Concurrent opposite effects of trichostatin A, an inhibitor of histone deacetylases, on expression of α-MHC and cardiac tubulins: implication for gain in cardiac muscle contractility

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PUMP FUNCTION OF THE HEART is intimately related to the ability of heart muscle to generate force and active shortening (46). A growing body of evidence, obtained from both clinical and experimental studies, indicates that between these two determinants of cardiac contractility, the ability of cardiac muscle to generate effective shortening velocity becomes a major defect in failing hearts (15, 27). Among the different biophysical and biochemical parameters studied, a shift in myosin isoform composition of the sarcomere, intrinsic properties of the cross bridges, as well as intra- and extracellular viscoelastic components contributing to cellular passive stiffness are considered to play major roles in determining shortening velocity of cardiac muscle fibers (7, 27, 49).

The myosin molecule of the cardiac sarcomere is composed of two myosin heavy chains (MHCs), α-MHC and β-MHC (35). These two MHC isoforms are differentially regulated during development, as well as in response to various pathophysiological stimuli, which has considerable physiological relevance to myocardial contraction. The α-MHC, which has high ATPase activity, accounts for faster shortening velocity of cardiac myofibers, and hearts with predominantly α-MHC have lesser systolic resistance and higher intrinsic capacity to generate active shortening. The β-MHC, which has low ATPase activity, leads to greater economy of force generation, and hearts with higher β-MHC have a greater intrinsic ability to generate force (1, 44). In rodents, β-MHC is the predominant isoform in the fetal heart, whereas α-MHC predominates in the adult heart. As the animal ages, levels of α-MHC become suppressed again. Unlike rodents, in the adult human heart, ~35% α-MHC mRNA and ~8% protein content are expressed (33, 36). In hearts with exercise-induced physiological hypertrophy, α-MHC levels are increased, which enhances cardiac output to meet increased metabolic demands. In stress-mediated pathological hypertrophy, β-MHC expression is elevated, allowing the heart to develop more force per unit of time (6). In failing hearts, however, regardless of the etiology of the disease, α-MHC has been found to be invariably decreased (6, 7, 33, 36). This is implicated to be a critical determinant of the diminished myofibrillar ATPase activity and reduced shortening velocity, thus leading to myocardial contractile dysfunction (22).

In addition, a change in viscoelastic components of the cell is also considered to play a major role in determining the shortening and relaxation velocity of cardiac myocytes. Both myofibrillar cytoskeleton, including change in expression of titin and desmin, and the extramyofibrillar microtubular network have been shown to influence passive stiffness (viscous drag) of the cell (16, 49). Several reports have indicated that during pathological hypertrophy a change in microtubule network density, via upregulated expression of tubulin isomers and/or increased stability of microtubules, creates a viscous drag force that hampers myocardial contractility (27, 42, 47, 49). Furthermore, Ishibashi et al. (18) showed that viscous-

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drag force imposed by densification of microtubules is directly proportional to the shortening velocity of myofibers; in certain pathological conditions, it also plays a role more prominent than myosin-isofrm shift in loss of myocardial contractility. From these reports, it has been postulated that any therapeutic strategy that can change expression of both of these components of cell contractility, that is, α-MHC and tubulin expression, is likely to have beneficial effects in protecting the heart when undergoing failure. However, no such strategy or agent that favorably influences the expression of both cardiac α-MHC and tubulin levels is known to date.

Toward this goal, we elected to analyze the effect of a histone deacetylase (HDAC) inhibitor trichostatin A (TSA) on the expression levels of α-MHC and tubulins in cardiac myocytes. TSA is a fermentation product of Streptomyces with anti-fungal properties and was found to be a reversible inhibitor of HDACs in vitro, as well as in vivo (54). It is a highly potent HDAC inhibitor, with an IC50 in the nanomolar range for most systems reported thus far. Because of its known pharmacology, it has come to be a “reference” substance in research aimed at changing the acetylation-deacetylation state of proteins for clinical as well as research applications. Post-translational acetylation-deacetylation of chromosomal histones has recently emerged as a key signal-responsive, epigenetic regulatory mechanism in cardiac growth control and gene expression. The histone acetylase and HDAC enzymes that mediate this process have been shown to functionally interact, directly or indirectly, with key cardiac myogenic transcription factors, such as serum response factor (SRF), myocyte enhancer factor 2 (MEF-2), and members of the GATA family (8, 9, 32). Mammalian HDACs are currently classified into three classes (class I, II, and III) according to homology with their yeast counterparts, Rpd3, HDA1, and Sir2, respectively (11). In the hypertrophied myocardium, HDACs have been shown to be important endpoint targets of cell signaling pathways involved in reinduction of the cardiac “fetal gene program” and reorganization of sarcomeres, hallmarks of cardiac hypertrophy. Activation of HDACs has been shown to have both antihypertrophic and prohypertrophic responses in cardiac myocytes. Although class II HDACs are considered to suppress prohypertrophic genes, the members of class I HDACs suppress the expression of antihypertrophic genes (55). Yet, the controlled general inhibition of HDACs was found to antagonize the hypertrophic response of different hypertrophic agonists in primary cultures of cardiac myocytes (2). Several HDAC inhibitors are recognized as potent inhibitors of cell growth and are in clinical trials for use as anticancer agents (50). It has also been suggested that HDAC inhibitors could be developed as tools in “transcriptional therapy” for reversal of the fetal gene program, for reverse remodeling of the heart undergoing failure, and/or for situations in which heart failure already exits. Therefore, it is becoming imperative that the molecular mechanisms of action of HDAC inhibitors, their cardioprotective functions, and their probable facilitation of improved cardiac performance are analyzed. In this study, we report that general inhibition of HDACs by TSA has an opposite effect on the expression of two key contractile parameters, that is, α-MHC and tubulins. It upregulates expression of the α-MHC gene, whereas it downregulates the expression of both α- and β-tubulin isoforms. To the best of our knowledge, this is the first strategy that changes both parameters in such a way that the combined effect of this change will be anticipated to enhance the active shortening and relaxation velocity of cardiac muscle fibers.

MATERIALS AND METHODS

Cell culture and transfection. Primary cultures of cardiac myocytes were prepared from 2-day-old neonatal rats as previously described (37). All animal protocols were approved by the University of Chicago Animal Care and Use Committee. After differential plating to eliminate fibroblasts, we further purified myocytes using a Percoll density gradient (Amersham Pharmacia Biotech, Piscataway, NJ) and plated at an average density of 4 × 105 per 100 mm2 on cell culture dishes precoated with 2% gelatin. Cells were grown in DMEM supplemented with 10% fetal bovine serum and 100 U/ml penicillin and 100 mg/ml streptomycin. The H9C2 cardiac myoblast cell line and C2C12 skeletal myoblast cell line were typically plated in Falcon six-well tissue culture dishes at a density of 3–5 × 105 cells/well and maintained in DMEM with 10% fetal bovine serum and penicillin-streptomycin combination. Differentiation of both cell types was attained by complete serum withdrawal for 48 h. Cells were transfected using lipofectamine reagent (Invitrogen Life Technologies, Carlsbad, CA) or TFX-20 transfection reagent (Promega, Madison, WI), according to the manufacturer’s protocol. pCMV-β-gal was used as a reference plasmid in all transfections. After 48 h, transfected cells were harvested, and cell lysates were prepared and assayed for CAT (CAT ELISA kit; Roche Diagnostics, Manheim, Germany), luciferase (luciferase assay system; Promega), or β-galactosidase expression (β-Gal staining kit; Invitrogen Life Technologies) and protein content (Bio-Rad protein reagent; Bio-Rad, Hercules, CA). CAT and luciferase activities for a given construct were corrected for the protein content of each extract and normalized to the β-galactosidase activity in the same cell extract.

Animal studies. Adult male Harlan Sprague Dawley rats weighing 230–280 g that had undergone thyroparathyroidectomy (both thyroid and parathyroid glands removed) were purchased from Harlan. Age- and weight-matched adult male Harlan Sprague Dawley rats were used as euthyroid controls. All animals were housed for 4 wk before use. Rats were treated with 500 µg/kg TSA or vehicle (DMSO) administered subcutaneously once daily for 2 wk. Body weight was monitored at the onset and end of the treatment. Serum 3,5,3’-triiodothyronine (T3) and thyroxine (T4) levels were measured from blood samples obtained on the day of death. Tissues were harvested, snap frozen, and stored at −80°C until utilized.

Plasmids used. The α-MHC promoter-CAT reporter constructs, i.e., −2,936 α-MHC-CAT, −1,698 α-MHC-CAT, and −1,283 α-MHC-CAT, and the early growth response factor 1 (EGR-1) expression construct pCMV-Egr-1 have been described earlier (13). The −368 α-MHC-CAT, thyroid receptor element (TRE) α-MHC-CAT, and α-MHC-delta TRE-CAT were kindly provided by Dr. Youngsook Lee (Department of Anatomy, University of Wisconsin, Madison, WI). Multimeric EGR-1 wild-type or mutant binding sites cloned adjacent to the thymidine kinase (TK) promoter-luciferase reporter gene were provided by Dr. Eugene Chen (Cardiovascular Research Institute, Morehouse School of Medicine, Atlanta, Georgia) (31). 5xCArG-Hsp/LacZ was cloned by ligating 5×CArG sites from pSRE-luc (Stratagene, La Jolla, CA) between the Xhol and HindIII sites of the LacZ reporter gene linked to the heat shock protein (hsp) 68 promoter (20). The clone was confirmed by sequencing as well as by restriction digestion.

Western analysis and coimmunoprecipitation of proteins. Unless otherwise specified, all common salts and reagents were obtained from Sigma (St. Louis, MO). Whole cell lysates from frozen tissues or from cultured cells were prepared in RIPA buffer and directly subjected to Western analyses or coimmunoprecipitation was followed by Western analyses according to the methods described before (10). Antibodies used were as follows: mouse monoclonal anti-rat cardiac

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α-MHC, i.e., BA-G5 hybridoma (ATCC, Manassas, VA); rabbit polyclonal anti-thyroid hormone receptor α-1 and anti-thyroid hormone receptor β-1 (Affinity Bio Reagents, Golden, CO); rabbit polyclonal panacetyl antibody, rabbit polyclonal anti-SRF, rabbit polyclonal anti-α-tubulin, and rabbit polyclonal anti-β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA); and mouse monoclonal anti-acetylated tubulin (Sigma-Aldrich, St. Louis, MO).

Cytoblot analyses. H9c2 cells were plated on 96-well tissue culture treated plates at a density of ~10^4 cells/well in DMEM containing 10% FBS and penicillin-streptomycin and allowed to attach overnight. Serum was completely withdrawn to induce differentiation. Forty-eight hours postdifferentiation, cells were subjected to increasing concentrations of TSA (0–300 nM) for an additional 24 h. Cells were washed twice in PBS and fixed with methanol (30 min on ice), further washed twice, and blocked with PBS containing BSA (0.1%) for 60 min at room temperature. Cells were incubated with a panacetyl mouse monoclonal IgG, developed against a mixture of chemically acetylated antigens, clone 4G12 (Upstate, Lake Placid, NY) (1:200 for 1 h at room temperature). Cells were then washed three times in PBS containing 1% BSA and then incubated with an anti-mouse horseradish peroxidase (HRP)-conjugated IgG (1:1500) as a secondary antibody. Cells were washed three times with PBS + 1% BSA. For signal detection, an enhanced chemiluminescent substrate, SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) was used in quantitative plate-based luminoimmunetry utilizing a Berthold Microplat Plus plate reader (Perkin-Elmer, Boston, MA).

RNA extraction and Northern blot analysis. Total RNA was extracted from control and TSA-treated rat hearts or cultured neonatal primary cardiomyocytes with TRizol reagent (Invitrogen Life Technologies) according to the method provided by the manufacturer. Northern blot analysis was performed as described earlier (10) with synthetic oligonucleotide probes complementary to the unique cis-untranslated sequences of the rat α-MHC and β-MHC mRNA. Sequences of the single-stranded oligonucleotide probes were as follows: 5’-GTG GGA TAG CAA CAG CGA GGC-3’ for rat α-MHC and 5’-GTT CTC AGG GCT TCA CAG GC-3’ for rat β-MHC.

Protein–DNA binding array analysis. We utilized a high-throughput, array-based analysis, i.e., TranSignal protein/DNA array (Panomics, Redwood City, CA) that permitted the identification of activated transcription factors in response to TSA treatment. Nuclear extracts from control or TSA-treated myocytes were obtained with a commercially available nuclear extraction kit (Panomics). The assay was conducted as per the manufacturer’s instructions. Briefly, a set of biotin-labeled DNA binding oligonucleotides provided by the manufacturer were preincubated with nuclear extracts (25 μg of total protein) obtained from either vehicle (DMSO) or TSA-treated (100 nM) primary neonatal rat cardiac myocytes. The protein-DNA complexes formed were separated from the unbound free probe by the agarose gel electrophoresis and by excising the relevant area. The complexes were hybridized to extracted probes at 42°C overnight. Membranes were washed with the provided wash buffer, blocked, incubated with provided streptavidin–HRP substrate, and exposed to Hyperfilm enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK). On chemiluminescence detection, autoradiograms were subjected to scanning densitometry using Scion Image for Windows analysis software (release beta 4.0.2), based on NIH Image for Macintosh by Wayne Rasband (NIH). Signal intensity adjusted for background density was determined for all factors.

In vivo transcription reporter array. The TranSignal transcription reporter array (Panomics) was used to study transcription factor activation in response to TSA treatment of rat cardiac myocytes, according to the manufacturer’s protocol. The overall principle involves the activation of transcription factors, subsequent binding to corresponding cis-acting elements, and activation of the transcription of a unique cis-element-tagged reporter sequence that can be detected with hybridization-based array technology. This system comprises transfection of cells simultaneously with multiple plasmids each with unique cis-acting DNA sequence elements upstream of a tagged reporter gene. Accordingly, we transfected neonatal rat primary cardiac myocytes with the manufacturer’s reporter array plasmid mix using TFX-20 transfection reagent. One group of cells was treated with TSA (100 nM), and the controls were treated with the DMSO solvent. Forty-eight hours later, cells were harvested, and total RNA was isolated with TRizol reagent (Invitrogen Life Technologies). Biotinylated cDNA probes were synthesized from control or treated cells by use of the reporter array primers provided by the manufacturer and biotin-dUTP and hybridized at 42°C overnight with provided blots and hybridization buffer. On washing, membranes were exposed to a streptavidin–HRP conjugate and detection buffer, followed by chemiluminescence and exposure to Hyperfilm enhanced chemiluminescence (Amersham Biosciences). Scanning densitometry was conducted as described above. Signal intensity adjusted for background density was determined for all factors.

Electromobility gel shift assay. Double-stranded oligonucleotides were 5’-end-labeled with T4 polynucleotide kinase (Promega) and [γ-32P]ATP. The binding reaction was carried out in a total volume of 25 μl containing 10,000 counts/min (0.1–0.5 ng) of the labeled DNA, 2–5 μg of the specified nuclear extract, and 1 μg of poly(dI-dC). The binding buffer consisted of (in mM) 10 Tris·HCl (pH 7.4), 100 NaCl, 0.1 EGTA, 0.5 dithiothreitol, 0.3 MgCl2, 8% glycerol, and 0.5 PMSF. After incubation at room temperature for 45 min, the reaction mixtures were loaded on 5% native polyacrylamide gels (44:1, acrylamide-bisacrylamide), and electrophoresis was carried out at 150 V in 0.5% TBE buffer in a cold room. For competition and antibody experiments, unlabeled competitor DNA or the antibody was preincubated with nuclear extracts at room temperature for 15–20 min in the binding buffer before addition of the labeled DNA probe. The probe α-MHC gene EGR-1 binding site sense strand is 5’-GTG GGG GTG-3’ (13).

Quantitative analysis of cellular microtubules vs. free tubulin. To isolate the free and polymerized fractions of tubulin, all of the assay components (e.g., samples, rotors, centrifuge tubes, and buffers) were maintained at 37°C throughout the isolation procedure. Control and α-tubulin–treated myocytes were harvested and lysed in a cell lysis-microtubule stabilization buffer of the following composition: 100 mM PIPES (pH 6.9), 5 mM MgCl2, 1 mM EGTA, 30% glycerol, 0.1% nonidet P-40, 0.1% Triton X-100, 0.1% Tween 20, 0.1% β-mercaptoethanol, 0.001% antifoam, 1 mM dithiorthiophenol, 1 mM GTP, 10 mM ATP, 10 μM Taxol, and 10 μM protease inhibitor cocktail (Cytoskeleton, Denver, CO). Cell homogenization and centrifugation were done at 37°C to maintain microtubule stability. On centrifugation at 100,000 g for 30 min, supernatants containing soluble tubulin were separated from the pellets containing microtubules (polymerized tubulin plus microtubule-associated proteins). The pellet was dissolved in double-distilled deionized water (ddH2O) of the same volume as the supernatant fraction, and calcium chloride was added, such that the final concentration was 200 μM. Pellets were dissociated by incubation on ice with frequent vortex for 1 h. Protein concentration of the supernatant, as well as pellet fractions, was determined with the detergent-compatible bicinchoninic acid reagent kit (Pierce, Rockford, IL). Equal proteins per well were loaded on 8% SDS-polyacrylamide gels, and Western analyses were conducted to determine α-tubulin, β-tubulin, and acetylated tubulin fractions associated with free as well as polymerized fractions.

Immunostaining and confocal microscopy. Neonatal rat cardiac myocytes were grown on 2% gelatin-coated coverslips. After treatment for required time periods, cells were washed with PBS (37°C), fixed with 3.7% para-formaldehyde at 37°C for 5 min, and rehydrated with 0.5% Triton X-100 in PBS. Cells were blocked with a 0.3% BSA solution and incubated with specified primary antibody at room temperature for 2 h. On three rapid washes, cells were treated with a rhodamine-conjugated secondary antibody at room temperature for
1 h. After three rapid washes, cells were mounted using the Slow-Fade antifade kit with DAPI (Molecular Probes, Eugene, OR). Cells were observed with a ×63 Planapo objective and photographed by a Zeiss Axioplan microscope equipped with a Photomatrix cooled charged-coupled device camera (Roper, Tucson, AZ). Cellular details in each field were obtained by Nomarski (differential interference contrast) imaging. For color fluorescence in each field, nuclear DAPI staining was visualized with 330- to 380-nm excitation and 460- to 470-nm emission filters, and rhodamine staining was visualized with a 556- to 580-nm excitation filter and 600- to 660-nm emission filters. Single images were processed by no-neighbor deconvolution, using Openlab 3 (Improvision, Coventry, UK) with digital confocal processing. All imaging was carried out in the Cancer Center Digital Light Microscopy Laboratory at the University of Chicago.

RESULTS

TSA induces hyperacetylation of cellular proteins. In our preliminary experiments, we determined the effects of TSA treatment on the rate of cell survival using trypan blue exclusion as a measure of cell viability. We observed that 100 nM TSA was well tolerated by both primary cultures of cardiac myocytes and H9C2 cells. The percent cell viability in TSA-treated cells, compared with controls, was 93.8 ± 4.2% and 96.4 ± 3.1% in primary myocytes and H9C2 cells, respectively. To determine the optimal amount of TSA that induces cellular hyperacetylation, we treated H9C2 cells with increasing concentrations of TSA. After 24 h of treatment, cytoblot analysis was performed with a panacetyl mouse monoclonal antibody. Results indicated a dose-dependent increase in cellular hyperacetylation in response to TSA treatment, with a maximal effect (96% increase above basal) at 100 nM TSA (Fig. IA). To demonstrate hyperacetylation of proteins in cultured cardiac myocytes, we performed Western analyses using a polyclonal panacetyl antibody (Santa Cruz Biotechnology), which recognizes acetylated histones. As shown in Fig. 1B, TSA treatment (100 nM) profoundly increased acetylated levels of H3 and H4 histones of cardiac myocytes. We also determined the efficacy of TSA treatment by measuring the whole cell HDAC activity, using an HDAC assay kit (Calbio-
TSA upregulates α-MHC gene expression. To determine the effect of TSA on α-MHC expression, primary cultures of cardiac myocytes, exhibiting spontaneous synchronous contractions, were treated either with TSA or vehicle (DMSO) for 48 h. Total RNA was extracted and analyzed by Northern blot analysis. As shown in Fig. 1, C and E, TSA consistently (n = 6 cultures) increased the steady-state levels of α-MHC mRNA by approximately three- to fourfold, without changing the levels of β-MHC mRNA (not shown). To determine whether the TSA-mediated mRNA induction resulted in increased α-MHC protein expression by TSA, we included cycloheximide (10 μM), a protein synthesis inhibitor, in both control and TSA-treated cells. RNA analysis of these cells resulted in no detectable signal for α-MHC mRNA (even on prolonged exposure) in either group of cells, indicating that on-going protein synthesis is required for the basal as well as TSA-mediated expression of α-MHC mRNA, consistent with our previous report (13).

To determine whether the TSA-mediated mRNA induction resulted in increased α-MHC protein, cells were harvested in RIPA buffer and subjected to Western analysis using a mouse monoclonal antibody against the rat cardiac α-MHC, i.e., BA-G5 hybridoma (ATCC). As shown in Fig. 1D, TSA treatment also enhanced the α-MHC protein expression by ~30% compared with nontreated controls.

We next examined whether TSA could induce the α-MHC expression in vivo. For these experiments, we utilized rats in which thyroid glands were surgically removed. Thyroidectomy in rats results in lack of circulating ligand and a general hypothyroid phenotype, which includes induction of the “fetal gene program” together with a shift in MHC isoforms from predominantly α-MHC to β-MHC expression (35). These rats provided us with a model to demonstrate any detectable in vivo increase in α-MHC levels. Accordingly, the thyroidectomized adult male rats were treated with TSA (500 μg/kg sc) or vehicle (DMSO) once daily for 2 wk. We found no adverse effects of TSA treatment on animal behavior, gross organ morphology, or heart weight-to-body weight ratio. The circulating T₃ (37.5 ng/dl) and T₄ (0.56 μg/dl) levels were significantly lower in thyroidectomized rats than in euthyroid (T₃, 49 ng/dl; T₄, 4.9 μg/dl) controls, as expected, and they remained unchanged due to TSA treatment. Also, we found no change in expression levels of thyroid receptors α₁ and β₁ in TSA-treated rats compared with controls. However, by Northern blot analysis, we found a three- to fourfold increase in α-MHC mRNA levels, with no change in β-MHC in TSA-treated thyroidectomized rats (Fig. 1F). Thus these results indicate that TSA treatment selectively increases the α-MHC expression, which is likely independent of cardiac thyroid hormone levels and circulating ligands.

Role of EGR-1 in TSA-induced expression of the α-MHC gene. To delineate the mechanisms involved in TSA-mediated α-MHC induction, we utilized a high-throughput, array-based analysis, i.e., TranSignal protein-DNA array (Panomics) that permitted us to identify TSA-activated transcription factors of cardiac myocytes. This array allowed us to study 54 transcription factors, including previously identified key regulators of the α-MHC gene transcription; however, it excluded many other potential cardiac muscle gene regulators, an inherent limitation of this assay. A set of biotin-labeled DNA binding oligos provided by the manufacturer were preincubated with nuclear extracts obtained from either vehicle (DMSO) or TSA-treated (100 nM, for 48 h) cultures of cardiac myocytes. This enabled the formation of DNA-protein complexes, which were then separated from the unbound free probe. The probes in the complexes were then extracted and hybridized to the TranSignal array membrane. The chemiluminescence signal obtained from this analysis was subjected to densitometry scanning using Scion Image program for Windows analysis software (NIH Image for Macintosh by Wayne Rasband, NIH). Complete results of this analysis are presented in Table 1. As indicated, of the 54 transcription factors, 33 were activated by TSA. We graded them arbitrarily on the basis of their level of activation: marginal activation in 15, moderate activation in 6, good activation in 5, very good activation in 1, and excellent activation in 5. The early growth response gene, EGR-1, was one of the factors that was most strongly activated by TSA.

We next sought to demonstrate whether the TSA-mediated activation of EGR-1 results in an in vivo functional effect. For this purpose, we utilized the TranSignal transcription reporter array system (Panomics), which unlike the transient transfection assay allows for the assessment of the net effect of interpathway cross talk among various transcription factors in vivo. The differences in adjusted signal densities between TSA- and vehicle-treated myocytes are shown in Table 2. Of the 24 factors studied for in vivo transcriptional activation, we observed 9 with significant increase, 6 with moderate increase, 2 with moderate decrease, 1 with significant decrease, and 6 with no detectable change. The results of this assay allowed us to determine the effect of TSA-dependent activation of transcription factors as well as the effects of their cross talk and interaction with other factors in cardiac myocytes. As shown in Table 2, TSA treatment of cardiac myocytes produced the net effect of a marked increase in EGR-1-dependent transcription, demonstrating a positive functional outcome of EGR-1 activation in vivo in response to TSA.

Previous studies from this laboratory (13) have shown that EGR-1 transactivates the α-MHC promoter involving an EGR-1 binding site, located between the −1,471- and −1,463-bp region of the gene. To demonstrate whether this EGR-1 binding site is involved in the TSA-mediated activation of the α-MHC gene expression, we performed mobility gel-shift analyses, using the EGR-1-binding site as a labeled probe and nuclear extracts from TSA-treated and untreated myocytes. As shown in Fig. 2, a slow-moving complex was formed that was competed by the unlabeled oligo and supershifted by the anti-EGR-1 antibody, indicating specificity of the complex. In addition, nuclear extracts from TSA-treated myocytes consistently demonstrated a higher intensity EGR-1 complex compared with controls, suggesting enhanced DNA-binding activity of EGR-1 following TSA treatment of cells.

We next performed transient transfection analyses to explore the functional role of EGR-1 in TSA-induced expression of the α-MHC gene. H9C2 cells were transfected with different...
EFFECT OF TSA ON CARDIAC MHC AND TUBULIN ISOFORM EXPRESSION

Table 1. TSA induces a marked activation of EGR-1 in cardiac myocytes as determined by high-throughput array-based analysis

<table>
<thead>
<tr>
<th>cis-Element/Transcription Factor</th>
<th>Increase in TSA-Induced Signal Intensity Above Vehicle Controls, arbitrary density units</th>
<th>Score Based on Adjusted Signal Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGR-1, GATA, NF-κB, Pax-5, Smad3/4</td>
<td>&gt; 200</td>
<td>&gt; ++ + + (+ excellent)</td>
</tr>
<tr>
<td>E2F-1</td>
<td>150–200</td>
<td>++ + + (+ very good)</td>
</tr>
<tr>
<td>c-Myc, CREB, MEF-1, Stat4, USF-1</td>
<td>100–150</td>
<td>++ + (good)</td>
</tr>
<tr>
<td>AP-1, AP-2, NF-1, NF-E2, HSE, MRE</td>
<td>50–100</td>
<td>++ (moderate)</td>
</tr>
<tr>
<td>ARE, Bm-3 (POU4F1), CDP, GRE, p53, RAR[DR-5], Smad SBE, Sp1, Stat3, Stat5, Stat5b/Stat6, TFIIID, TR, TR[DR-4], VDR[DR-3]</td>
<td>5–50</td>
<td>+ (marginal)</td>
</tr>
<tr>
<td>C/EBP, Ets, Ets-1/PEA3, FAST-1, GAS/ISRE, HNF-4, IRF-1, MEF-2, c-Max, NFATc, NF-E1 (YY1), Oct-1, Pbx1, Pit 1, PPAR, PRE, RXR[DR-1], SIE, SRE (SREBF), Stat1 p84/p91</td>
<td>&lt; 5</td>
<td>– (no detectable difference)</td>
</tr>
</tbody>
</table>

Nuclear extracts from control or trichostatin A (TSA)-treated (100 nM, 48 h) primary cultures of cardiac myocytes were processed according to the manufacturer’s instructions (see MATERIALS AND METHODS). All signal intensities were determined using Scion Image for Windows analysis software. Threshold signal intensity (on 8 min of exposure of autoradiogram) twice the intensity of background was scored as a positive signal. Signal intensity adjusted for background density was determined for all factors. Measurable activation was detected in 33 of 54 factors studied, which represents 61.11% of factors in the array. From adjusted-density individual signals, the following arbitrary scoring system was implemented: < 5, no detectable change; 5–50, marginal (+); 50–100, moderate (+ +); 100–150, good (++ +); 150–200, very good (++ ++ +); and > 200, excellent (++ ++ ++ +). Early growth response factor 1 (EGR-1), a known intermediary in a broad range of cellular signaling and a positive regulator of α-myosin heavy chain (MHC) transcription, was one of the factors graded excellent. Early growth response factor 1 (EGR-1), a known intermediary in a broad range of cellular signaling and a positive regulator of α-myosin heavy chain (MHC) transcription, was one of the factors graded excellent.

Table 2. Endogenous EGR-1-mediated transcription is activated by TSA, as determined by the TranSignal transcription reporter array

<table>
<thead>
<tr>
<th>Enhancer Sequence</th>
<th>Change in TSA-Induced Signal Intensity From Vehicle Controls, arbitrary density units</th>
<th>Score Based on Adjusted Signal Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGR, NF-κB, E2F, Ets, AP-1 (2), RARE, GRE, TR, NFAT</td>
<td>&gt; + 25</td>
<td>(+ +) Significant increase</td>
</tr>
<tr>
<td>CRE, ISRE, p53, RXXR, ERE, GATA</td>
<td>+ 5 TO + 25</td>
<td>(+) Moderate increase</td>
</tr>
<tr>
<td>VDR</td>
<td>&lt; − 25</td>
<td>(− −) Significant decrease</td>
</tr>
<tr>
<td>Oct-1, Stat3</td>
<td>− 5 TO − 25</td>
<td>(−) Moderate decrease</td>
</tr>
<tr>
<td>GAS (Stat1), c-Myc, YY1, AP-1 (1), SRE, C/EBP</td>
<td>+/− 5</td>
<td>No detectable change</td>
</tr>
</tbody>
</table>

Enhancer sequences are available from the manufacturer provided and/or cited literature. Cardiac myocytes were transfected with manufacturer (Panomics)-provided reporter array plasmid and maintained with or without TSA (100 nM) for 48 h. Total RNA was isolated, and biotinylated cDNA probes were synthesized and hybridized with manufacturer-provided blots (see MATERIALS AND METHODS). Threshold signal intensity (on 8 min of exposure) twice the intensity of background was scored as a positive signal. Signal intensity adjusted for background density was determined for all factors. The difference in adjusted signal densities between TSA-treated and untreated control myocytes were quantified for each factor. Of the 24 factors studied based on scanning densitometry and an arbitrary scale, we detected 14 to be positively activated, representing 56% of factors studied; 4 to be negatively activated (12.5% of factors studied), and 6 with no detectable change (25% of factors studied).
not the mutant sites. To confirm that this effect was attributed by activation of the endogenous EGR-1 gene, we also examined the EGR-1 levels of cardiac myocytes by Western analysis. As shown in Fig. 4B, TSA substantially increased the expression of endogenous EGR-1 gene in a concentration-dependent manner. Thus, collectively, these results demonstrate a key role of EGR-1 in mediating the TSA-dependent activation of the α-MHC gene expression.

Potential upstream involvement of SRF in TSA-mediated increase in α-MHC expression. A critical role of SRF in regulation of the EGR-1 gene expression has been well established with five different functionally active SRF binding sites characterized on its promoter. These sites play a pivotal role in EGR-1 gene activation in response to various growth stimuli (47). To demonstrate whether there is an upstream involvement of SRF in TSA-induced EGR-1 expression, we also examined the effect of TSA on a SRF-dependent promoter-reporter gene having five SRF-binding sites (5xCArG-Hsp/LacZ). As shown in Fig. 5, A and B, TSA substantially increased (~5-fold) the reporter gene activity of the 5xCArG-Hsp/LacZ plasmid in a concentration-dependent manner. To examine whether this effect was contributed by induction of the endogenous SRF levels, we measured SRF levels in TSA-treated and untreated cells by the Western analysis using a SRF-specific antibody. As shown in Fig. 5C, TSA treatment of cells resulted in a marked increase (3- to 4-fold) in the expression of SRF protein. Because EGR-1 expression is SRF dependent, it is likely that TSA-induced increase in SRF is an upstream mechanism that contributes to the activation of the EGR-1 promoter by TSA.

TSA-mediated changes in cardiac microtubules. In addition to α-MHC, a change in microtubular network dynamics has been shown to regulate myocardial shortening velocity (18). Tubulins exit as nonpolymerized (free) or polymerized heterodimers of α- and β-tubulins. These two isoforms differ in their amino acid compositions and are regulated independently in a tissue-specific manner (24). In cardiac myocytes, both isoforms are expressed and developmentally regulated (38, 41). TSA treatment of myocytes reduced the expression of both α- and β-tubulin isoforms in a concentration-dependent manner (Fig. 6, A and B). However, the TSA-mediated downregulation...
of β-tubulin expression was more severe than that of α-tubulin, i.e., 40% and 75% reduction of α- and β-tubulin levels in response to 300 nM TSA, respectively. Because tubulins are considered targets of HDACs, we also examined the change in acetylation state of tubulins by TSA. As shown in Fig. 6C, TSA profoundly increased acetylation of total tubulins in a concentration-dependent manner. In this experiment, we also consistently observed two additional bands of lower molecular mass (25 and 37 kDa) below the tubulin bands; however, the nature of these bands is unknown to us at present (Fig. 6C).

During cardiac hypertrophy or failure, an increased myocyte microtubule network density, via upregulated expression of tubulins and/or increased stability of microtubules, has been implicated in ventricular contractile dysfunction (18). Because we observed downregulation of both tubulin isoforms by TSA, we next asked whether TSA could also alter microtubules network density induced by hypertrophy agonists. Accordingly, we treated primary cultures of cardiac myocytes with ANG II (100 ng/ml) for 48 h, which resulted in hypertrophy of myocytes, as determined by cell morphology, increased sarcomere organization, and induction of the fetal gene program (results not shown). ANG II treatment of cells also induced (77–80%) the expression of α- and β-tubulin isoforms, compared with untreated controls (Fig. 7). However, when cells were treated with ANG II together with TSA (100 nM) and harvested 48 h later, no significant induction of tubulin expression was observed, suggesting that TSA was able to prevent ANG II-mediated induction of both tubulin isoforms (Fig. 7, A and B). Experiments carried out to examine acetylation of tubulins showed that the ANG II treatment and the resultant hypertrophy had no noticeable effect on the content of the acetylated tubulins. Also, the combined treatment of ANG II plus TSA resulted in levels of acetylated tubulin that were not different from TSA alone, suggesting that acetylation of tubulins has no role in their changed expression levels.

We next determined whether TSA alters the fraction of α- and/or β-tubulins that associate with the polymerized microtubules. Free and polymerized tubulins from myocyte cultures of different treatment groups were fractionated as described in MATERIALS AND METHODS. Equal amounts of proteins from the free and polymerized pools of tubulins were resolved by SDS-PAGE and analyzed by Western analysis using a rabbit polyclonal anti-EGR-1 antibody. Bottom: equal protein loading in individual lanes. NS, nonspecific.

Fig. 4. TSA activates EGR-1-mediated transcription from a heterologous promoter. A: schematic representation of the 3×EGR-1 wild-type and mutated (mt) thymidine kinase (TK)-luciferase (Luc) promoter-reporter constructs. H9C2 cells were grown on 6-well plates and transfected with the indicated promoter-reporter plasmid with or without increasing concentrations of the pCMV-EGR-1 expression plasmid. In each case, a β-galactosidase expression plasmid was included to monitor transfection efficiency. Cells were either treated with TSA (100 nM) or vehicle for 48 h. Results are presented as means ± SE from at least 3 separate experiments. B: TSA induces expression of the endogenous EGR-1 gene. Cells were treated with increasing concentrations of TSA (100 and 300 nM) or vehicle (C) for 48 h. Whole cell lysate was prepared and analyzed by Western analysis using a rabbit polyclonal anti-EGR-1 antibody. Bottom: equal protein loading in individual lanes. NS, nonspecific.
copy. As shown in Fig. 9, in control cardiac myocytes stained for acetylated tubulin, the microtubules appeared as a network of loose loops, with some perinuclear tubular structures. The microtubule reticular pattern appeared random with no specific alignment with myofibrils and was distributed throughout the cytoplasm (similar organization was seen in cells stained with a rhodamine-tagged β-tubulin antibody, results not shown). Every cell in any given field had a unique distribution pattern, probably indicating the highly dynamic state of microtubules.

Treatment of cells with TSA significantly increased the levels of acetylated tubulin; however, we found no detectable change in the organization of microtubules compared with control myocytes. Similarly, when cells were stained for total β-tubulin, we could detect a significant TSA-mediated reduction in tubulin expression, with no discernable effects on microtubule structures (results not shown). In myocytes with ANG II-induced hypertrophy, most microtubules were arranged in dense arrays parallel to each other and to the long axis of the cell (Fig. 9B). TSA noticeably reversed the ANG II-induced density of microtubules as well as the linear arrays of tubulin (Fig. 9C), to a profile that was similar to that for control cells or cells treated with TSA alone. Thus, together, these results demonstrated that an increased proportion of α- and β-tubulins associated with microtubular fraction in hypertrophied myocytes is reversed by TSA treatment of cells.

DISCUSSION

The main intent of this study was to examine the effects of HDAC inhibition on the two major determinants of myocardial contractility, i.e., α-MHC and tubulin expression, as well as cardiac microtubule composition. Our results indicate that the general inhibition of HDAC by TSA increases the α-MHC expression in vitro as well as in vivo, whereas it downregulates the expression of α- and β-tubulin isoforms. We also show that TSA concomitantly decreases the fraction of α- and β-tubulins associated with polymerized microtubules in hypertrophied cardiac myocytes. Additionally, it reverses the structurally dense parallel array configuration of the microtubular network, a main characteristic of ANG II-induced cardiac hypertrophy. Based on the documented importance of α-MHC composition and microtubule density in regulating the cardiac contractility, we believe that the opposite effect of TSA on these two variables might result in enhanced myocardial contractile function.

HDAC inhibition and subsequent chromatin decompaction by histone acetylation have been shown to activate a very specific subset of genes but not generalized gene activation (Ref. 28 and references therein). In this study, by utilizing both in vitro and in vivo model systems where α-MHC levels were suppressed, we were able to demonstrate a significant induction of the α-MHC gene expression on HDAC inhibition by
TSA, without changing the levels of β-MHC. This effect was dependent on new protein synthesis, as the addition of cyclohexamide (a protein synthesis blocker) inhibited TSA-mediated upregulation of the α-MHC gene expression in the cultured myocytes. Results obtained from transfection assays indicated that TSA involved transcriptional mechanisms for induction of the α-MHC gene activity. Previously, several different transcription factors have been identified that bind to an upstream promoter region of the α-MHC gene and control its high level of expression in cardiac myocytes. These include

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**Fig. 6.** TSA treatment represses the expression of cardiac tubulins. Primary cultures of cardiac myocytes were treated with TSA (100 and 300 nM) or DMSO (vehicle) for 48 h, and the expression levels of total α-tubulin (A), β-tubulin (B), and acetylated tubulin (C) were determined by Western analysis. Constant protein loading/lane was ensured by Coomassie blue staining of parallel gels (not shown). Membranes with transferred proteins were probed with either a mouse monoclonal α-tubulin antibody that recognizes total cellular α-tubulin or the rabbit polyclonal β-tubulin antibody that recognizes all subisoforms of β-tubulin or a monoclonal mouse anti-acetylated tubulin antibody that recognizes acetylated tubulins. Relative signal intensity (relative increase above background) was quantified by scanning densitometry and plotted as means ± SE of 3 separate experiments.

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**Fig. 7.** TSA prevents ANG II-induced expression of cardiac tubulins. Cardiac myocytes were treated with TSA (100 nM) and ANG II (100 ng/ml) either alone or together for 48 h. C, control cells treated with vehicle (DMSO). Expression of tubulins was determined with isoform-specific antibodies as described in Fig. 6. Representative Western blots of total α-tubulin (A), β-tubulin (B), and acetylated tubulin (C) are shown. Histogram represents mean ± SE (n = 3) of signal intensity of different bands in Western blots as quantified by scanning densitometry.
GATA4, SRF, MEF-2, and the well-studied thyroid receptors, which have profound effects on α-MHC gene expression (12, 34). The transcription activity of each of these factors is regulated, directly or indirectly, by their association with histone acetyltransferases and HDACs (8, 9, 32). For the regulation of thyroid receptor activity, the presently accepted paradigm is that ligand-bound thyroid receptors recruit coactivator complexes containing histone acetyltransferases, whereas ligand-unbound thyroid receptors recruit HDACs to modify acetylation/deacetylation of the core histones in specific sites on gene promoters, resulting in the altered target gene expression (23). Despite a concerted effort at promoter analysis, we were unsuccessful in demonstrating an obligatory role of the TREs in mediating the TSA effect on the α-H9251-MHC gene promoter in our cell culture system. It should be, however, noted that, at the chromatin level when α-MHC gene is located in its natural milieu, it is possible that some of the TSA effect is contributed by involvement of TRES. Sharma and Fondell (43) reported that the recruitment of thyroid receptor-p300 complex to TRES of certain gene promoters rapidly induces histone acetylation, leading to change in chromatin structure. This is possible in other large thyroid receptor-associated protein co-regulatory complexes that in turn alter the activity of RNA-polymerase II, leading to potentiation of gene transcription. Therefore, despite the lack of evidence for a role for TRES in the induction of α-MHC gene expression by TSA in the isolated cell system, TRES may still have a role in the induction of the endogenous α-MHC gene.

In our subsequent high-throughput array-based analyses for transcription factor activation and resultant transcriptional effects in cardiac myocytes, we found a key role for EGR-1 in mediating the TSA effect. EGR-1 is a 533-amino acid-long nuclear phosphoprotein with three zinc finger domains. EGR-1 is constitutively expressed at high levels in the heart, brain, and lung (47). It has been implicated in diverse pathophysiological responses and functions as an intermediary factor that couples changes in the environment to adaptational changes in the expression of many target genes. EGR-1 has been also shown to play a pivotal role in cardiac and vascular system development. It has been identified as a positive regulator of the α-MHC gene promoter (13). Also, there are reports showing a strong positive correlation between the levels of EGR-1 and activation of the endogenous α-MHC gene expression (25, 26, 47). EGR-1 binds to a GC-rich DNA sequence (CGC-CCCCGC) and has been shown to compete with Sp1 to bind to this motif (5). By utilizing the double-stranded α-MHC gene EGR-1 binding site as a probe, we have shown an increased DNA binding activity from the nuclear extracts of TSA-treated myocytes, compared with untreated controls. Whether EGR-1/Sp1 competition occurs on the α-MHC gene promoter is not known. However, if it does, it will be relevant in explaining the transactivation effect of EGR-1 by changing the activity of Sp1, which has been shown to be responsive to HDAC inhibition leading to gene activation (30, 45). In the present study, by utilizing several additional approaches, including the protein-DNA array, transcription reporter array, cotransfection assays, heterologous promoter-reporter assays, and Western analyses, we concluded that overall TSA-mediated upregulation of EGR-1 expression is necessary for the activation of α-MHC gene expression.

Experiments designed to elucidate the further upstream mechanism activating the EGR-1 gene led us to examine the effect of SRF on the gene expression. The EGR-1 promoter has five well-characterized SRF binding sites, which play a central role in growth factor-dependent activation of the EGR-1 gene. Previously, we as well as others have shown that SRF by itself
is under the control of cofactors having histone acetyltransferase and HDAC activity (9, 19). SRF directly interacts with the class II HDAC, HDAC4, which results in profound repression of SRF transcription activity (9). This is consistent with our observation in this study that the HDAC inhibition by TSA leads to SRF activation. SRF has been also been shown to have an essential role in the induction and maintenance of the cardiac myogenic program (4). In the proximal promoter region of the α-MHC gene, at least two functional SRF-binding sites (CArG boxes) have been identified (34). However, in our transfection analyses, we did not find a direct involvement of SRF in the activation of α-MHC gene expression by TSA. Our compiled results are highly indicative of a key role for EGR-1, with potential upstream involvement for SRF in the TSA-mediated activation of the α-MHC gene promoter activity.

In addition to the role of cardiac MHC isoforms, the components of the myofibrillar and extramyofibrillar cytoskeleton have been shown to influence the cell contractile function by changing the viscosity and passive stiffness of the cell. The extramyofibrillar cytoskeleton in eukaryotic cells is composed of an intertwined network of three classes of filamentous biopolymers, including microtubules, actin-containing microfilaments, and intermediate filaments. Tubulins and the microtubular network form the basis of many common cellular functions, including the structural basis for cell mitosis, organelle placement, exocytosis, receptor recycling, organization, and the activity of plasma membrane channels (21, 40). A role of microtubules in the induction of myogenic program has also been suggested where it is thought to be involved in elongation and alignment of myoblasts before fusion into myotubes and then in the formation and organization of myofibrils (39). Moreover, during the assembly of sarcomeres, microtubules are considered to serve as templates for myosin thick filaments to align. Subsequently, they undergo dynamic depolymerization and are replaced by actin-containing thin filaments. In the heart, the total tubulin content is developmentally regulated. In neonatal cardiac myocytes, the total tubulin content represents 0.08% of the total cell protein and it declines to 0.01% in adult hearts. In other cell types, tubulin comprises 0.2–10% of the total cell protein. Despite this relatively low level of expression of tubulin in adult hearts, its unique function in cardiac myocytes has been shown to regulate cell contractility (53). During cardiac hypertrophy or failure, tubulin expression and microtubule organization have been shown to change dramatically (3, 27, 48, 49, 53). Evidence even shows that, during hypertrophy, a change in microtubular density contributes far more than the MHC isoform shift in the myocardial contractile dysfunction (18). Results presented here demonstrate a significant downregulation of total α- and β-tubulin levels in response to HDAC inhibition by TSA. TSA also significantly reversed the tremendous increase in tubulin expression in response to ANG II-induced cardiac cell hypertrophy, which is predictive of improved cell contractile function.

Recently, α-tubulin was identified as a novel cytoplasmic substrate for a class II HDAC, i.e., HDAC6 (17). HDAC6 is strongly inhibited by TSA, with corresponding intracellular increases in acetylated forms of both free and polymerized α-tubulin, consistent with our findings in primary cultures of cardiac myocytes. The role of tubulin acetylation in microtubule dynamics is still debated. Initially, it was thought that acetylation might promote tubulin polymerization and increase stability of microtubules in different cellular milieu (29).
However, it has now become clear that increased microtubule stability is not caused by hyperacetylation (40). Rather, microtubules are acetylated after assembly, and acetylation appears to be a marker for how long the microtubules have been available to serve as a substrate for the tubulin acetyltransferase. Unmodified tubulin essentially retains the same ability to assemble into microtubules as acetylated unpolymerized tubulin (29). Some studies have indicated that, once stable, microtubules are subsequently posttranslationally modified, a change that interferes with their further growth (14). The increased levels of acetylated tubulin by TSA, whereas not affecting microtubule stability, might mediate a functional difference in tubulins by altering their protein-protein interaction ability. Importantly, we observed that, in hypertrophied myocytes, TSA markedly decreased the total α- and β-tubulin fractions associated with polymerized microtubules. This is likely the result of diminished available pools of free tubulin in response to HDAC inhibition.

How does TSA regulate expression of tubulin? Although the exact mechanism of TSA-mediated decrease in tubulin expression is not known, by using the monoclonal anti-acetylated tubulin antibody, we consistently detected two additional lower bands in TSA-treated samples (~37 and ~25 kDa, Fig. 6C), suggesting that they might have resulted from some active proteolysis of tubulin. In this context, it is worth noting that the tubulin-specific carboxy-peptidase enzyme that mediates protein detyrosination cleaves tubulin at a COOH-terminal tyrosine residue, leaving the glutamine (Glu) tubulin (named for its newly exposed COOH-terminus residue) fragment. This process is reversible with tubulin-tyrosine ligase, which adds a tyrosine residue to the COOH-terminus of the Glu-23 tubulin, leading to reformation of tyrosine tubulin (51, 52). Interestingly, Glu-tubulin subsets have been demonstrated to overlap with the acetylated subset of tubulin in differentiated cells; in fact, acetylated tubulin has been considered to be a target for tubulin carboxy peptidases (51). Thus it is likely that acetylated tubulin becomes a target for some active proteolytic process. However, it is also likely that the lower bands that we observed originate from nonspecific proteins, whose expression is highly induced by TSA. Future experiments designed to explore the mechanism of TSA effect on tubulin expression should be able to address this issue, which is beyond the scope of this paper. The present consensus is that increased cardiac myocyte microtubule network mass via upregulated expression of tubulin isoforms and/or increased microtubule density plays an important role in ventricular contractile dysfunction through viscous loading of active myofilaments. There is an emergent correlation between lowered left ventricle fractional shortening to the degree of microtubule protein expression and polymerization both in animal models of pressure overload hypertrophy and subsequent heart failure and in patients with failing hearts. The data presented here show that the two components that have profound consequences in the contractile function, that is, myosin isoform shift and the extramyofilament cytoskeletal proteins such as tubulins, are targets of TSA treatment. It appears that HDAC inhibition in cardiac myocytes mediates changes to these contractile components in a manner that is independently predictive of gain in contractility. Thus the present report, although it improves our understanding of epigenetic regulation of cardiac myocytes, provides the basis for testing the effect of HDAC inhibition on myocardial contractility. These studies together with the increasing reports of antihypertrophic effects of HDAC inhibition will have important therapeutic implications in the management of heart failure.

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REFERENCES