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Abstract

Cytoskeletal reorganization has been shown to participate in cellular remodeling and in the alterations of mechanical function of isolated cardiomyocytes during pressure overload hypertrophy. Post-translational modifications of tubulin towards stabilization of microtubules have also been described in animal models of compensatory hypertrophy, but the status of the microtubules network in end stage heart failure is not clearly established. Using a rat model of congestive heart failure (CHF) induced by aortic banding, we studied the expression of α- and β-tubulin, as well as their post-translational modification and distribution in the soluble and polymerized fraction by immunoblotting. We found an accumulation of α- and β-tubulin protein content specifically in the soluble fraction with no change in the polymerized fraction. Amongst the several variants of α-tubulin examined, only detyrosinated Glu-tubulin and deglutamylated Δ_2-tubulin levels were selectively increased during heart failure. Glu-tubulin accumulated in the polymerized fraction while Δ_2-tubulin levels were increased in the soluble fraction in CHF hearts. These results show that a profound remodeling of the microtubule network occurs in heart failure. This remodeling suggests an increase in the stability of the microtubule network which is discussed in terms of possible functional consequences. (Mol Cell Biochem **237**: 39–46, 2002)

Key words: heart failure, microtubules, post-translational modifications; α- and β-tubulin

Introduction

Microtubules constitute a dynamic network of α- and β-tubulin dimers which assemble and disassemble with both fast (dynamic microtubules) and slow (stable microtubules) kinetics [1]. Recent studies suggest that the microtubule network is involved in many cellular functions such as growth, signaling pathways, intracellular trafficking, protein synthesis, and organelles interactions [2].

In cardiac myocytes, contraction and stretch increase the amount of mRNA and β-tubulin protein, an effect mimicked by α-adrenergic stimulation and potentiated by angiotensin II, two stimuli involved in cell growth [3]. Reciprocally, increases in tubulin and microtubule contents reduce the beating rate of cardiac myocytes [4]. In addition, taxol, a microtubule-stabilizing agent, increases the viscous component of the passive stiffness of isolated myocytes [5]. Thus, the total tubulin (and/or microtubule) content may alter the contractility of normal cardiac myocytes.

The involvement of the microtubule network in cell growth and contractile dysfunction of hypertrophied cardiomyocytes was first suggested by Tsutsui *et al*. [6]. It is well know that cell architecture is modified and/or disorganized during various cardiac diseases, and cytoskeletal protein alterations are known to participate in cardiac myopathy, hypertrophy and failure [7, 8]. Increases in microtubule content and up-regulation of tubulin mRNA and proteins were observed during cardiac hypertrophy and failure, both in animal models and

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human biopsies [8, 9]. In diseased hearts, the reduction of contractility was shown to parallel the accumulation of microtubules and could be corrected by microtubule-depolymerizing treatments [10, 11]. The participation of microtubules in the cardiomyocyte contractile dysfunction has been characterized in ventricular hypertrophy [12] and heart failure [13–16] although the generalization of these observations is still a matter of debate [7, 13, 14, 17, 18].

Although it appears that the microtubule network is increased with intense growth of the cardiac myocyte, its composition may also change in diseased states. Indeed, the α-tubulin subunit can undergo a variety of post-translational modifications [19]. Acetylation of Lys40 or C-terminal detyrosination (yielding Glu-tubulin) are reversible modifications that correlate with an increased stability of the microtubules in neonatal cardiac myocytes [19–22]. The irreversible loss of the C-terminal Glu residue of Glu-tubulin yields Δ_2-tubulin which is a marker of long-lived microtubules [23]. Importantly, Glu-tubulin and Δ_2-tubulin levels were found to increase in an animal model of right ventricular hypertrophy [24]. These observations raised the possibility that changes in post-translational modifications of tubulin, rather than (or in addition to) the total amount of tubulin, might also participate in cellular dysfunction during heart failure.

In the present study, our goal was to examine the status of microtubules network in end stage heart failure. We demonstrate that profound post-translational modifications of cardiac tubulin occur in the left ventricle (LV) of rats in severe congestive heart failure (CHF).

Materials and methods

Experimental model

Thoracic aortic stenosis was induced in male Wistar rats (60–70 g) weaned by placing a stainless steel hemoclip of 0.6 mm width around the ascending aorta as previously described [25]. This investigation was carried out in accordance with the Helsinki Recommendations for Humane Treatment of Animals during Experimentation. Seven months after surgery, mortality was ~ 70% in the CHF group. Animals were anesthetized with an intraperitoneal injection of urethane (0.2/100 g).

Myocardial homogenates

For Western blot analysis, fresh left ventricle (LV) specimens were homogenized in microtubule stabilizing buffer of the following composition: 50% glycerol, 5% dimethyl sulfoxide, 10 mmol/L sodium phosphate, 0.5 mmol/L EGTA, 0.5 mmol/L $MgSO_4$ (pH 6.95), 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL pepstatin, 1 mmol/L PMSF and centrifuged (100,000 g, 25°C, 15 min). The supernatants corresponded to free tubulin fraction. The pellets were resuspended at 0°C in microtubule depolymerizing buffer: 0.25 mol/L sucrose, 10 mmol/L sodium phosphate, 0.5 mmol/L $MgSO_4$ (pH 6.95) 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL pepstatin, 1 mmol/L PMSF. After 1 h at 0°C, microtubules were centrifuged at 100,000 g (4°C, 15 min), and the supernatants corresponded to the initially polymerized tubulin fractions. No tubulin was detected in the supernatant when the pellet was re-extracted in the depolymerizing buffer.

Western blot analysis

Ten percent SDS-PAGE were loaded with identical protein amounts of the free and the polymerized tubulin fractions. The polyclonal antibody to Glu tubulin and monoclonal antibody to Δ_2-tubulin were generous gifts from Dr. L. Paturle-Lafanechere (Laboratoire du Cytosquelette, INSERM U366, Grenoble, France). The Tyr tubulin (TUB-1A2), α-tubulin (DM1 A), β-tubulin (TUB 2.1) and acetylated tubulin (clone 6-11B-1) monoclonal antibodies were purchased from Sigma. Samples were probed with the appropriate primary antibody (2 h), followed by incubation with horseradish peroxidase conjugated anti rabbit or antimouse IgG secondary antibody (1 h), and revealed with an enhanced chemiluminescent substrate (ECL kit, Amersham). Light emission was detected using films (Kodak Biomax Light-1, Sigma) and bands were quantified using an image-analysis system (Biorad) and normalized relative to the actin band that occurred at 42 kDa.

Statistical analysis

Values are presented as means ± S.E.M. An unpaired Student's *t*-test was used to determine the statistical difference of means between sham-operated and CHF groups. Differences were considered significant when $P < 0.05$.

Results

Anatomical data

The anatomical characteristics of CHF rats and sham-operated rats are summarized in Table 1. CHF animals exhibited lower body weight and increased heart weight. The increase in the absolute and relative weights of both left and right ventricles, as well as lungs, indicated severe heart failure in these animals. This was reinforced by anatomical indexes of cardiac decompensation such as ascites, congestion, pleural effusions, and edema. Moreover, as shown in our earlier study [25], left ventricular end diastolic pressure is dramatically

Table 1. Characteristic of CHF and sham-operated rats

Anatomical data	Sham n = 15	CHF n = 13	p*
Body weight (BW), g	674 ± 17	488 ± 19	< 0.001
Heart weight (HW), g	1.5 ± 0.05	3.3 ± 0.19	< 0.001
Right ventricle (RV), g	0.3 ± 0.01	0.58 ± 0.03	< 0.001
Left ventricle (LV), g	1.0 ± 0.02	1.87 ± 0.12	< 0.001
Tibia length (TL), cm†	4.7 ± 0.02	4.59 ± 0.04	n.s.
HW/TL, mg/cm†*	324 ± 10	717.4 ± 37.2	< 0.001
RVW/TL, mg/cm†	58 ± 3	126 ± 8	< 0.001
LVW/TL, mg/cm†	217 ± 5.1	406 ± 25	< 0.001
LuW/TL, mg/cm†	376 ± 13	705 ± 69	< 0.001
LiW/TL, g/cm†	4.5 ± 0.2	3.6 ± 0.2	< 0.003

Data show the means ± S.E.M. *compared with sham. n = number of animals. †n = 11 for sham. n.s. – non significant.

increased in this model (from 10–62 mm Hg) confirming the occurrence of severe heart failure in this model.

Tubulin contents

α-Tubulin was identified as two bands (~ 55 and ~ 60 kDa) in cardiac ventricles from both sham-operated and CHF rats (Fig. 1A). As shown in Fig. 1B, the total amount of α-tubulin was increased ≈ 1.6-fold in CHF as compared to sham-operated hearts (Fig. 1B). Because α-tubulin occurs as an heterodimer with β-tubulin, we also examined the total amount of cardiac β-tubulin. As shown in Fig. 1C, two bands of β-tubulin were also observed in sham and CHF LV. Total β-tubulin significantly increased ≈ 1.3 fold in hearts of CHF as compared to sham animals (Fig. 1D). Thus, both α- and β-tubulin levels were increased during severe heart failure induced by aortic stenosis.

Next we examined the distribution of a-tubulin in the free soluble and polymerized fractions. As shown in Fig. 2, α-tubulin was found in both fractions, but at higher amounts in the free soluble one. Most interestingly, a dramatic increase in a -tubulin level was observed in the free soluble fraction during heart failure while the amount of α-tubulin in the polymerized fraction remained unchanged (Fig. 2B). Thus, the increase in total α-tubulin observed in CHF rats is due to a selective increase of the free soluble form.

Truncated isoforms of tubulin

We then examined the post-translational modifications of α-tubulin in CHF and sham-operated hearts. First, the amount of full-length tubulin was evaluated using a Tyr-tubulin specific antibody which recognized a single band (at 55 kDa) in both sham-operated and CHF hearts. As shown in Table 2, there was no significant difference between the average

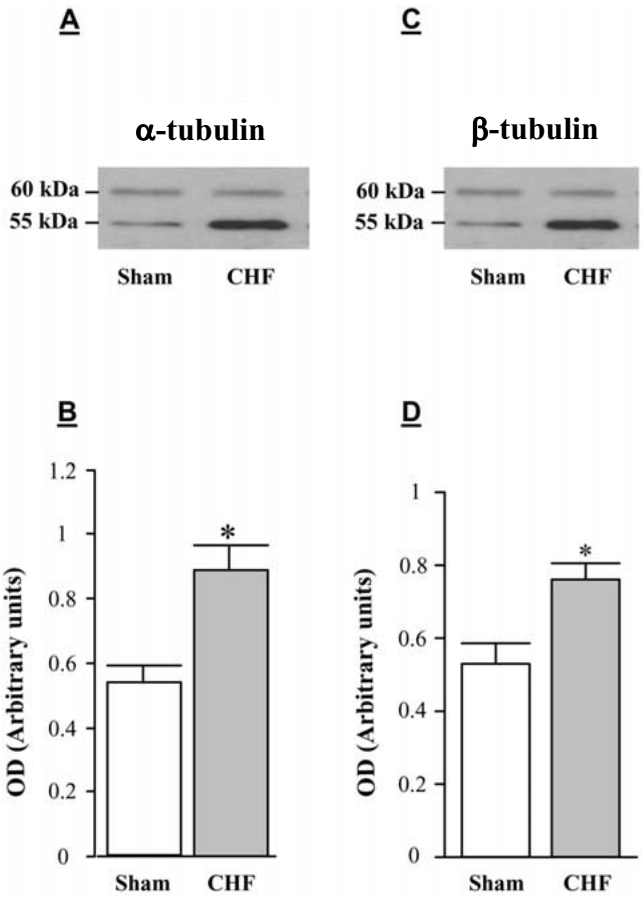

Fig. 1. α- and β-tubulin in heart failure. (A) Representative Western blot of α-tubulin in sham-operated and CHF hearts. Equal amounts (15 μg) of LV homogenates were loaded in each lane and probed with the anti α-tubulin antibody. Two bands are observed (~ 55 and ~ 60 kDa). (B) Summary of the total α-tubulin contents in LV from CHF (filled bars) and sham-operated rats (empty bars). (C) Representative Western blot of β-tubulin in sham-operated and CHF hearts. Equal amounts (15 μg) of LV homogenates were loaded in each lane and probed with the anti β-tubulin antibody. Two bands are observed (~ 55 and ~ 60 kDa). (D) Summary of the total β-tubulin contents in LV from CHF (filled bars) and sham-operated rats (empty bars). In (B) and (D), the bars are the means ± S.E.M. of 12 experiments; significant differences are indicated as * ($p < 0.05$).

amounts of Tyr-tubulin in failing and non-failing hearts. Second, the amount of Glu-tubulin was evaluated using a polyclonal anti Glu-tubulin antibody. As shown in Fig. 3A, this antibody recognized two bands, at 55 and 60 kDa in both sham-operated and CHF hearts. Most interestingly, there was a dramatic increase in the amount of Glu-tubulin in CHF rats. On average (Fig. 3B), Glu-tubulin was ~ 3 times higher in LV from CHF rats as compared to sham-operated animals. As expected, Glu-tubulin was exclusively present in the polymerized fraction of α-tubulin in both sham and CHF hearts (Figs 4A and 4B), and its level increased by up to 4-fold during heart failure (Fig. 4B).

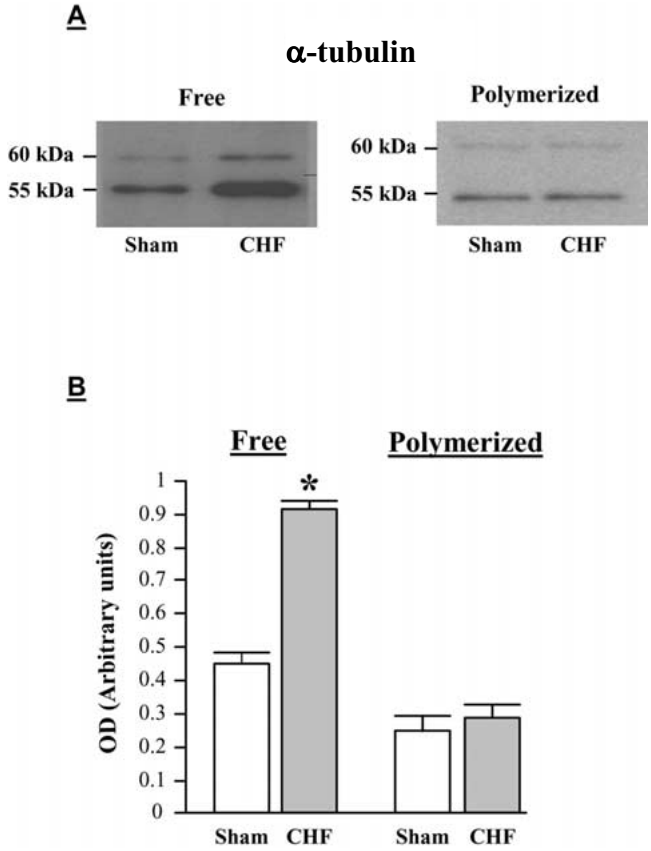

Fig. 2. Free and polymerized pools of α-tubulin in heart failure. (A) Representative Western blot of the free soluble and polymerized fractions of α-tubulin in sham-operated and CHF hearts. Equal amounts (15 μg) of LV homogenates were loaded in each lane and probed with the anti α-tubulin antibody. (B) Mean distribution of α-tubulin in the free and the polymerized fractions of CHF (filled bars) and sham operated hearts (empty bars). Bars are the means ± S.E.M. of 6 experiments.

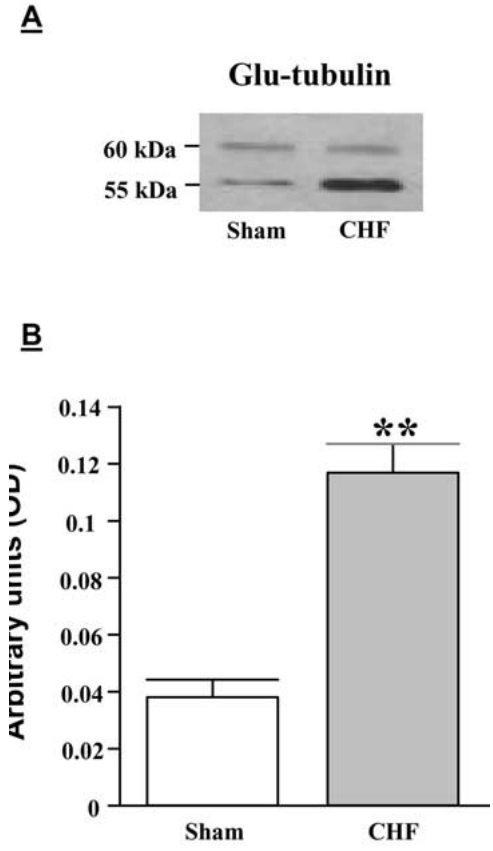

Fig. 3. Glu-tubulin in heart failure. (A) Representative Western blot of Glu-tubulin in sham-operated and CHF hearts. Equal amounts (15 μg) of LV homogenates were loaded in each lane and probed with the polyclonal anti Glu-tubulin antibody. Two bands are observed (~ 55 and ~ 60 kDa). (B) Summary of the total Glu-tubulin contents in LV from CHF (filled bars) and sham-operated rats (empty bars). Bars are the means ± S.E.M. of 12 experiments; significant differences are indicated as ** ($p < 0.01$).

Finally, the amount of Δ_2-tubulin was evaluated using a polyclonal anti Δ_2-tubulin antibody. As shown in Fig. 5A, this antibody recognized two bands, at ~ 55 and ~ 60 kDa. Δ_2-Tubulin was detected in LV of both sham-operated and CHF rats (Fig. 5A). However, its total level increased ~ 2-fold in heart failure (Fig. 5B). Further analysis of the repartition of Δ_2-tubulin between the free soluble and polymerized fractions showed that Δ_2-tubulin was present in both fractions with a somewhat higher amount found in the soluble fraction (Figs 6A and 6B). Surprisingly, in CHF rats, the free soluble Δ_2-tubulin form increased dramatically (~ 3-fold) while the polymerized form significantly decreased (Fig. 6B). Thus, the increase in total Δ_2-tubulin observed in CHF rats is due to a selective increase of the free soluble form.

Table 2. Total contents in acetylated tubulin and Tyr-tubulin in rat heart homogenates

	Sham-operated (n = 12)	Chronic heart failure (n = 12)	p*
Acetylated tubulin	0.16 ± 0.03	0.19 ± 0.05	n.s.
Tyr-tubulin	0.45 ± 0.03	0.43 ± 0.02	n.s.

Data show the means ± S.E.M. *compared with sham. n = number of animals. n.s. – non significant.

Acetylated tubulin

The above post-translational modifications of α-tubulin occurred exclusively at the C-terminal end of the protein. However, a post-translational modification of α-tubulin may also take place within the N-terminal region, by acetylation of Lys40, and this modification was shown to contribute to an increased stability of the microtubules in neonatal cardiac myocytes [19–22]. Since this post-translational modification

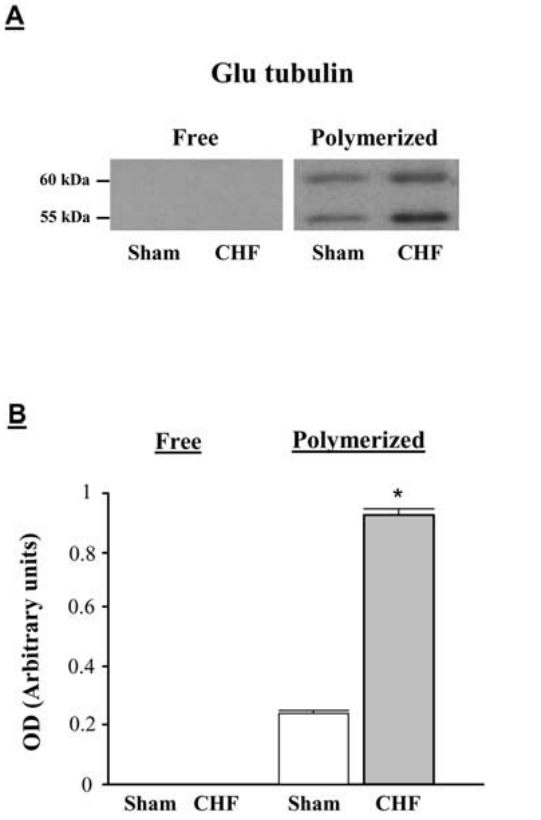

Fig. 4. Free and polymerized pools of Glu-tubulin in heart failure. (A) Representative Western blot of the free and polymerized fractions of Glu-tubulin in sham-operated and CHF hearts. Equal amounts (15 µg) of LV homogenates were loaded in each lane and probed with the polyclonal anti Glu-tubulin antibody. (B) Mean distribution of Glu-tubulin in the free and the polymerized fractions of CHF (filled bars) and sham operated hearts (empty bars). Bars are the means ± S.E.M. of 12 experiments; significant differences are indicated as ** ($p < 0.01$).

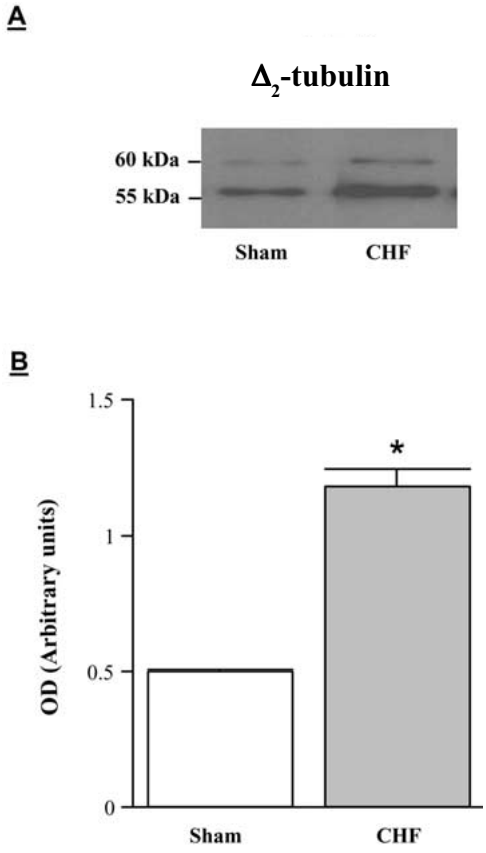

Fig. 5. Δ_2-tubulin in heart failure. (A) Representative Western blot of Δ_2-tubulin in sham-operated and CHF hearts. Equal amounts (15 µg) of LV homogenates were loaded in each lane and probed with the polyclonal anti Δ_2-tubulin antibody. Two bands are observed (~ 55 and ~ 60 kDa). (B) Summary of the total Δ_2-tubulin contents in LV from CHF (filled bars) and sham-operated rats (empty bars). Bars are the means ± S.E.M. of 4 experiments; significant differences are indicated as * ($p < 0.05$).

is independent and can be additive with those that occur at the C-terminal end of the protein, we examined the acetylated form of α-tubulin in heart failure. Using an acetylated tubulin specific monoclonal antibody, a single band (at 55 kDa) was recognized in both sham-operated and CHF hearts. As shown in Table 2, there was no difference in the total amount of protein found in sham-operated and CHF rats. Thus, the post-translational modifications of α-tubulin that take place during heart failure are limited to the C-terminal region of the protein.

Discussion

The results of the present study show that, in end stage heart failure, there is an important accumulation of total tubulin which is limited to the soluble fraction. However, although the polymerized tubulin fraction was not increased there was an important remodeling of the network via specific post-translational modifications of tubulin, strongly suggesting an overall increase in the stability of the microtubule network in heart failure. This is achieved by a combination of an increased Glu-tubulin content in the polymerized fraction and an increased Δ_2-tubulin content in the soluble fraction.

In the present study, heart failure was induced by pressure overload of the left ventricle and maintained for a prolonged period of time, in order to closely mimic the human clinical setting. Anatomical and clinical data clearly evidenced severe heart failure in this model. Seven months after surgery, end diastolic pressure was greatly increased although collagen content was not significantly altered, suggesting that other factors might participate in increased diastolic tension [25].

We found that cardiac a-tubulin level consistently increased in the LV from CHF rats. This is in agreement with other reports in animal models of cardiac hypertrophy, heart failure, and in biopsies of diseased human hearts [8, 9]. How-

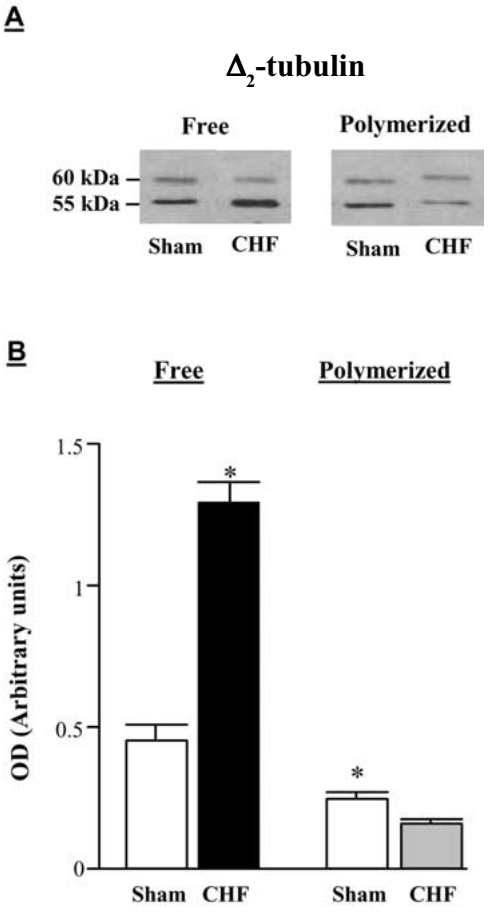

Fig. 6. Free and polymerized pools of Δ_2-tubulin in heart failure. (A) Representative Western blot of the free and polymerized fractions of Δ_2-tubulin in sham-operated and CHF hearts. Equal amounts (15 μg) of LV homogenates were loaded in each lane and probed with the polyclonal anti Δ_2-tubulin antibody. (B) Mean distribution of Δ_2-tubulin in the free and the polymerized fractions of CHF (filled bars) and sham operated hearts (empty bars). Bars are the means ± S.E.M. of 6 experiments; significant differences are indicated as * ($p < 0.05$) and ** ($p < 0.01$).

ever, at the late stage of heart failure, this excess of α-tubulin was not incorporated into microtubules, but accumulated in the soluble tubulin pool. Similarly, and consistent with the fact that tubulin proteins exist as heterodimers, β-tubulin levels were also elevated in CHF animals, and also accumulated in the non-polymerized form (data not shown). This is in contrast with the increase in polymerized tubulin observed in a canine model of decompensated pressure overload [14]. However, unchanged microtubules network in end stage heart failure may reflect a further stage of cytoskeleton remodeling following the transitory increase in microtubule density associated with cellular growth [18, 26].

The organization of the microtubule network is not only determined by the amount of tubulin, but also by its dynamics. Some of the post-translationally-modified tubulin that normally occur in stable polymers were affected in CHF hearts, as indicated by a dramatic increase in Glu- and Δ_2-tubulin levels. Meanwhile, acetylated, tyrosinated (see Table 2), polyglutamylated, Tyr-phosphorylated, and nitrotyrosinated tubulin levels were unchanged (data not shown).

The observed variation in the amount and distribution of truncated α-tubulin isoforms is unlikely to initiate at the late stage of heart failure since both Glu- and Δ_2-tubulin levels were already found to increase in a model of right ventricular hypertrophy [24]. Interestingly, while Glu-tubulin increased as expected in the polymerized fraction, Δ_2-tubulin decreased in this fraction and accumulated in the soluble fraction, participating in the abnormal compartmentalization of tubulin isoforms in CHF.

The accumulation of Glu-tubulin could originate either from an increased tubulin-carboxypeptidase activity or a decreased tubulin-tyrosine-ligase activity [21]. Ligase is unlikely to play a major role since this enzyme is only active on free Glu-tubulin subunits which were not detected in the soluble pool. Carboxypeptidase activity is known to be modulated during neuronal growth [27] and to decrease after birth in cardiac myocytes [22]. Since components of the fetal microtubular network are up-regulated during cardiac hypertrophy [28] an increased carboxypeptidase activity could account for the increased Glu-tubulin levels in CHF hearts. In addition, the observation that Glu-tubulin accumulates in the polymerized fraction only suggests an increased stability of the microtubules. Consistent with this hypothesis, MAP4, a microtubule associated protein which stabilizes microtubules, was shown to be up-regulated during cardiac hypertrophy [24].

The increase in total Δ_2-tubulin in CHF hearts might result from the excessive Glu-tubulin amounts that should be diverted from the soluble pool to avoid detrimental effects. Indeed, the injection of exogenous soluble Glu-tubulin disrupted (rather than strengthened) the interactions between microtubules and intermediate filaments in fibroblasts [29]. In addition, the accumulation of Δ_2-tubulin in the soluble fraction might play a beneficial role in CHF myocytes as free tubulin heterodimers can regulate various cell functions such as the binding of the heterochromatin protein 1 homologue to the nuclear envelope [30] or the activity of heterotrimeric G-proteins [31].

What could be the functional significance of microtubule remodeling in CHF? Since Δ_2-tubulin is irreversibly truncated, microtubules containing Glu-tubulin, rather than Δ_2-tubulin, might be a better reservoir of modifiable tubulin. In addition, the presence of Glu-tubulin in microtubules is thought to mediate specific interactions, yet unknown, during cardiac development [22]. By analogy with what occurs in fibroblasts, kinesin binding to Glu-tubulin might play a critical role in the interaction between microtubules and vimentin intermediate filaments [30]. Interestingly, vimentin,

along with vinculin and desmin, are known to increase in human heart failure, and might thus buffer the increased strain imposed to the failing heart [9]. The likely functional counterpart of this strengthening of the connections between microtubules and intermediate filaments is an impairment in cell shortening, and an increase in the passive stiffness of the cardiac tissue, thus participating in the adverse effects of cardiac remodeling. Indeed, the dramatic enhancement in end diastolic pressure observed in our model can not be explained by an increase in collagen content [25]. Thus, the altered microtubule network may participate in the changes in elastic properties of the myocardium in heart failure [8, 17, 18].

Remodeling of the microtubule network during end-stage heart failure may also have broader consequences. Indeed, microtubule integrity is required for calcium signaling and β-adrenergic response in cardiac myocytes [33]. In addition, changes in the microtubule network are likely to modify the role of microtubule associated proteins (MAPs) in the signal transduction pathways involving for example mitogen-activated protein-kinase or NFκB [32]. Furthermore, since the cytoskeleton appears to determine metabolic interactions between intracellular organelles, alterations in the cytoskeleton and/or cell architecture might be involved in the regulation of mitochondrial function and oxygen consumption [34, 35].

Clearly, the detailed role of microtubules during end-stage heart failure will require more investigations. However, since the microtubule network undergoes significant changes in cardiac hypertrophy, and is further modified during end-stage heart failure, we hypothesize that they are likely to participate in both the compensatory and the detrimental events that occur during this disease.

Acknowledgements

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