Increased microtubule density, through viscous loading of active myofilaments, causes contractile dysfunction of hypertrophied and failing pressure-overloaded myocardium, which is normalized by microtubule depolymerization. We have found this to be based on augmented tubulin synthesis and microtubule stability. We show here that increased tubulin synthesis is accounted for by marked transcriptional up-regulation of the β1- and β2-tubulin isoforms, that hypertrophic regulation of these genes recapitulates their developmental regulation, and that the greater proportion of β1-tubulin protein may have a causative role in the microtubule stabilization found in cardiac hypertrophy.

When under pathological circumstances the heart is forced to eject blood against an increased impedance, the terminally differentiated cardiac muscle cell, or cardiocyte, responds by hypertrophic growth (1). The resultant increase in muscle mass constitutes the basic compensatory cardiac response to sustained hemodynamic overloading, but this initial compensation is frequently vitiated by a progressive decline in cardiocyte contractile function (2), so that congestive heart failure ensues.

We have found that this cardiocyte contractile defect is caused by increased density of the cellular microtubule network (3), which imposes a viscous load on the shortening sarcomeres during contraction (4). Thus, microtubule depolymerization in hypertrophied cardiocytes restores normal cellular contractile function, and induced microtubule hyperpolymerization in normal cardiocytes causes these cells to exhibit the same contractile abnormality found in hypertrophied cells (3).

The αβ-tubulin heterodimer-microtubule system is in a dynamic steady state. Therefore, in attempting to uncover the cause of increased microtubule density in hypertrophied cardiocytes, we focused on increased tubulin synthesis (5) and thus microtubule formation as well as on increased stability of the microtubules once formed. With respect to the latter, we have indeed found marked stabilization of the microtubule network in hypertrophied cardiocytes associated with a substantial increase in the predominant microtubule-associated protein of the heart, MAP4 (6). Although recent data (7, 8) put into question the previously accepted role of MAP4 in microtubule assembly and stability for some cell types, the muscle-specific variant of MAP4 appears to play a role in striated muscle (9). Given that MAPs and the expressed proteins of the β-tubulin multigene family exhibit coordinate developmental regulation (10) and that the latter may via their isoform-variable carboxy-terminal domain confer differing MAP binding affinity and microtubule stability after assembly (11), the question of whether there is differential regulation of the members of the β-tubulin multigene family during cardiac hypertrophy assumed pivotal importance.

EXPERIMENTAL PROCEDURES

Right Ventricular Pressure Overloading—Pressure overload hypertrophy of the feline right ventricle (RV) was created (12) by placement of a 2.9-mm inner diameter band around the proximal pulmonary artery. Because the RV mass increase stabilizes by 2 weeks after a step increase in load (5), at 2 weeks after surgery intravascular pressures were measured in these and in control cats; values in the systemic circulation were the same for both groups. The heart was then removed, RV mass was determined, and Ca2+-tolerant quiescent cardiocytes were isolated enzymatically from the RV and left ventricle (LV) separately (13). All operative procedures were carried out under full surgical anesthesia; all procedures and the care of the cats were in accordance with institutional guidelines. At 2 weeks, RV systolic pressure was doubled, and there was a 59% increase in the ratio of RV to body weight; the mass of the normally loaded same animal control LV was unchanged.

Western Blotting—Peptide synthesis and coupling were performed as described (14) with minor modifications. The peptides underlined in Fig. 1 were synthesized, purified, keyhole limpet hemocyanin-conjugated via glutaraldehyde cross-linking, and injected into rabbits. Sera were monitored on slot blots using bovine serum albumin-conjugated peptides until high antibody activity was achieved; the IgG fractions were then purified using covalently coupled peptide columns. Bm9 fragments of the human hβ1, mouse mβ2, and human hβ2 (14) genes, which encode the carboxyl-terminal 100 amino acids of β1-, β2-, and β4-tubulin as noted here, respectively, were ligated into the pET-28 histidine-tagged expression vector (Novagen). Cultures were induced to express for 1.5 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were then spun down, resuspended in binding buffer, and lysed with a French press. The resultant fusion proteins, after isolation on a Ni2+-chelation column (Novagen), were used to verify the monospecificity of each of the site-directed polyclonal antibodies. Because of potentially differing antibody affinities, aliquots of the purified fusion proteins for β1-, β2-, and β4-tubulin were resolved on SDS-PAGE and silver-stained; loading was adjusted to produce equal tubulin band intensities. For the subsequent immunobLOTS, fusion protein loading normalized in this manner was then used as an internal standard for analysis of the tubulin present in the samples, where the ratio of integrated optical density for the single band for each sample to that for each fusion protein was estimated.

Northern Blotting—To generate isoform-specific cDNA probes, the 3'-untranslated region of each β-tubulin isoform gene was cloned from the cat. β1- and β4-tubulin were cloned from a cat cdNA library (Stratagene) by PCR, polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid.
corresponding to the underlined region of each β-tubulin isoform shown in the upper panel, which uses the one-letter amino acid code, were used to prepare site-directed polyclonal antibodies. Fusion proteins corresponding to the β1, β2, and β4 isoforms were then used, as shown in the lower panel, to determine antibody specificity. These fusion proteins, with concentrations adjusted to produce comparable staining intensities, were loaded onto each lane of the 12.5% SDS-PAGE before immunoblotting and visualization by enhanced chemiluminescence (DuPont). Each isoform-specific antibody bound strongly to the corresponding fusion protein, but there was no detectable antibody cross-reactivity.

![Image of immunoblots of β-tubulin isoforms in RV and LV myocardium from a control cat and a RV pressure-overloaded cat 2 weeks after pulmonary artery banding.](image1)

**FIG. 2.** Immunoblots of β-tubulin isoforms in RV and LV myocardium from a control cat and a RV pressure-overloaded cat 2 weeks after pulmonary artery banding. Both free (lanes 1 and 3) and polymerized (lanes 2 and 4) tubulin fractions, prepared and validated as before (6), were probed with the β1-, β2-, or β4-tubulin-specific antibodies or, for total β-tubulin, with an antibody denoted as “common β,” which recognizes an epitope common to all β-tubulin isoforms (DM1B, Amersham Pharmacia Biotech). Equal proportions of the free and polymerized samples were loaded onto the two lanes for each ventricle, and an equal amount of protein as determined by a bicinchoninic acid assay (Pierce) was loaded for the RV and LV samples. The LV pressure of both cats was normal and equivalent.

![Image of a table comparing fusion proteins and peptides.](image2)

**Table 1.** Fusion proteins and corresponding peptides used for antibody generation, and validation of the specificity of three of these antibodies. Polyclonal antibodies were generated using the peptides corresponding to the underlined region of each β-tubulin isoform shown in the upper panel, which uses the one-letter amino acid code, were used to prepare site-directed polyclonal antibodies. Fusion proteins corresponding to the β1, β2, and β4 isoforms were then used, as shown in the lower panel, to determine antibody specificity. Each isoform-specific antibody bound strongly to the corresponding fusion protein, but there was no detectable antibody cross-reactivity.
laminin-coated coverslips for 45 min and then, after 1 h of exposure to either 37 or 8 °C, extracted for 1 min in 1% Triton X-100 in microtubule stabilization buffer (16), washed three times in the same buffer, and fixed for 30 min with 3.7% formaldehyde, all at 25 °C. After blocking with 10% donkey serum in 0.1M glycine, the cardiocytes were incubated overnight at 4 °C both with a 1:200 dilution of our rabbit b1-tubulin-specific antibody and with a 1:500 dilution of a mouse monoclonal antibody (B-5-1-2, Sigma), which recognizes all native a-tubulin isoforms (17), followed by both Cy3-labeled anti-rabbit IgG and Cy5-labeled anti-mouse IgG (Jackson ImmunoResearch) secondary antibodies. Micrographs were acquired as single 0.7-μm confocal sections taken at the level of the nuclei (LSM GB-200, Olympus).

Cardiac Developmental Expression of b-Tubulin—Pregnant and 1-, 20-, and 90-day-old postpartum Sprague-Dawley rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). The 15-day-old embryonal and 1-day-old neonatal rats were decapitated, the hearts were removed, and the atria and great vessels were trimmed away. About 30 embryonal hearts and 50 mg of ventricular myocardium from the other stages were homogenized in 500 μl of lysis buffer (10 mM Tris, 0.5 mM dithiothreitol, 1 mM sodium vanadate, 1% sodium dodecyl sulfate, pH 7.4), boiled for 5 min, and centrifuged at 16,000 × g at room temperature for 10 min; the supernatants were saved. For the subsequent 12.5% SDS-PAGE, an equal amount of protein (25 μg) as determined by a bicinchoninic acid assay (Pierce) was processed with SDS-sample buffer and loaded for each sample; immunoblotting was done with the same antibodies as those specified in Fig. 1. Three samples from each stage were studied with confirmatory results.

RESULTS AND DISCUSSION

The five b-tubulin isoform proteins whose expression was examined in this model, classified according to Cleveland (14, 18), are shown in Fig. 1. Site-directed polyclonal antibodies were generated against each of these b-tubulin isoforms using synthetic peptides having the sequences underlined in Fig. 1. After preparing the appropriate fusion proteins, the specificity of the peptide column-purified b1, b2, and b4 antibodies was validated, as also shown in Fig. 1. No reactivity of the b3 and b5 antibodies with homogenates from either normal or hypertrophied myocardium was detected despite strong reactivity of each antibody with the respective bovine serum albumin-conjugated peptide.

Immunoblots of normal feline hearts showed that b4-tubulin expression is greatly preponderant (see below). As shown in Fig. 2, by taking advantage of the much higher affinity of the b1- and b2- as opposed to the b4- and common (all isoforms)
β-tubulin antibodies and by varying blot exposure times, it was possible to visualize all four classes of β-tubulin in the same homogenates, where the samples from the control and RV

dehydrated for 0 (lane 5) and 18 (lane 6) h. These findings were confirmed in cardiocytes from another cat with 2 days of RV pressure overloading.

This increase was accounted for in its entirety by increases in hypertrophy hearts were treated identically. In the hypertrophied RV, despite the marked increase in both free and polymerized isoform-common β-tubulin, there was little change in

β-tubulin isoforms and isoform-common total β-tubulin in RV and LV cardiocytes from a cat RV pressure-overloaded 2 weeks earlier. Cardiocytes were isolated from each ventricle and treated with 5 μg/ml actinomycin D. Northern blots show message levels for each isoform and for total β-tubulin in RV and LV cardiocytes isolated from each ventricle and treated with 5 μg/ml actinomycin D and 10 μM colchicine and incubated for 0 (lane 5) and 4 (lane 6) h. These findings were confirmed in cardiocytes from another cat with 2 days of RV pressure overloading.

When the time course of these changes was examined, where the pressure-overloaded RV was compared with the normally loaded LV in five cats at each time of 2 days, 1 week, and 2 weeks after an increase in RV load, by 1 week there were increases in both the polymerized and free β-tubulin fractions, with the greater increase being in the polymerized fraction. Of singular interest, as shown by the immunoblot summary data in Fig. 3, solely for β1-tubulin in the hypertrophied RV there was a disproportionate increase in the microtubule-assembled pool. That is, at 2 weeks of RV pressure overloading the increase of β1-tubulin in the microtubule-assembled pool was about twice that in the unassembled heterodimer pool. Although functional significance has been ascribed to differential β-tubulin isoform expression in the male germ line of Drosophila (19, 20) and has been inferred from heterogeneous cellular isoform distributions (e.g., Ref. 21), the present data represent the first such direct evidence in vertebrates, and this is in a context of potential consequence to an important human disease state.

The two cytosolic lanes were loaded equally as were the two cytoskeletal lanes; the loading ratio of the soluble versus cold-stable fractions was constant. The same result was obtained in the hearts from four further RV pressure-overloaded cats. The right panels show double label immunofluorescence confocal micrographs of cardiocytes isolated from the RV and LV of a cat heart 2 weeks after RV pressure overloading. Prior to fixation, the two upper cells were maintained at 37 °C and the two lower cells at 8 °C for 1 h; the latter condition causes selective depolymerization of labile microtubules (6). These cells were double-stained for β1-tubulin (red) and for α-tubulin (green), where β1- and α-tubulin primary antibodies were followed by species-specific fluorochrome-conjugated secondary antibodies; areas of coincident decoration by both primary antibodies range in color from orange to yellow. The inset at the lower right corner of each micrograph is a magnified view of a segment of a single microtubule. Cardiocyte preincubation with the β1-tubulin peptide abolished β1-tubulin labeling. When this protocol was repeated using the β2-tubulin antibody, very little microtubule decoration was apparent, and it was not obviously selective for cold-stable microtubules (data not shown).

FIG. 5. Message stability for β-tubulin isoforms and isoform-common total β-tubulin in RV and LV cardiocytes from a cat RV pressure-overloaded 2 weeks earlier. Cardiocytes were isolated from each ventricle and treated with 5 μg/ml actinomycin D. Northern blots show message levels for each isoform and for total β-tubulin at 0 (lane 1) and 18 (lane 4) h after actinomycin D treatment. Message half-life averaged ~6 h for each mRNA species examined in this and three further cats with 2 weeks of RV pressure overloading. Additional RV and LV cardiocytes were exposed simultaneously to both 5 μg/ml actinomycin D and 10 μM colchicine and incubated for 0 (lane 5) and 4 (lane 6) h. These findings were confirmed in cardiocytes from another cat with 2 days of RV pressure overloading.

FIG. 6. β-Tubulin isoform localization via immunoblotting and confocal microscopy. The left panels show an immunoblot analysis of β-tubulin isoforms in soluble cytosolic and cold-stable cytoskeletal fractions of the RV and LV of a cat heart 2 weeks after RV pressure overloading. The two cytosolic lanes were loaded equally as were the two cytoskeletal lanes; the loading ratio of the soluble versus cold-stable fractions was constant. The same result was obtained in the hearts from four further RV pressure-overloaded cats. The right panels show double label immunofluorescence confocal micrographs of cardiocytes isolated from the RV and LV of a cat heart 2 weeks after RV pressure overloading. Prior to fixation, the two upper cells were maintained at 37 °C and the two lower cells at 8 °C for 1 h; the latter condition causes selective depolymerization of labile microtubules (6). These cells were double-stained for β1-tubulin (red) and for α-tubulin (green), where β1- and α-tubulin primary antibodies were followed by species-specific fluorochrome-conjugated secondary antibodies; areas of coincident decoration by both primary antibodies range in color from orange to yellow. The inset at the lower right corner of each micrograph is a magnified view of a segment of a single microtubule. Cardiocyte preincubation with the β1-tubulin peptide abolished β1-tubulin labeling. When this protocol was repeated using the β2-tubulin antibody, very little microtubule decoration was apparent, and it was not obviously selective for cold-stable microtubules (data not shown).
the β1 and β2 isoforms. This pattern was found in additional studies to be maintained for up to 6 months after the hypertrophic response was complete (data not shown).

Expression of β-tubulin isoform transcripts was then examined by Northern blot analysis. Fig. 4, where the alternate polyadenylation site of β1-tubulin (22) produces two transcripts, shows that the pattern of β-tubulin isoform expression on the mRNA level mimics that seen on the protein level. That is, the amount of β4-tubulin mRNA was equivalent in the RV and LV of control cats and changed very little in either ventricle during the development of RV hypertrophy. There was, however, a striking and persistent up-regulation of β1- and β2-tubulin mRNA in the hypertrophying RV.

To gain some insight into the relationship of mRNA levels to protein levels for these β-tubulin isoforms, we examined mRNA stability in cardiocytes from the hypertrophied RV and the control LV of pulmonary artery-banded cats. Because it has not proved possible to isolate transcriptionally active nuclei from these cells, we used actinomycin D to inhibit RNA polymerase in fresh primary cultures of these cells and then measured the rate of decline of mRNA levels for the β-tubulin isoforms and for total β-tubulin by Northern analysis. Lanes 1–4 of Fig. 5 show that mRNA stability estimated in this manner is quite similar in hypertrophied RV versus control LV both for the three β-tubulin isoforms examined and for isoform-common total β-tubulin. The same result was obtained in the RV versus LV of normal cats (data not shown). Thus, increased β1- and β2-tubulin protein in hypertrophied myocardium results from increased transcription of these genes.

The concurrent up-regulation of β-tubulin on both the protein and the message levels, which we have found to persist indefinitely in myocardium hypertrophying in response to a pressure overload (5), appears to contravene the co-translational negative feedback control that tubulin exerts on its own rate of synthesis (23) via reduced mRNA stability. That is, the stability of ribosome-bound β-tubulin mRNA is controlled by co-translational binding of either β-tubulin itself or an intermediary factor to the amino-terminal β-tubulin tetrapeptide as it emerges from the ribosome. This binding then activates an RNase or causes ribosomal stalling with the result in either case being accelerated β-tubulin mRNA degradation, such that the net effect is an inverse relation between β-tubulin protein concentration and β-tubulin mRNA half-life. A breakdown of this regulatory control of tubulin synthesis, which might selectively affect different β-tubulin gene products, could explain increased microtubule density in hypertrophied cardiocytes. Thus, we used colchicine to acutely increase the concentration of tubulin heterodimers in control and hypertrophied cells and observed the effect of this intervention on tubulin mRNA stability. Lanes 5 and 6 of Fig. 5 show that to an equivalent degree for both control and hypertrophied cardiocytes, colchicine-induced microtubule depolymerization accelerates the rate of mRNA degradation for all three β-tubulin isoforms and for total β-tubulin. Further, in intact cats given 1 mg/kg colchicine intravenously with or without 2 or 7 days of RV hypertrophy, where α- and β-tubulin mRNAs are markedly increased in hypertrophied RVs (5), β-tubulin mRNA levels had decreased equivalently 4 h later in normal and hypertrophied RVs and in the control LVs to 25.5 ± 1.7% of the respective control values for cats not given colchicine. Thus, these data demonstrate that
the co-translational regulatory mechanism for controlling tubulin mRNA stability is intact in normal and hypertrophied terminally differentiated cardiocytes, such that, again, there is an authentic increase in the transcription of the β1- and β2-tubulin genes in cardiac hypertrophy. However, they also strongly suggest that such co-translational control is exerted as a rate-dependent rather than a concentration-dependent function of the cytosolic concentration of tubulin heterodimers, a finding having general rather than cardiocyte-restricted implications.

A central goal of this study was to determine whether up-regulation of specific β-tubulin isoforms during cardiac hypertrophy was discovered, such changes in gene expression have functional significance. That is, the cardinal alterations of the extramyofilament cytoskeleton of hypertrophied cardiocytes, in terms of inducing contractile dysfunction, are interrelated increases in the quantity and stability of the microtubule network. If there were increased expression of specific β-tubulin genes, the protein products would directly account for the persistent increases in both free and polymerized tubulin. However, if one or more of the up-regulated isoforms conferred greater stability on the microtubules once assembled, this would contribute to increased density of the microtubule network via a mechanism independent of augmentation of the heterodimer pool. Thus, because stable microtubules are resistant to cold-induced depolymerization (6), we measured β1-, β2-, and β4-tubulin in the cold-stable cytoskeletal fraction of normal and hypertrophied myocardium and cardiocytes. The left panels of Fig. 6 show that although the proportion of β2- or β4-tubulin in this fraction is very low for normal or hypertrophied myocardium, a significant proportion of β1-tubulin is found in the cold-stable cytoskeletal fraction, and this is more pronounced for the hypertrophied RV. The right panels of Fig. 6 show that the microtubule array of the hypertrophied cardiocyte is more cold-stable than that of the control cardiocyte, that microtubules of the hypertrophied cardiocyte incorporate more β1-tubulin than those of the control cardiocyte, and that this latter finding is especially pronounced in the cold-stable microtubules of the hypertrophied cell. These findings are consistent with densitometric analysis of tubulin isoforms, because the data in Fig. 7 show first in normal RVs and LVs a greater proportion of β1-tubulin in the cold-stable microtubule fraction and second a selective further shift solely of β1-tubulin to this fraction in the hypertrophied RV. Nonetheless, there is not necessarily an exact correspondence between microtubule cold stability and microtubule stability in vivo, such that these correlative data do not constitute proof that β1-tubulin-enriched microtubules are more stable in the intact cardiocyte of the heart in situ. We are therefore testing this point directly via adenovirus-mediated overexpression of β1-tubulin in isolated cardiocytes and via cardiac-targeted β1-tubulin overexpression in transgenic mice.

Because many protein isoforms normally expressed in the developing heart and then down-regulated in the adult heart are re-expressed after hemodynamic hypertrophic stimulation and because the specificity of these isoform switches may become important to understanding transcriptional regulation during cardiac hypertrophy, we examined cardiac developmental regulation of the β-tubulin multigene family in hearts extirpated from a developmentally timed series of rats that were subjected to immunoblot analysis of β1-, β2-, and β4-tubulin as well as total isoform-common β-tubulin. Fig. 8 shows that during embryonic day 15 and postpartum day 90 there is a rather modest decrease in total β-tubulin and a very minor increase in β4-tubulin. However, both β1- and β2-tubulin peak at postpartum day 1 and decline to very low levels in the adult heart.

In common with the questionable functional significance of many other cardiac isoform switches wherein hypertrophy recapitulates phylogeny (1), these data do not imply a role for altered β-tubulin isoform expression in the generative processes either of cardiac hypertrophy or of cardiac development. Rather, the functional significance of the hypertrophic cardiac β-tubulin isoform switch described here is presently of known consequence only in terms of the resultant disordered contractile function (3). However, hypertrophic expression of the β-tubulin multigene family clearly does recapitulate its developmental expression, which, again, may provide eventual insight into β-tubulin transcriptional control mechanisms common to these two phases of cardiocyte growth.

Apart from the intrinsic interest of these observations, the major impetus for this study was to ascertain their basis in terms of the augmented tubulin quantity (5) and microtubule stability (6) found in the pressure overload-hypertrophied heart. Although our finding of MAP4 up-regulation in cardiac hypertrophy (6) may well be important to the latter phenomenon, it does not directly explain the former. Thus, the possibility that increased expression of one or more members of the β-tubulin multigene family might explain both the greater quantity of tubulin and the greater microtubule stability, either directly via differing intrinsic properties or indirectly via differing MAP4 affinities (11), was quite intriguing. The data in this study indeed show that whereas expression of the predominant cardiac β-tubulin isoform is but little affected, there is marked up-regulation of two ordinarily minor cardiac β-tubulin isoforms. In addition to the possibility that this explains augmented tubulin quantity, selective localization of the β1-tubulin isoform to stable microtubules may also explain augmented microtubule stability. Finally, the fact that hypertrophic regulation of β-tubulin mimics its developmental regulation may provide the eventual insight required to understand transcriptional regulation of the β-tubulin gene family during cardiac hypertrophy.

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