JCI The Journal of Clinical Investigation

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J Clin Invest. 1990;85(4):1206-1214. https://doi.org/10.1172/JCI114554.

Research Article

Cardiac hypertrophy produced in vivo by pressure overload is characterized by selective up-regulation of the fetal/neonatal beta-cardiac myosin heavy chain (MHC) isogene. However, a molecular signal for beta-MHC isogene induction has not been identified. We examined cardiac MHC isogene expression in a cell culture model for hypertrophy. alpha-MHC and beta-MHC iso-protein and iso-mRNA levels in cultured cardiac myocytes were quantified during hypertrophy stimulated by the alpha 1-adrenergic agonist, norepinephrine (NE). beta-MHC iso-protein content was increased 3.2-fold vs. control (P less than 0.001), whereas alpha-MHC isoprotein content was not changed significantly (1.4-fold vs. control, P = NS). MHC iso-mRNA levels were quantified by nuclease S1 analysis, using a single oligonucleotide probe. NE increased beta-MHC iso-mRNA content by 3.9-fold vs. control (P less than 0.001), but there was no change in alpha-MHC iso-mRNA (1.1-fold vs. control, P = NS). The NE-stimulated increase in beta-MHC iso-mRNA preceded in time the increase in beta-MHC isoprotein accumulation. The EC50 for NE induction of beta-MHC was 40 nM, and pharmacologic experiments indicated alpha 1-adrenergic receptor specificity. alpha-MHC isogene expression was predominant in control myocytes (68% alpha-isoprotein and 60% alpha-iso-mRNA). In contrast, beta-MHC expression was equal to alpha-MHC or predominant after treatment with NE (51% beta-isoprotein and 69% beta-iso-mRNA). Thus, alpha 1-adrenergic receptor stimulation increases the cellular contents of beta-MHC iso-mRNA and beta-MHC isoprotein during hypertrophy of […]

The Cardiac β-Myosin Heavy Chain Isogene Is Induced Selectively in α_1-Adrenergic Receptor-stimulated Hypertrophy of Cultured Rat Heart Myocytes

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Abstract

Cardiac hypertrophy produced in vivo by pressure overload is characterized by selective up-regulation of the fetal/neonatal β-cardiac myosin heavy chain (MHC) isogene. However, a molecular signal for β-MHC isogene induction has not been identified. We examined cardiac MHC isogene expression in a cell culture model for hypertrophy. α-MHC and β-MHC isoprotein and iso-mRNA levels in cultured cardiac myocytes were quantified during hypertrophy stimulated by the α_1-adrenergic agonist, norepinephrine (NE). β-MHC iso-protein content was increased 3.2-fold vs. control ($P < 0.001$), whereas α-MHC isoprotein content was not changed significantly (1.4-fold vs. control, P = NS). MHC iso-mRNA levels were quantified by nuclease S1 analysis, using a single oligonucleotide probe. NE increased β-MHC iso-mRNA content by 3.9-fold vs. control ($P < 0.001$), but there was no change in α-MHC iso-mRNA (1.1-fold vs. control, P = NS). The NE-stimulated increase in β-MHC iso-mRNA preceded in time the increase in β-MHC isoprotein accumulation. The EC_{50} for NE induction of β-MHC was 40 nM, and pharmacologic experiments indicated α_1-adrenergic receptor specificity. α-MHC isogene expression was predominant in control myocytes (68% α-isoprotein and 60% α-iso-mRNA). In contrast, β-MHC expression was equal to α-MHC or predominant after treatment with NE (51% β-isoprotein and 69% β-iso-mRNA). Thus, α_1-adrenergic receptor stimulation increases the cellular contents of β-MHC iso-mRNA and β-MHC isoprotein during hypertrophy of cultured neonatal rat cardiac myocytes, but does not change the levels of α-MHC iso-mRNA or isoprotein. The effect on β-MHC is mediated primarily at the level of mRNA steady-state level (pretranslational). Activation of the α_1-adrenergic receptor is the first identified molecular signal for increased β-MHC isogene expression in a model of cardiac hypertrophy. (*J. Clin. Invest.* 1990. 85:1206–1214.) adrenergic receptor • cell culture • contractile protein • gene expression • myocardial hypertrophy

Introduction

Two sarcomeric myosin heavy chain (MHC)[1] isogenes, designated α and β, are known to be expressed in cardiac muscle (1, 2). The proteins encoded by these genes associate in pairs with identical light chains to form three myosin isoforms. The myosin isoforms are designated V1 (αα), V2 (αβ), and V3 (ββ) in order of decreasing electrophoretic mobility and myosin ATPase activity (3). In certain species, the relative proportions of α- and β-MHC iso-mRNA and isoprotein vary during development and in myocardial hypertrophy.

β-MHC expression is dominant during fetal life; both α- and β-MHC are expressed equally shortly after birth; and α-MHC is predominant in adulthood (4–6). In myocardial hypertrophy produced by pressure overload, there is marked up-regulation of β-MHC iso-mRNA and isoprotein relative to α-MHC iso-mRNA and isoprotein (7–11). One study has indicated that this change in isogene predominance can be accounted for by a preferential increase in β-MHC isogene expression, with no change in α-MHC (11).

The differences in ATPase activity of the myosin isoforms suggest that up-regulation of β-MHC in hypertrophy may be functionally significant (12, 13). Furthermore, it has been shown that β-MHC induction in pathological hypertrophy produced by pressure overload can be reversed by exercise training, a stimulus for physiological hypertrophy (14–16). This observation has raised the possibility that the molecular mechanisms mediating β-MHC expression are activated only in certain types of hypertrophy.

The molecular signals responsible for selective induction of the β-MHC isogene in pressure overload hypertrophy are unknown. Thyroid hormone has been shown to regulate expression of the cardiac MHC isogenes in vivo and in culture, by stimulating directly α-MHC iso-mRNA accumulation and inhibiting β-MHC iso-mRNA accumulation (3, 4, 6, 17–22). However, no putative signal for β-MHC isogene up-regulation has been identified.

We have established a cell culture model to investigate the molecular events associated with signal-mediated cardiac myocyte hypertrophy (23, 24). Using this system, we have shown previously that α_1-adrenergic receptor stimulation by the catecholamine norepinephrine (NE) produces myocyte enlargement without hyperplasia in primary cultures of neonatal rat ventricular myocytes (25, 26). This hypertrophy model was used to examine MHC isogene expression.

Cultured cardiac myocytes were treated with NE, and α- and β-MHC iso-mRNA and isoprotein levels were quantified. The results show that α_1-adrenergic receptor stimulation increases selectively the cellular content of β-MHC iso-mRNA and isoprotein during hypertrophy. There is no change in the levels of α-MHC iso-mRNA or isoprotein. The close correlation observed between the level of β-MHC mRNA and its corresponding protein provides evidence that α_1-adrenergic receptor-linked control of MHC expression in culture is mediated at a pretranslational level. Activation of the α_1 receptor is the first identified molecular signal for increased β-MHC isogene expression in a model of cardiac hypertrophy.

Preliminary reports of this work have been published in abstract form: (1983. *Circulation.* 68:III-85; 1986. *Circulation.* 74:II-418; 1986. *Clin. Res.* 34:16A).

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Received for publication 26 May 1989 and in revised form 28 November 1989.

1. *Abbreviations used in this paper:* LV, left ventricle; MHC, myosin heavy chain; NE, norepinephrine.

Methods

Cell culture. Primary cultures of neonatal rat heart muscle cells were established as described previously (26). In brief, cells were obtained from the hearts of day-old rats by trypsinization and plated in MEM medium (Hanks' salts) with 5% calf serum. After 12 h in culture, cells were transferred to serum-free medium supplemented with transferrin and insulin (each 10 μg/ml). Cells were maintained in 100- or 35-mm culture dishes at a density of 100–150/mm^2. Contaminating nonmuscle cells were kept at < 10% by preplating and addition of 0.1 mM bromodeoxyuridine to the medium though day 3 of culture. For some experiments, cardiac myocytes from day 18 rat fetuses were prepared and maintained in culture in the same manner as neonatal myocardial cells.

On day 3 or 4 of culture, adrenergic agents (agonists and antagonists) or their vehicle, ascorbic acid (100 μM final) were added to the dishes. The final concentration of the adrenergic agents was 2 μM, except as noted below for dose-response measurements. NE is stable under these conditions for at least 72 h (25, 26). Cell number was determined by counting under the microscope (24). Cells were harvested at various times after additions for quantitation of the cell contents of total RNA, MHC iso-mRNAs, total protein, total MHC protein, and MHC isoproteins.

Pure cultures of cardiac nonmuscle cells were prepared as follows (24). The preplates containing a predominance of nonmyocytes were scraped with a rubber policeman, and the detached cells were allowed to reattach overnight. The medium containing unattached cells (myocytes) was removed, and fresh medium with 5% calf serum and no bromodeoxyuridine was added. The dishes were scraped again, and the cell suspension was distributed into culture dishes. After the nonmyocytes had proliferated to nearly confluent densities, they were harvested for preparation of RNA.

Total cell RNA. The culture dishes were rinsed with cold phosphate-buffered saline (PBS). Total cell RNA was extracted using 3 M LiCl and 6 M urea precipitation (27, 28) and quantified by ultraviolet (UV) absorbance.

MHC iso-mRNA analysis by nuclease S1 protection assay. A single oligonucleotide probe was used in a nuclease S1 mapping analysis to quantify both α- and β-MHC iso-mRNAs in the same total cell RNA sample (29) (Fig. 1 *A*). The method is similar to one used by others (7) but does not require a specific MHC plasmid. The probe was a 61-base synthetic oligonucleotide prepared by the University of California, San Francisco, Biomolecular Resource Laboratory on a System 1 DNA synthesizer (Beckman Instruments, Inc., Palo Alto, CA) and purified by electrophoresis. The sequence of the probe was as follows: 5′-CCG TGT CTC TTC ATT CAG GCC CTT GGC GCC AAT GTC ACG GCT CTT GGC CCG CAG CTT GTT G-3′. This probe, 3′ end-labeled with [α-^{32}P *dideoxy*]ATP (Amersham Corp., Arlington Heights, IL), was designed to be complementary to a 41-nucleotide common coding sequence at the carboxyl end of both the α- and β-MHC iso-mRNAs. The probe was also complementary to the final 15 nucleotides in the coding sequence of β-MHC iso-mRNA, which diverge significantly from those of α-MHC iso-mRNA (1). The oligonucleotide also contained six nucleotides at its 5′ end which were not complementary to either MHC iso-mRNA. Hybrids formed with α-MHC iso-mRNA would protect 41 nucleotides from S1 digestion, whereas those formed with β-MHC iso-mRNA would protect 56 nucleotides.

For S1 analyses, labeled probe was hybridized in molar excess to amounts of total RNA from equal numbers of cells in control and treated groups of a given experiment (~ 1 × 10^6 cells; ~ 10–25 μg of RNA). Probe from a single labeling reaction was used in parallel assays on RNA from each group of a given experiment. The hybridization was carried out in 10 μl total volume containing 300 mM NaCl, 20 mM tricine, 1 mM EDTA, and sufficient carrier nonhomologous RNA (day 18 chick liver RNA) so that each annealing contained at least 25 μg RNA (30). Hybridizations were performed for 20 h at 52°C. Nuclease S1 digestion was conducted for 60 min at 37°C with 1,500 U of enzyme (Miles Laboratories, Inc., Naperville, IL) in 200 μl of 100 mM NaCl, 30 mM sodium acetate (pH 4.5), 3 mM $ZnCl_2$, and 20 μg/ml denatured salmon sperm DNA. The digestion products were separated on a 10% polyacrylamide/8.3 M urea sequencing gel as described (31). An autoradiograph of the gel was obtained by exposure to x-ray film for 16–18 h. The amounts of α- and β-MHC iso-mRNA were quantified by counting the radioactivity of the appropriate bands excised from gels in a scintillation counter (see legend to Fig. 1 *B*). The radioactive counts in gel bands corresponding to α-MHC mRNA and β-MHC mRNA increased in a linear manner as the amount of total myocyte RNA used in S1 assays was increased between 2 and 25 μg (data not shown).

The nuclease S1 assay was validated by analysis of total cell RNA extracted from rat tissues with known relative levels of α- and β-MHC iso-mRNAs. Total cell RNA was extracted from the ventricles of normal, hyperthyroid, and hypothyroid 2–4-mo-old Sprague-Dawley rats. Hyperthyroid and hypothyroid rat ventricles contain predominantly α-MHC iso-mRNA and β-MHC iso-mRNA, respectively (6, 32). Hyperthyroidism was induced using 4 μg of L-thyroxine per 100 g of body weight per day for 8 d, delivered though a subcutaneous osmotic pump (Alzet osmotic minipump, Alza Corp., Palo Alto, CA) (33). Rats made hypothyroid by thyroidectomy were obtained from Charles River Breeding Laboratories Inc. (Boston, MA) and were maintained for 2 wk on 1% calcium lactate in the drinking water. During the 2-wk interval, body weights indicated a cessation of growth in the thyroidectomized rats. The animals were killed by ether anesthesia; the ventricles were removed and rinsed in cold PBS; and total cell RNA was prepared as described above. At the time of sacrifice, 0.5 ml of blood was drawn and assayed for serum concentration of thyroxine (T_4) by radioimmunoassay. The serum T_4 level in rats receiving L-thyroxine was 8.3±1.0 μg/dl (n = 6) (normal = 5.9±0.5 μg/dl), confirming a hyperthyroid status (33). The serum T_4 level in thyroidectomized rats was below the measurable range (< 1.5 μg/dl, n = 5). Nuclease S1 analyses of ventricular RNA were performed in the same manner as described for the cultured cells (Fig. 1 *B*).

Fig. 1 *B* shows that after hybridization of the oligonucleotide probe to RNA from normal adult rat left ventricle, two sets of bands were detected, which migrated with the mobility expected for a S1-protected probe hybridized to α- and β-MHC iso-mRNAs. The "signature" of multiple bands at each position results from nibbling by the S1 endonuclease (34) and was consistent from experiment to experiment. No MHC mRNA protection was seen using RNA from pure nonmyocyte cultures or from rat liver (Fig. 1 *B*), which do not express sarcomeric MHC. The relative abundances of α- and β-MHC iso-mRNAs in the different rat ventricles were as follows (mean±SE): normal adult (n = 8), α = 80±2%, β = 20±2%; hyperthyroid (n = 9), α = 90±1%, β = 10±1%; hypothyroid (n = 8), α = 6±1%, β = 94±1%; neonatal (n = 3), α = 68±2%, β = 32±2%. These MHC iso-mRNA levels were in good agreement with reported values (6, 32). These data, and the linearity of the relationship of counts in gel bands to amount of total cell RNA assayed, established that S1 analysis employing the oligonucleotide probe could be used to measure changes in the steady state abundance of α- and β-MHC iso-mRNAs.

Northern blot analysis was used to confirm that the probe hybridized to a single RNA species of the size expected for MHC mRNA (Fig. 1 *C*). For Northern blots, 10-μg samples of total cell RNA were size-fractionated on 1% agarose gels containing 22 mM morpholinopropane sulfonic acid (pH 7.0), 5 mM sodium acetate, 1 mM EDTA, and 6.3% formaldehyde (35). The RNA was electrophoretically transferred to nylon membranes (Gene Screen, New England Nuclear, Boston, MA), using the procedures recommended by the manufacturer, and crosslinked to the filter by UV irradiation (36). RNA blots were prehybridized for 1 h at 52°C in 10 ml of 35% deionized formamide, 1% essentially fatty acid-free crystalline bovine serum albumin, 0.2 M $NaHPO_4$ (pH 7.2), 1 mM EDTA, and 7% sodium dodecyl sulfate (SDS) (36). The probe (100–200 ng) was labeled at the 5′ end using T4 polynucleotide kinase (37) to a specific activity of 2–4 × 10^8 cpm/μg,

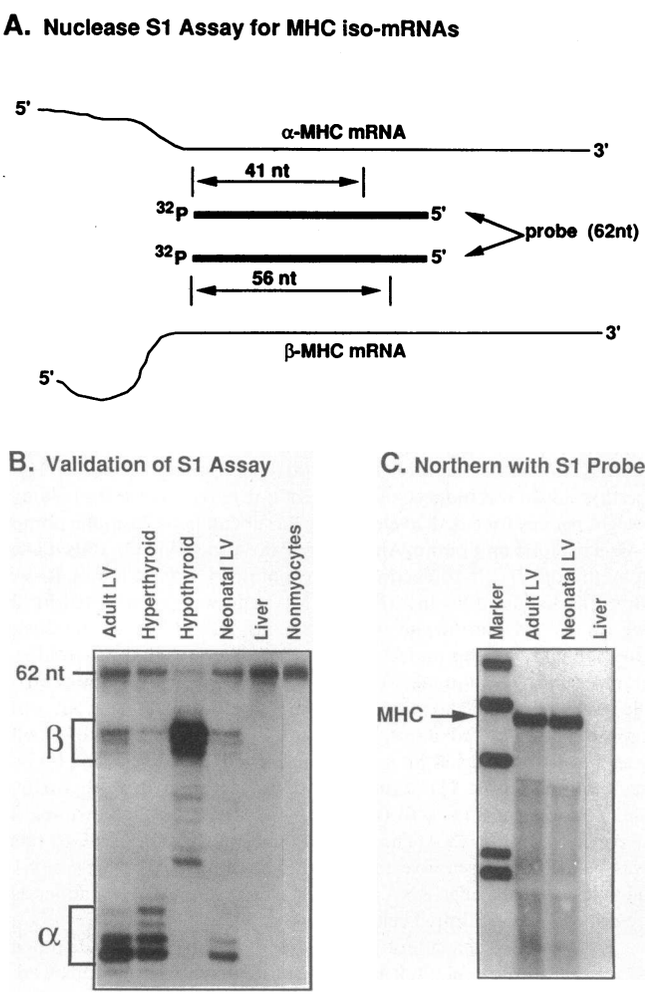

Figure 1. MHC iso-mRNA analysis by nuclease S1 protection assay. (*A*) A single 61-base synthetic oligonucleotide probe was labeled with [^{32}P-*dideoxy*]ATP at the 3′ end and used for quantitative nuclease S1 analysis. The probe was designed to hybridize to the 3′-translated end of the α- and β-MHC iso-mRNAs, in a region where sequence is initially shared (41 nucleotides [*nt*]) and then diverges (the next 15 nucleotides in the 3′ direction). When annealed to total cell RNA from heart tissue or cultured myocytes, hybrids formed with α-MHC iso-mRNA would protect 41 nucleotides from S1 digestion, whereas those formed with β-MHC iso-mRNA would protect 56 nucleotides. Six nucleotides at the 5′ end of the probe were not complementary to either iso-mRNA. (*B*) Labeled probe was hybridized with 10 μg of total RNA from normal adult rat left ventricle (LV) (*Adult LV*), hyperthyroid adult rat LV (*Hyperthyroid*), hypothyroid adult rat LV (*Hypothyroid*), day 1 neonatal rat ventricle (*Neonatal LV*), adult rat liver (*Liver*), and pure cardiac nonmyocyte cultures (*Nonmyocytes*). After digestion with nuclease S1, two sets of bands are detected in cardiac muscle RNA which migrate with the mobility expected for S1 protected probe hybridized to α- and β-MHC iso-mRNAs. The "signature" of multiple bands at each position was consistent and resulted from nibbling by the S1 endonuclease (34). The bands excised for determination of β-MHC were 54–56 nucleotides; and, for α-MHC, 38–41 nucleotides. When the amount of β-MHC iso-mRNA was large, bands of smaller size than the signature β-MHC bands were detected on the gels (see *Hypothyroid*). The counts in these smaller size bands were < 5% of those in the β-MHC bands at 54–56 nucleotides. The proportions of α- and β-MHC iso-mRNAs in the different heart tissues are similar to those reported by others (see data in Methods). No sarcomeric MHC mRNA is detected in RNA from liver or cultured cardiac nonmyocytes. (*C*) 10 μg of total RNA from normal adult rat LV, neonatal (day 1) rat LV, and adult rat liver was size-fractionated in agarose, blotted, and hybridized with the oligonucleotide probe, ^{32}P-labeled at its 5′ end. The *Marker* lane contains end-labeled lambda phage DNA cut with Hind III. The band sizes shown are 9,350, 6,630, 4,370, 2,280, and 1,980 nucleotides. A single band is detected in adult and neonatal rat LV RNA migrating at 6,300 nucleotides, the size expected for MHC mRNA. There is no hybridization of probe to RNA from liver.

and added directly to the prehybridization fluid (4–8 × 10^6 cpm/ml). Hybridization was for 18–20 h at 52°C. The blots were washed two to three times for 10 min each at 25°C in 500 ml of 0.15 M $NaHPO_4$ (pH 7.2), and 1% SDS, then an additional 2–5 min at 52°C. Autoradiography was performed with intensifying screens at −70°C for 16–20 h. When blots of total cell RNA from rat cardiac tissues were probed with the oligonucleotide used in the nuclease S1 analysis, a single band was detected migrating at 6,300 nucleotides (Fig. 1 *C*), the size expected for MHC mRNA (38). No hybridization of the probe to liver RNA was detected.

Total cell protein. The cultured cells were rinsed with cold PBS and dissolved in 0.1% SDS. Total cell protein was measured by the method of Bradford (39), using crystalline bovine serum albumin as a standard (24).

Total MHC protein. Total MHC protein content was determined by densitometry, after separation of MHC using SDS-PAGE. After two rinses with cold PBS, cells were dissolved in 150 μl of 0.1% SDS. 50 μl of a solution containing 8 mM Tris (pH 6.8), 2% SDS, 10% (vol/vol) glycerol, 5% 2-mercaptoethanol, 0.025% bromophenol blue, 8 mM EDTA, 4 mM phenylmethylsulfonyl fluoride (PMSF), 400 μM leupeptin, and 400 kIU/ml aprotinin was added, and the extract was heated at 100°C for 2 min. Samples were either used immediately or stored at −70°C. No differences were observed in the electrophoretic pattern between fresh and stored samples. Polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (40). 40 μl of sample (representing extract from an equivalent number of cells among groups in a given experiment) was added to each lane of an 8% gel. Electrophoresis was done at 25°C using constant voltage (150 V) for 4–5 h. The gels were stained with Coomassie Brilliant Blue R, then destained in 10% acetic acid (40). The MHC bands were quantified by densitometry, using a model GS 300 densitometer (Hoefer Scientific Instruments, San Francisco, CA) equipped with a model 3390A integrator (Hewlett-Packard Co., Palo Alto, CA) for determination of peak area. Each MHC band was scanned three times at spaced intervals across the band. The amount of MHC in pg was determined by comparison with a standard curve derived from three concentrations of rabbit skeletal muscle myosin (Sigma Chemical Co., St. Louis, MO) run in duplicate on each gel. Samples were in the linear range of the regression curve defined by the myosin standards ($r \geq 0.9$). The band representing MHC was well resolved from fibronectin.

Myosin isoproteins. Myosin isoproteins were quantified by densitometry, after separation using nondenaturing gel electrophoresis (41). Cells were rinsed with PBS and harvested in 3 ml of a low salt buffer containing 60 mM NaCl, 3 mM NaH_2PO_4, 2 mM EGTA, and 1 mM PMSF. The samples were centrifuged at 3,000 rpm for 10 min at 4°C. The pellet was resuspended in 2 ml of the low salt buffer, and centrifuged in the same manner for 10 min. The resulting cell pellet was extracted for myosin at 4°C in 400 μl of a solution containing 100 mM $Na_4P_2O_7$ (pH 8.8), 2 mM EGTA, 1 mM PMSF, and 2 mM 2-mercaptoethanol (41). The suspension was centrifuged at 10,000 *g* for 60 min at 4°C. The supernatant was collected, mixed with an equal volume of glycerol and stored at −20°C. Myosin isoproteins were separated in 4% polyacrylamide gels prepared with 20 mM sodium pyrophosphate, 1 mM EDTA, 2 mM cysteine, and 10% (vol/vol) glycerol at pH 8.8 (41). Each gel was loaded with 3.5–7.0 μg of extracted myosin protein.

Electrophoresis was performed in a model GE-214LS (Pharmacia, Inc., Piscataway, NY) using a recirculating buffer (20 mM sodium pyrophosphate, pH 8.8) at 2°C. Gels were run at a constant voltage of 14 V/cm for 16 h, stained for protein with Coomassie Brilliant Blue R as described for SDS-polyacrylamide gels, and destained for 12–16 h.

The relative amounts of V1, V2, and V3 myosin were quantified by densitometry (42). It was assumed that V1, V2, and V3 myosin contain $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$ MHC monomers respectively (3). Therefore, the percent of each MHC isoprotein was quantified using the following formula: % α-MHC = % V1 + ½% V2; and % β-MHC = % V3 + ½% V2 (6). These percent values were multiplied by the total MHC protein content per cell in the same sample, as determined by the SDS-PAGE analysis described above, to yield per cell content in picograms of α-MHC isoprotein and β-MHC isoprotein.

Data analysis and statistics. Each group in each experiment consisted of two to four culture dishes. Protein data (picograms) and RNA data (picograms or counts per minute) were corrected for cell number in each experiment. Values for treated groups were normalized to the control value (vehicle-treated) in each experiment. Results are expressed as the mean±SE. The difference between two groups was tested by the unpaired Student's t test. Differences among more than two groups were tested by analysis of variance with Newman-Keuls test for multiple sample comparison. Treated/control ratios were tested for deviation from unity by calculation of confidence intervals (43). Statistical significance was defined as $P < 0.05$.

Results

We examined MHC isogene expression during cardiac myocyte hypertrophy stimulated by the α_1-adrenergic agonist NE. On the third or fourth day after plating, neonatal rat heart myocytes maintained in low-density, serum-free culture were treated with either 2 μM NE or vehicle (control). After 48–72 h, MHC isoprotein and iso-mRNA contents were quantified as described in Methods. There were no differences in the results with treatment on day 3 or 4 of culture and for 48 or 72 h. Myocyte numbers and the proportion of nonmyocytes (< 10%) were not altered by any treatment (data not shown), in agreement with previous results (24–26).

NE increases β-MHC isoprotein content. Treatment of the cultured myocytes with NE increased selectively the cellular levels of β-MHC isoprotein (Figs. 2 and 3). NE increased β-MHC isoprotein by 3.2±0.2-fold times control ($P < 0.001$, n = 10). There was no significant change in the myocyte content of α-MHC isoprotein. After treatment with NE, the levels of α-MHC isoprotein were 1.4±0.2-fold times control (P = NS, n = 10) (Fig. 3). The ca. threefold increase in β-MHC isoprotein induced by NE was significantly greater ($P < 0.001$) than the NE-stimulated increase in total cell protein, a measure of myocyte hypertrophy (24, 26). Total myocyte protein was increased by 1.6±0.1-fold times control ($P < 0.001$, n = 7) (Fig. 3). Thus, the effect of NE on β-MHC protein accumulation exceeded its generalized effect on cell growth. Since NE upregulated only β-MHC isoprotein, total MHC protein (α and β) was increased by only 2.0±0.1-fold vs. control ($P < 0.01$, n = 13) (Fig. 3).

NE increases β-MHC iso-mRNA content. To determine whether NE induced a preferential accumulation of β-MHC isoprotein by altering mRNA steady-state level, the myocyte contents of α- and β-MHC iso-mRNA were measured by quantitative S1 nuclease analysis (Figs. 3 and 4). NE increased β-MHC iso-mRNA by 3.9±0.5-fold times control ($P < 0.001$, n = 15). α-MHC iso-mRNA content was not changed by NE. α-MHC iso-mRNA content in NE-treated cells was 1.1±0.1-fold times control (P = NS, n = 15) (Fig. 3). The ca. fourfold

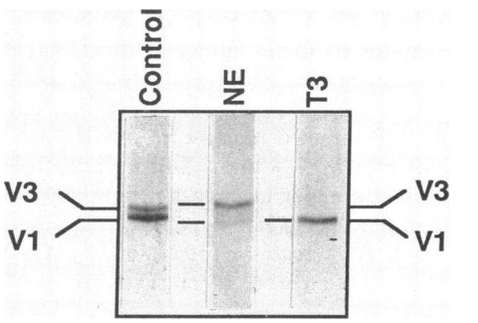

Figure 2. Myosin isoproteins in neonatal myocyte cultures. Neonatal myocytes were treated on culture day 4 with vehicle (control, 100 μM ascorbic acid), NE 2 μM, or T_3 20 ng/ml for 72 h. Myosin isoproteins in equal amounts of myosin extracted from control and treated cells were separated on nondenaturing gels, as described in Methods. Control cultures demonstrate a predominance of the V1 ($\alpha\alpha$) isomyosin (64% V1 by densitometric analysis of the gel shown). After treatment with NE, the V3 ($\beta\beta$) isoform becomes predominant (62% V3 in the example shown). Myocytes treated with T_3 contain only V1 isomyosin.

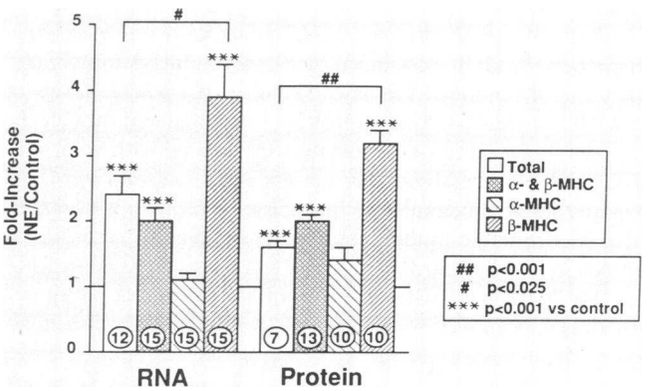

Figure 3. NE-induced increases in myocyte contents of total protein and RNA and MHC isoproteins and iso-mRNAs. Equal numbers of cultured neonatal myocytes were harvested for assay after 48–72 h of treatment with 2 μM NE or vehicle. Cell contents of total RNA, total protein, total MHC protein (α and β), and the α- and β-MHC iso-mRNAs and isoproteins were determined as described in Methods. Total MHC mRNA was calculated as the sum of the α- and β-MHC iso-mRNAs. Bars are the mean±SE of the NE-treated/control ratio in each experiment. Values for RNA are on the left side of the figure, and values for protein are on the right side. (*Open bars*) Total RNA or protein; (*hatched bars*) total (α and β) MHC mRNA or protein; (*bars with wide diagonal marking*) α-MHC iso-mRNA or isoprotein; and (*bars with narrow diagonal marking*) β-MHC iso-mRNA or isoprotein. The number of separate experiments is circled within the bars. Control values were as follows: total RNA = 10.8±0.6 pg/cell; total protein = 476±39 pg/cell; total MHC protein = 13.5±1.7 pg/cell; β-MHC isoprotein = 4.4±0.5 pg/cell; α-MHC isoprotein = 9.2±1.1 pg/cell. MHC mRNA contents were in counts per minute. ***$P < 0.001$ vs. control; ##, #$P < 0.001$ and < 0.025, respectively, for the comparisons indicated.

increase in β-MHC iso-mRNA content was significantly greater ($P < 0.025$) than the NE-induced increase in total cell RNA. Total cell RNA was increased by 2.4±0.3-fold vs. control ($P < 0.001$, $n = 12$) (Fig. 3). The close correlation between the magnitude of the NE-induced increase in β-MHC iso-mRNA and its cognate protein (3.9- and 3.2-fold vs. control, respectively), suggests that a major mechanism by which NE produces β-MHC isoprotein accumulation is pretranslational. Similarly, the NE-stimulated increases in total (α and β) MHC mRNA and total MHC protein were identical (each 2-fold times control) (Fig. 3).

NE reverses MHC isoform predominance in cultured neonatal myocytes. Control cultured neonatal myocytes expressed predominantly α-MHC isoprotein and α-MHC iso-mRNA (Figs. 2, 4, and 5). α-MHC isoprotein was 68% of total MHC protein in control cells ($n = 10$), and α-MHC iso-mRNA was 60% of total MHC mRNA ($n = 15$) (Fig. 5). Thus, the control cultured myocytes displayed a MHC isoform phenotype similar to the neonatal hearts from which they were derived (68% α-MHC iso-mRNA, see Methods).

The selective effect of NE on β-MHC isoprotein accumulation produced a switch from a predominance of α-MHC isoprotein in control cultures (68% α) to equal proportions of α-MHC and β-MHC isoproteins in NE-treated cultures (51% β) (Figs. 2 and 5). NE treatment reversed the relative abundance of the MHC iso-mRNAs in cultured myocytes from a predominance of α-MHC iso-mRNA in control cells (60% α) to a predominance of β-MHC iso-mRNA after NE (69% β) (Figs. 4 and 5).

Receptor specificity of β-MHC iso-mRNA and isoprotein induction. Separate pharmacologic experiments demonstrated that the NE-induced increases in cultured myocyte levels of β-MHC iso-mRNA and isoprotein were mediated though the α_1-adrenergic receptor. Myocytes were treated with vehicle or NE (2 μM) in the absence or presence of terazosin (2 μM), an α_1-adrenergic antagonist, or propranolol (2 μM), a nonselective β-adrenergic antagonist. Terazosin blocked the NE-induced increase in cardiac myocyte level of β-MHC iso-mRNA by 94±7% ($n = 5$), whereas propranolol blocked this response by only 23±13% ($n = 5$). The effect of the adrenergic antagonists on β-MHC isoprotein accumulation paralleled the effect on β-mRNA. Terazosin completely inhibited the NE-stimulated increase in β-MHC isoprotein (100±15% inhibition, $n = 5$). Propranolol, on the other hand, inhibited β-MHC isoprotein accumulation by only 22±17% ($n = 3$). There was no significant change in α-MHC iso-mRNA or isoprotein under any condition, and no change in α- or β-MHC iso-mRNA or isoprotein with antagonist treatment alone (data not shown).

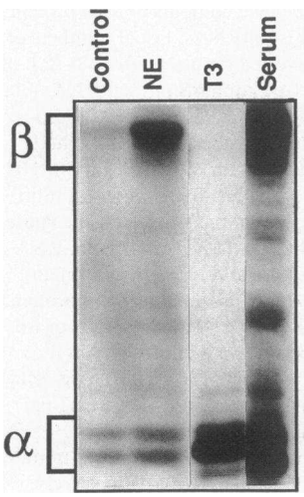

Figure 4. MHC iso-mRNAs in neonatal myocyte cultures. Neonatal cardiac myocytes were treated on culture day 3 with vehicle (*Control*), NE 2 μM, T_3 20 ng/ml, or 5% serum for 72 h. RNA from equal numbers of cells was taken for S1 nuclease analysis of MHC iso-mRNAs. In control myocytes, α-MHC iso-mRNA is predominant. NE increases β-MHC iso-mRNA, but α-MHC iso-mRNA is changed little. After stimulation with T_3, β-MHC iso-mRNA is not detected; and α-MHC iso-mRNA is increased. Serum treatment up-regulates both MHC iso-mRNA species.

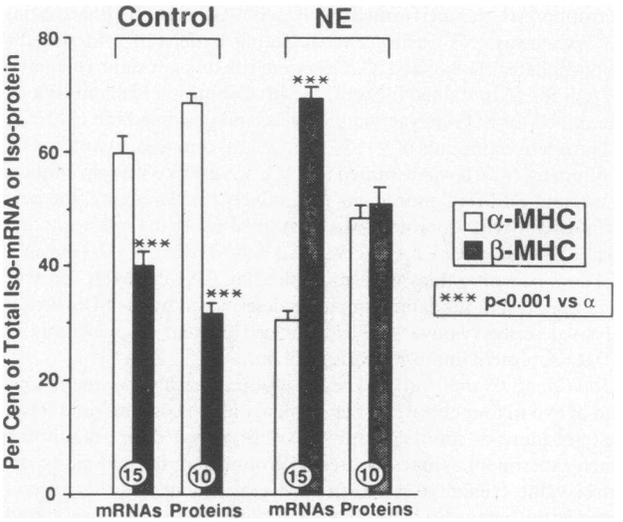

Figure 5. NE reverses MHC isoform predominance. Cultured neonatal myocytes were treated with 2 μM NE or vehicle for 48–72 h, and MHC iso-mRNAs and isoproteins were quantified. The percentage of total for each iso-mRNA or isoprotein was calculated. Each bar is the mean±SE. Control cultures are on the left, and NE-treated cultures are on the right. (*Open bars*) α-MHC; (*solid bars*) β-MHC. The number of experiments is circled within the bars. In control cultures, α-MHC is the predominant isoform. β-MHC is predominant or equal to α-MHC after treatment with NE. ***$P < 0.001$ vs. α-MHC.

Dose response and time course of β-MHC induction. In dose-response experiments, NE at 0.02 μM produced a significant increase in the level of β-MHC iso-mRNA (Fig. 6, *right*). The induced increase in β-MHC iso-mRNA was maximal at 2 μM NE, and the EC_{50} was ~ 40 nM. Time course experiments with 2 μM NE demonstrated a significant increase in cellular level of β-MHC iso-mRNA at 12 h, and a plateau at 48 h (Fig. 6, *left*). The NE-induced increases in β-MHC iso-mRNA preceded the increases in β-MHC isoprotein.

Specificity of β-MHC up-regulation by NE. To determine whether the increased level of β-MHC iso-mRNA with unchanged α-MHC iso-mRNA was a specific response to NE or a generalized response to myocyte growth factors, cells were treated with triiodothyronine (T_3) or calf serum. Both serum and T_3 produce myocyte hypertrophy in this culture system (24, 44). After 72 h in the presence of 20 ng/ml T_3, the steady-state level of β-MHC iso-mRNA decreased to nearly undetectable levels, whereas that of α-MHC iso-mRNA increased ~ 10-fold (Fig. 4). There were similar changes in the relative levels of the MHC isoproteins (Fig. 2). Treatment of cells with 5% calf serum for 72 h produced a marked increase in the levels of both α- and β-MHC iso-mRNA in comparison with

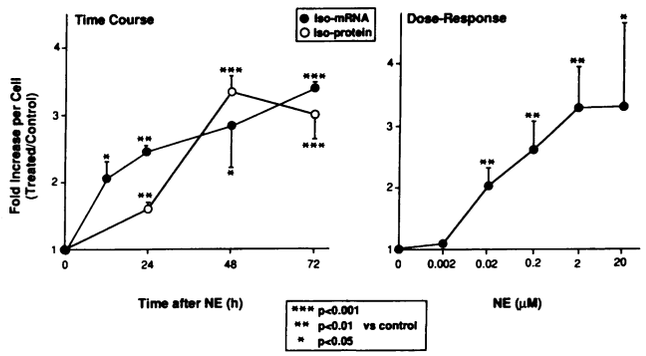

Figure 6. Time course and dose-response relationship for induction of β-MHC by NE. For determination of the time course of β-MHC induction by NE (*left*), cultured neonatal myocytes were treated with 2 μM NE or vehicle (control) for 12–72 h. At each time point, MHC iso-mRNAs and isoproteins were quantified as described in Methods. Each value is the mean±SE of the ratio of β-MHC iso-mRNA (●) or isoprotein (○) in NE-treated cells vs. control cells (n = three to eight experiments for each time point). NE did not change α-MHC iso-mRNA or isoprotein at any time point (data not shown). In the control cells, α- and β-MHC isoprotein contents were similar to those given in the legend to Fig. 3 and did not change over the time studied (data not shown). In dose-response experiments (*right*), myocytes were treated with vehicle or increasing concentrations of NE for 48–72 h, and MHC iso-mRNAs were quantified by nuclease S1 analysis. Each point is the mean±SE NE-treated/control ratio for β-MHC iso-mRNA content per cell (n = three experiments for each NE dose, except 0.002 μM, where n = one experiment). NE did not change α-MHC iso-mRNA at any of the concentrations tested (data not shown). ***, **, *$P < 0.001$, < 0.01, and < 0.05 vs. control, respectively.

controls (ca. sevenfold) (Fig. 4). Thus, the selective effect of NE on β-MHC isogene expression was different from that of two other stimuli which produce cardiac myocyte hypertrophy in this system.

NE increases β-MHC in cultured fetal rat heart myocytes. Cultured heart myocytes from day 18 rat fetuses had a response to NE similar to that of the cultured neonatal heart myocytes. Control cultured fetal cardiac myocytes contained a predominance of β-MHC iso-mRNA (64±4% β, n = 4), in contrast with control cultured neonatal myocytes, in which α-MHC iso-mRNA predominated (see above). Total RNA was 8.5±1.3 pg/cell in the control fetal myocytes. Stimulation of the fetal heart myocytes with 2 μM NE for 72 h produced an increase in total RNA content, one measure of cell size (45), of 2.2±0.2-fold times control ($P < 0.005$, n = 4). NE also induced an increase in fetal myocyte β-MHC iso-mRNA content of 2.6±0.5-fold vs. control ($P < 0.025$, n = 4). α-MHC iso-mRNA content was not changed by NE (0.9±0.2-fold times control, P = NS, n = 4). This selective increase in β-MHC iso-mRNA after treatment of the fetal cardiac myocytes with NE was the same as the response of the neonatal cardiac myocytes, although the magnitude of the increase was somewhat less marked. Therefore, day 18 cultured fetal heart myocytes responded to NE by an increase in size and by a further increase in the steady-state level of β-MHC iso-mRNA.

Discussion

The central finding of this study is that the cellular contents of β-MHC iso-mRNA and isoprotein are increased selectively and specifically in α_1-adrenergic receptor-stimulated hypertrophy of cultured neonatal rat heart myocytes. Treatment with NE did not change the levels of α-MHC iso-mRNA or isoprotein. The NE-stimulated increase in β-MHC isogene expression, without alteration of α-MHC, was distinctly different from the pattern of MHC isogene regulation produced by two other hypertrophic growth stimuli, T_3 and serum. Furthermore, the NE-induced increases in β-MHC iso-mRNA and isoprotein were significantly greater than the increases in total RNA or protein content, two indices of cell hypertrophy.

Activation of the α_1-adrenergic receptor is the first identified molecular signal for increased expression of the β-MHC isogene. In certain species, expression of the β-MHC isogene is characteristic of the fetal myocardium and the myocardium subjected to pressure overload. In myocardial hypertrophy due to pressure overload in vivo, there is an increase in the proportion of β-MHC iso-mRNA and isoprotein relative to α-MHC (7–11). In agreement with a study of hypertrophy in vivo (11), our results indicate that a shift in MHC isoform predominance can be accounted for by a preferential increase in β-MHC isogene expression, with no change in α-MHC.

The selective up-regulation of β-MHC isogene expression by NE parallels and extends our earlier observation in the same model system that skeletal α-actin iso-mRNA is increased preferentially to cardiac α-actin iso-mRNA by α_1-adrenergic stimulation (28, 46). Like β-MHC, skeletal α-actin, a fetal/neonatal isoform of α (sarcomeric)-actin, is reexpressed in myocardial hypertrophy induced by pressure overload (47). Thus, α_1-induced hypertrophy of cardiac myocytes in culture is associated with selective up-regulation of at least two contractile protein isogenes characteristic of neonatal and/or fetal development and hypertrophy produced by pressure overload.

The molecular mechanisms that link the hemodynamic stimulus of increased pressure to the complex events of cardiac myocyte hypertrophy and preferential expression of fetal/neonatal contractile protein isogenes are not known. The cell culture model used in this study cannot be compared directly to hypertrophy in vivo. However, since early developmental contractile protein isogenes are up-regulated selectively both in pressure overload in vivo and in response to NE in culture, it is possible that the α_1-adrenergic receptor, or the intracellular mechanisms it controls, may play a role in some types of myocardial hypertrophy in vivo. Recent work has revealed both spatial and temporal heterogeneity in β-MHC and skeletal α-actin expression during pressure-load hypertrophy in vivo, and it has therefore been speculated that different signals and/or regulatory mechanisms may be involved in induction of these two genes (48). Our demonstration that both β-MHC and skeletal α-actin can be up-regulated by the same molecular mechanism (present study, 28, 46) provides a starting point for study of the additional factors that may modulate the expression of one or both isogenes to produce the nonsynchronous accumulation seen in vivo.

Induction of early developmental isogenes is not found in all types of hypertrophy in vivo, such as hypertrophy produced

by exercise training or thyroid hormone excess (7, 14–16, 49). Similarly, hypertrophied cardiac myocytes in culture have different MHC isoform phenotypes which depend on the specific hypertrophic stimulus. Thus, the culture model provides direct support for the idea that different types of myocardial hypertrophy in vivo may be regulated by diverse molecular mechanisms. Since hypertrophy produced by exercise training is characterized by induction of α-MHC (14–16), the intracellular mechanisms activated by the α_1-adrenergic receptor may not be involved in the hypertrophy of exercise training.

In our previous study of α-actin isogene switching, gene expression was quantified only by assay of the α-actin iso-mRNAs (28). In the present study of cardiac MHC isoforms, measurement of protein content provided evidence that the increased β-MHC iso-mRNA was translated into protein. This result is important, since preliminary data from our laboratory suggest that certain hypertrophic stimuli can increase β-MHC iso-mRNA without a commensurate increase in β-MHC isoprotein (50). Two findings suggest that the increase in β-MHC iso-mRNA in response to α_1-adrenergic stimulation can account for the increase in β-MHC isoprotein. First, the NE-stimulated increase in β-MHC iso-mRNA preceded the increase in β-MHC isoprotein. Secondly, the magnitude of β-MHC isoprotein induction was very similar to that of β-MHC iso-mRNA. Additional translational and posttranslational mechanisms may be involved, but do not appear necessary to explain the changes observed.

It was not investigated whether the increase in β-MHC iso-mRNA level was due to changes in isogene transcription rate or mRNA processing or stability. However, the rapid induction of β-MHC iso-mRNA within 12 h after treatment with NE is consistent with an effect on β-MHC isogene transcription. Stimulation of the α-adrenergic receptor increases skeletal α-actin iso-mRNA content with a similar time course (28) and induces transcription of the skeletal α-actin isogene (46).

The intracellular mechanism or mechanisms that couple α_1-adrenergic receptor activation to β-MHC isogene expression in the cultured myocytes are unknown. Contractile activity does not appear to be required for β-MHC induction, since the cultured myocytes in this system are quiescent with α_1-adrenergic stimulation (26). Indirect evidence implicates protein kinase C in signal transduction. Stimulation of the α_1-adrenergic receptor activates protein kinase C in the cultured myocytes (51), perhaps via diacylglycerol generated from membrane phospholipids (52–54). Furthermore, phorbol myristate acetate also activates myocyte protein kinase C (51) and produces selective up-regulation of β-MHC iso-mRNA (Waspe, L. E., Ordahl, C. P. and P. C. Simpson, unpublished results, 50). In other systems, protein kinase C is thought to regulate transcription via post-translational modification of transcription factors (55–59). It is possible that a transcription factor interacting with a response element or elements on the β-MHC isogene is activated by protein kinase C. Since β-MHC, skeletal α-actin, and, to a lesser extent, cardiac α-actin are regulated by α_1-adrenergic stimulation, these three genes may share common response elements that are not present on α-MHC. This hypothesis can now be tested.

The mechanisms involved in α_1-induced up-regulation of β-MHC isogene expression appear to be present as early as day 18 of fetal life, since β-MHC iso-mRNA content was increased selectively by NE in cultured day 18 fetal rat heart myocytes. A previous study did not find an effect of adrenergic agonists on MHC iso-mRNA expression in cultured fetal rat heart myocytes, although coupling of adrenergic receptors to second messenger systems was demonstrated (17). This difference from our findings might be due to the high level of β-MHC expression in the control cultures used for this prior study (17).

Acknowledgments

The authors thank Dr. Neal White, University of California, San Francisco, for myosin protein analysis; Dr. Lawrence Gavin, University of California, San Francisco, and San Francisco Veterans Administration Medical Center, and Ms. Marie Moeller for assistance with preparation of hyperthyroid rats and for serum T_4 determinations; and Ms. Clarissa Bush and Mr. Wesley Webb for their expertise in cell culture.

This work was supported by the United States Public Health Service (grants HI-31113, HL-42150, and HL-35561), the Veterans Administration Research Service, and the American Heart Association, California Affiliate. Dr. Waspe is the recipient of a National Research Service Award (HL-07508), and Dr. Simpson is a Clinical Investigator of the Veterans Administration.

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