characterized role in mediating acute increases in contractility of cardiac and smooth muscle (36), the receptor has now been shown to have trophic effects in a variety of cell types. α_1 -Adrenergic stimulation is mitogenic for cultured hepatocytes (39), smooth muscle cells (40), and mouse 3T3 and bovine endothelial cells (41); and it induces expression of the c-myc proto-oncogene in cultured cardiac myocytes (42). It will be interesting to see whether these diverse effects on contractility, cell growth, and gene expression are mediated through common or distinct intracellular pathways.

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