T-Tubule Remodeling During Transition From Hypertrophy to Heart Failure

Sheng Wei,* Ang Guo,* Biyi Chen,* William Kutschke, Yu-Ping Xie, Kathy Zimmerman, Robert M. Weiss, Mark E. Anderson, Heping Cheng, Long-Sheng Song

Rationale: The transverse tubule (T-tubule) system is the ultrastructural substrate for excitation–contraction coupling in ventricular myocytes; T-tubule disorganization and loss are linked to decreased contractility in end stage heart failure (HF).

Objective: We sought to examine (1) whether pathological T-tubule remodeling occurs early in compensated hypertrophy and, if so, how it evolves during the transition from hypertrophy to HF; and (2) the role of junctophilin-2 in T-tubule remodeling.

Methods and Results: We investigated T-tubule remodeling in relation to ventricular function during HF progression using state-of-the-art confocal imaging of T-tubules in intact hearts, using a thoracic aortic banding rat HF model. We developed a quantitative T-tubule power (TTpower) index to represent the integrity of T-tubule structure. We found that discrete local loss and global reorganization of the T-tubule system (leftward shift of TTpower histogram) started early in compensated hypertrophy in left ventricular (LV) myocytes, before LV dysfunction, as detected by echocardiography. With progression from compensated hypertrophy to early and late HF, T-tubule remodeling spread from the LV to the right ventricle, and TTpower histograms of both ventricles gradually shifted leftward. The mean LV TTpower showed a strong correlation with ejection fraction and heart weight to body weight ratio. Over the progression to HF, we observed a gradual reduction in the expression of a junctophilin protein (JP-2) implicated in the formation of T-tubule/sarcoplasmic reticulum junctions. Furthermore, we found that JP-2 knockdown by gene silencing reduced T-tubule structure integrity in cultured adult ventricular myocytes.

Conclusions: T-tubule remodeling in response to thoracic aortic banding stress begins before echocardiographically detectable LV dysfunction and progresses over the development of overt structural heart disease. LV T-tubule remodeling is closely associated with the severity of cardiac hypertrophy and predicts LV function. Thus, T-tubule remodeling may constitute a key mechanism underlying the transition from compensated hypertrophy to HF. (Circ Res. 2010;107:520-531.)

Key Words: T-tubule ■ myocardial remodeling ■ hypertrophy ■ heart failure ■ confocal microscopy

T he transverse tubules (T-tubules) are orderly invaginations of surface membrane along the Z-line regions, with regular spacing (~2 μm) along the longitudinal axis of mammalian ventricular myocytes. The widely distributed, highly organized T-tubule system is essential for rapid electric excitation, initiation and synchronous triggering of sarcoplasmic reticulum (SR) Ca2+ release, and, therefore, coordinated contraction of each contractile unit throughout the entire cytoplasm. The T-tubule system is thus an important determinant of cardiac cell function.1-3 Heart failure (HF) is characterized by reduction of myocyte contractile function and defects in Ca2+ handling (eg, blunted and dyssynchronous SR Ca2+ release) in myocytes from HF models (including human).4-6 The cause of Ca2+ handling defects in HF is likely multifaceted, with contribution from reduced SR Ca2+ content, ryanodine receptor (RyR) hyperphosphorylation, and change in action potential shape, among other factors.7-10 Recent studies from several groups have provided compelling evidence supporting a concept that T-tubule structural remodeling is directly linked to SR Ca2+ release dysfunc-
tion in myocytes from animal models of HF\textsuperscript{11–16} and in artificially denubulated myocytes.\textsuperscript{17,18} Although there is no doubt that T-tubule remodeling correlates with Ca\textsuperscript{2+} handling defects, it is unknown whether T-tubule alterations are an early or late event in HF. The lack of studies of T-tubule changes before HF onset is thus a critical knowledge gap in understanding HF. It is also not clear whether there is a relationship between T-tubule structure and cardiac function during the progression from hypertrophy to HF. In addition, little is known about the potential molecular mechanisms underlying T-tubule remodeling in heart disease.

We investigated T-tubule structure and LV function during progression of cardiomyopathy in a thoracic-aortic banding (TAB) Sprague–Dawley rat model of pathological left ventricular (LV) afterload. We adapted our confocal imaging system to examine the in situ epicardial T-tubule structure from Langendorff perfused intact hearts. A quantitative and sensitive method (T-tubule power index, \(TT_{\text{power}}\)) was developed to characterize the overall integrity of the T-tubule system. Surprisingly, we found that T-tubule remodeling starts before echocardiographically detectable LV systolic dysfunction during compensated hypertrophy, and is seen as patchy T-tubule loss in local regions of the LV, as well as global T-tubule reorganization. With disease progression, T-tubule structural remodeling penetrates from the left to the right ventricle. \(TT_{\text{power}}\) is closely associated with LV systolic function during disease progression, and corresponds to the loss of junctophilin (JP-2) expression. Moreover, in vitro gene silencing experiments show that JP-2 knockdown reduces T-tubule integrity in cultured ventricular myocytes. These data support the hypothesis that T-tubule remodeling is a critical early event marking the transition from compensated hypertrophy to decompensated HF during the progression of HF.

**Methods**

Animal experiments were performed according to the protocol approved by the University of Iowa Institutional Animal Care and Use Committee. Male Sprague–Dawley rats (\(\sim 60\) g) were subjected to pressure overload by TAB surgery.\textsuperscript{19} Transsthoracic echocardiograms were performed in the University of Iowa Cardiology Animal Phenotyping Core Laboratory, using a Sonos 5500 Imager (Phillips Medical Systems, Andover, Mass).\textsuperscript{20} In situ confocal imaging of epicardial myocyte T-tubule structure on intact heart, power spectrum analysis of T-tubule structure, Western blotting protein quantification, and JP-2 knockdown experiments in cultured mouse adult myocytes are described in detail in the expanded Methods section in the Online Data Supplement, available at http://circres.ahajournals.org.

**Results**

**Echocardiographic Assay of Cardiac Function**

TAB rats developed hypertrophy or HF at around 8 to 12 weeks, as determined by echocardiography (Online Table I). TAB rats with compensated hypertrophy exhibited significant increases in heart weight (HW), heart weight to body weight ratio (HW/BW), LV mass, LV mass to body weight ratio (LV mass/BW), and lower end diastolic volume to LV mass ratio (vol/mass) but normal end diastolic volume, normal end systolic volume, and highly comparable ejection fraction (EF), in comparison with those of sham-operated control rats. In contrast, HF rats displayed enlarged hearts with markedly higher HW/BW ratio and vol/mass ratio, massive increase in end systolic and diastolic volumes, and therefore significant reduction in EF (52.8\(\pm\)2.3\%, \(N=14\) HF rats versus 81.8\(\pm\)1.2\%, \(N=7\) sham rats, \(P<0.001\)). Figure 1A shows typical echocardiographic images at end systole and end diastole from different stages of TAB rats and sham control. We divided HF rats into early and advanced HF groups: those rats with EF higher or equal to the median EF (54.4\%) were assigned to early HF group (with a mean EF=59.4\(\pm\)1.8\%, \(N=7\)), and those rats with EF lower than the median EF were assigned to advanced HF group (with a mean of 45.0\(\pm\)4.4\%, \(N=7\)) (Figure 1B).

In addition, pericardial effusion was often observed in HF rats (7 of 29 TAB rats). Pulmonary edema was apparent in HF but not in hypertrophy rats, because the lung weight to body weight ratio was significantly increased in failing rats (11.1\(\pm\)1.0 mg/g, ranging from 5.5 to 16.2 mg/g, \(N=14\) HF rats versus 4.4\(\pm\)0.1 mg/g, \(N=7\) hypertrophy rats and 4.3\(\pm\)0.2 mg/g, \(N=5\) sham rats, \(P<0.001\) between HF and hypertrophy or between HF and sham group). Furthermore, there was a significant difference in lung weight to body weight ratio between early HF and advanced HF group (8.4\(\pm\)1.0 mg/g versus 13.8\(\pm\)0.7 mg/g, \(P<0.01\)).

**In Situ T-Tubule Imaging in Langendorff-Perfused Hearts**

We performed in situ T-tubule imaging on intact hearts using a Langendorff perfusion system adapted to laser scanning confocal microscopy. This approach provides high resolution imaging of myocytes within \(\sim 70\) \(\mu\)m depth of the epicardium. Each frame covered a 202\(\times\)202 \(\mu\)m\(^2\) area and contained 10 to 20 well-demarcated myocytes (Figure 2A). Figure 2B shows a 3D image of the T-tubule system reconstructed from 25 stacks of T-tubule images acquired sequentially at 0.2 \(\mu\)m interval. This in situ imaging of T-tubule

**Non-standard Abbreviations and Acronyms**

<table>
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<th>Description</th>
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<tr>
<td>EC</td>
<td>Excitation–contraction</td>
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<tr>
<td>EF</td>
<td>Ejection fraction</td>
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<tr>
<td>HF</td>
<td>Heart failure</td>
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<td>HW/BW</td>
<td>Heart weight/body weight ratio</td>
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<td>JP-2</td>
<td>Junctophilin-2</td>
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<tr>
<td>LV</td>
<td>Left ventricle/ventricle</td>
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<tr>
<td>RV</td>
<td>Right ventricle/ventricle</td>
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<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
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<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
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<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TAB</td>
<td>Thoracic aortic banding</td>
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<tr>
<td>TT(_{\text{power}})</td>
<td>Transverse-tubule power</td>
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<td>T-tubule</td>
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\(P<0.01\).
system on Langendorff-perfused intact hearts avoids possible damage of myocyte membranes and biased loss of cardiac myocytes during enzymatic isolation (see discussion), providing a novel, high resolution imaging assay for studying the T-tubule system in normal and diseased hearts.

The most important characteristics of myocyte T-tubule system are the highly organized, periodic striations with almost identical spacing ($\approx 2\mu m$ distance). Figure 2C shows an example of power spectrum analysis of T-tubule signals from Figure 2A. The dominant frequency at $\approx 0.5\mu m^{-1}$ corresponds to the anticipated spatial distance of $\approx 2\mu m$, representing T-tubule spacing in intact heart. We used the peak power value (in arbitrary units) at the dominant frequency as a quantitative index of T-tubule integrity ($TT_{power}$). On average, $TT_{power}$ of sham-operated healthy hearts was $2.08\pm 0.03$ (N=7 hearts). In contrast, atrial cells from control rat hearts, which had sparse irregular T-tubule system (both left and right atria), did not show a dominant peak in FFT power spectrum (Online Figure I).

Impairment of T-Tubule Integrity Begins Before LV Systolic Dysfunction

We next sought to determine the stage at which T-tubules begin to remodel after TAB, and the features of early T-tubule remodeling. Surprisingly, T-tubule remodeling began early at the compensated hypertrophy stage before LV dysfunction (Figure 3B; EF=77%). At the onset, this process was manifested as subcellular T-tubule loss limited to discrete local regions in some LV myocytes (arrows in Figure 3B). By using $TT_{power}$ as a sensitive method to quantify the overall T-tubule integrity, we were able to detect subtle T-tubule structural reorganization, even in those with no visually discernible change in the regularity of T-tubule network (Figure 3B). Specifically, this reorganization was detected as a significant reduction in the average $TT_{power}$ from in situ LV images (Figure 3E; $2.08\pm 0.03$, N=7/sham LV versus $1.66\pm 0.07$, N=12 hearts/hypertrophy LV, $P<0.01$) (see also below). Our findings strongly suggest that T-tubule remodeling is an important early event that occurs in the LV at the stage of compensated hypertrophy, even though there is no ventricular dysfunction (as detected by echocardiography).

Progressive T-Tubule Deterioration During HF Progression

Next, we sought to investigate how T-tubule remodeling evolves during the transition from hypertrophy to HF in intact heart. Figure 3C and 3D shows representative T-tubule...
Images at different stages during HF progression. As the hypertrophied heart became decompensated with compromised LV function (Figure 3C; EF=61%), the T-tubule system showed a marked increase in the irregularity of T-tubule structure. At the advanced HF stage, the T-tubule system was severely disrupted, with dramatic loss of T-tubule signals and loss of the regular striated pattern (Figure 3D; EF=49.5%). This phenomenon was consistently observed in all of the hearts examined in each group. We defined images with \( \text{TTpower} \leq 1.3 \) as having a massively disrupted T-tubule system, and compared images across different stages. We found that from sham to advanced HF the percentages of acquired images from the LV with massive T-tubule disruption were 0% (0 of 70), 10% (13 of 129), 76% (62 of 81), and 86% (67 of 78) in sham, hypertrophy, and early and advanced HF, respectively. On average, \( \text{TTpower} \) showed a continuous, decreasing function in LV myocytes during HF development (Figure 3E). These data demonstrate that the LV T-tubule system undergoes progressive deterioration during the developmental course from hypertrophy to HF.

Delayed T-Tubule Remodeling in Right Ventricular Myocytes in Pressure Overload–Induced Cardiomyopathy

Normally, the T-tubule system of LV and right ventricular (RV) myocytes in healthy heart appears to be indistinguishable and displays near identical \( \text{TTpower} \) (Figures 3A and 4A). Pressure overload–induced cardiac hypertrophy attributable to TAB starts from the LV. During the compensated hypertrophy stage, LV myocytes showed loss of T-tubules with significantly reduced \( \text{TTpower} \), but the T-tubule structure in RV myocytes remained unaffected (Figure 4A and 4B). This indicates differential properties between LV and RV T-tubule remodeling in response to pressure overload at the hypertrophy stage. Interestingly, with the progression of disease T-tubule remodeling spreads from the LV to the RV. Figure 4 displays RV T-tubule images from hearts at different stages. As shown in Figure 4C, RV myocyte loss of T-tubules and their regular organization is first detectable at the early HF stage. However, at the advanced stage of HF (Figure 4D), the RV myocyte T-tubule system also showed severe disruption, similar to that seen in the LV. The percentages of RV T-tubule images with massive disruption (\( \text{TTpower} \leq 1.3 \)) were 0% (1 of
Global Reorganization of T-Tubule System in TAB-Induced Hypertrophy and HF

The fact that a fraction of myocytes display massive T-tubule disruption during the progression of TAB-induced hypertrophy and HF raises an intriguing question: Is there a subset of myocytes that are more vulnerable to the pressure overload stress, or are all myocytes globally affected by the stress, in...
terms of T-tubule remodeling? To discriminate between these 2 possibilities, we analyzed the histogram distribution of TT_power from individual images of both LV and RV at different disease stages.

Under control conditions, TT_power in either LV or RV myocyte populations exhibited a single-mode (centered at 2.0 to 2.3), bell-shaped distribution (Figure 5A and 5E), suggesting that TT_power from all myocytes belong to one single population in the unstressed hearts. After the TAB stress, the entire histogram of LV TT_power shifted leftward in hypertrophied hearts (centered between 1.7 to 2.0; Figure 5B). Nevertheless, the histogram retained its single-mode configuration, suggesting that the entire population of myocytes undergo T-tubule remodeling in a similar
fashion. In particular, it is noteworthy that the highest LV $TT_{\text{power}}$ ($\approx 2.6$) was decreased from 16% in sham to 1% in the hypertrophic hearts (Figure 5B). These data strongly suggest that, in addition to overt subcellular loss of T-tubules in a fraction of myocytes, the entire population of epicardial myocytes undergoes global T-tubule remodeling (in a manner that may not be detectable visually) early at the hypertrophy stage in response to TAB stress. As the disease progressed to early and advanced HF, we found that the $TT_{\text{power}}$ histogram further shifted leftward while retaining a single mode of distribution in the LV (Figure 5C and 5D), further supporting the notion that the entire myocyte population examined behaves similarly during the progression of cardiomyopathy. Similar results were obtained with RV myocytes, except that the leftward shift of $TT_{\text{power}}$ histogram in RV showed a clear delay than that of LV myocytes (Figure 5E and 5F). Taken together, these findings demonstrate global T-tubule structure reorganization at hypertrophy (LV) and HF stages (LV and RV) in myocyte response to pressure overload stress.

T-Tubule Remodeling Correlates With LV Function

The role of the T-tubule system in excitation–contraction (EC) coupling, particularly in triggering synchronous SR Ca$^{2+}$ release and the consequence of T-tubule remodeling in isolated failing myocytes (eg, dysynchrony of SR Ca$^{2+}$ release), is well recognized. However, the functional relationship between T-tubule structure and global LV systolic function is not well understood. We examined the correlation between the LV mean $TT_{\text{power}}$ (which represents the overall T-tubule status of myocardium, assuming the mid- and endocardium myocytes share similar patterns in T-tubule remodeling during HF progression) with the corresponding cardiac morphometrics and performance. We found that LV $TT_{\text{power}}$ displayed a strong negative correlation ($R^2=0.80$, $P<0.001$) with HW/BW ratio (Figure 6A), an important index of cardiac hypertrophy, suggesting T-tubule remodeling is closely associated with the development of hypertrophy. Moreover, we found that LV $TT_{\text{power}}$ correlated with LV EF in a positive linear function ($R^2=0.68$, $P<0.001$; Figure 6B), suggesting that...
such T-tubule remodeling exerts an adverse effect on contractile function. Furthermore, we showed that the relationship between LV TT_power and end systolic volume ($R^2=0.57$, $P<0.001$; Figure 6C) and end diastolic volume ($R^2=0.38$, $P<0.001$; Figure 6D) was nicely fitted with a power function. These results reveal a heretofore unappreciated structure-function correlation between subcellular T-tubule remodeling and whole-animal cardiac performance during disease development.

**JP-2 Downregulation in TAB Hearts**

To investigate the molecular mechanism of T-tubule remodeling in pressure overload cardiomyopathy, we examined the protein level of JP-2, a member of the junctophilin protein family important for the formation of T-tubule–SR junctions,21 in LV tissues from frozen hearts. There was a progressive decrease in JP-2 protein level in LV tissues, starting from hypertrophy stage (Figure 7A and 7B). To examine whether JP-2 loss contributes to T-tubule remodeling observed in pressure overload disease, we used recombinant lentivirus carrying JP-2 short hairpin (sh)RNA to silence JP-2 expression in cultured adult mouse ventricular myocytes. After 64 hours of transfection, JP-2 shRNA knocked down the expression level of JP-2 to 51% of that of myocytes transfected with control vectors containing a scrambled shRNA sequence (control) (Figure 8A and 8B). JP-2 shRNA–infected myocytes displayed a significant reduction in T-tubule power compared to the control group (Figure 8C and 8D; $n=43$ and 57 cells for scramble control and JP-2 shRNA groups, respectively, $P<0.01$), with a leftward shift in the T-tubule power histogram (Figure 8E and 8F). These data indicate that JP-2 downregulation may be involved in T-tubule remodeling in pressure overload cardiomyopathy.

**Discussion**

In the present study, using TAB rat model in combination with state-of-the-art confocal imaging technique and a novel method to analyze T-tubule integrity, we examined the developmental changes of myocyte t–tubule system during the progression of hypertrophy and HF. The major findings of our study are as the following: (1) myocytes at the compensated hypertrophy stage already display disruptive T-tubule remodeling, characterized by discrete local T-tubule loss and global T-tubule structural reorganization; (2) this deleterious structure remodeling progressively worsens as the disease develops, with massive T-tubule disruption at advanced HF; (3) a differential T-tubule remodeling process was observed between LV and RV myocytes; (4) T-tubule structural remodeling is highly correlated with the degree of hypertrophy; and (5) the severity of T-tubule disorganization is strongly correlated with global LV function and loss of JP-2 expression.

**In Situ Confocal Imaging of T-Tubule Structure**

The T-tubule system is a specialized membrane network, playing important roles in membrane excitation, SR Ca$^{2+}$ release activation, muscle contraction, and signal transduction.22 Early studies on T-tubule structure were based on electron microscopy. Electron microscopy studies with high spatial resolution have provided important information about T-tubule structure in normal myocardium23,24 and in hypertrophied heart.25 However, the sophisticated electron microscopy technique cannot provide a complete picture of the T-tubule system. Recently, several groups have used confocal microscopy to investigate T-tubule
structure in health and disease. All of these studies were performed in single isolated myocytes. Enzymatic dissociation of myocytes may impair the T-tubule membrane of healthy cells. It is also very likely that those myocytes with severely damaged T-tubule membrane may be more fragile because of enzyme digestion, mechanical stirring, Ca^2+ unloading and reloading during myocyte isolation process. Thus, it is possible that enzymatic isolation may yield predominantly (relatively) healthy myocytes and myocytes isolated from healthy hearts may display varying degrees of T-tubule change (see figure 2A in the article by Heinzel et al). Taken together, these factors intrinsic to the isolation of cardiomyocytes are an obstacle to identifying subtle changes in T-tubule membrane structure, as we observed here in hypertrophied hearts. Membrane damage attributable to cell-isolation techniques could explain the inconsistent results reported previously in human failing hearts. In this study, we examined myocyte T-tubule structure using a novel in situ confocal imaging technique combined with Langendorff-perfused hearts. In doing so, we were able to avoid damage to the T-tubule system linked to myocyte dissociation. Additionally, this technique allows us to visualize a large number of myocytes collectively and to measure myocytes from different epicardial regions in situ. It also allowed measurement of early, subtle alterations and severe disruption of T-tubule structure (Figures 3 and 4).

(Ultra)structural T-Tubule Remodeling in Heart Diseases

In animals, He et al first identified a significant loss of T-tubule density (without T-tubule disorganization) in ventricular myocytes from a dog model with pacing-induced HF. Subsequently, other studies (including ours) using other animal HF models have reported profound T-tubule remodeling (loss and/or disorganization) in single isolated failing myocytes. These other animal models include spontaneous hypertensive rats with overt HF, mouse myocardial infarction, pig myocardial infarction, and sheep rapid pacing HF model. In human, early reports with histological examination in failing heart tissue sections showed T-tubule dilation with an increase or decrease in the density of T-tubules. Other preliminary reports also presented inconsistent findings. A very recent study using both scanning ion conductance microscope and confocal microscope helped resolve the discrepancy in early reports. T-tubule loss was pronounced in failing ventricular myocytes from all HF patients (ischemic heart disease, idiopathic dilated cardiomyopathy, and hypertrophic obstructive cardiomyopathy). From a spectrum of etiologic settings in both human and animal models, these studies had drawn similar conclusions, indicating that T-tubule remodeling is a principal problem in many heart diseases, especially at end-stage HF. However, all of these studies (including...
those human studies and our previous studies were performed in myocytes at advanced HF stage. Our present findings showed that T-tubule remodeling occurs before the onset of HF, suggesting T-tubule remodeling is not a secondary modification after HF, rather an important early event during HF progression.

**T-Tubule Remodeling in the Transition From Hypertrophy to HF**

The deleterious consequence of T-tubule remodeling on EC coupling in HF has been carefully explored in several elegant studies. T-tubule loss and/or disorganization has been directly linked to dysynchronous Ca\(^{2+}\) sparks, reduced and slowed Ca\(^{2+}\) transients during EC coupling in failing myocytes. Using spontaneously hypertensive rats in HF, we showed that T-tubule disorganization led to an increase in orphaned RyRs and loss of local control of Ca\(^{2+}\) release, resulting in decreased EC coupling efficacy and increased dysynchrony of SR Ca\(^{2+}\) release in failing myocytes. A recent study from Sipido’s group reported similar findings in an ischemic cardiomyopathy pig model where T-tubule loss was associated with reduced synchrony of Ca\(^{2+}\) release and reduced efficiency of coupling Ca\(^{2+}\) influx to Ca\(^{2+}\) release. Very recently, T-tubule remodeling and Ca\(^{2+}\) release dysfunction were also found in atrial myocytes from a rapid-paceing induced sheep HF model. These experimental findings were also supported by computer modeling, in which T-tubule reorganization can reduce the synchrony of Ca\(^{2+}\) spark production and lead to the appearance of late Ca\(^{2+}\) sparks and greater nonuniformity of intracellular Ca\(^{2+}\).

It is surprising that hypertrophied heart with normal global function already showed some degree of T-tubule remodeling. This might be related to large cardiac reserve function: no apparent cardiac functional phenotype even when straining. Specifically, cardiac hypertrophy in response to pressure overload involves many compensatory, molecular and biochemical mechanisms that may account for compensating the negative effect of T-tubule loss on SR Ca\(^{2+}\) release and myocyte contractility, resulting into a maintained myocardial function in hypertension. However, these compensating mechanisms ultimately are apparently ineffective for balancing the defects caused by more severely damaged T-tubule system as structural heart disease progresses. The progressive deterioration of T-tubule structure throughout the disease development and the strong correlation between T-tubule remodeling and LV function support the notion that T-tubule remodeling is a critical factor during the transition from compensated hypertrophy to HF.

**Molecular Mechanisms Underlying T-Tubule Remodeling in TAB-Induced Cardiomyopathy**

JP-2 is a member of the junctophilin protein family important for the formation of junctional membrane complex (ie, the cardiac dyad) between T-tubule membrane and SR. JP-2 mutations were associated with human hypertrophic cardiomyopathy and JP-2 knockout mice have defective cardiac dyads, altered intracellular calcium transients, and embryonic lethality. JP-2 mRNA expression was reduced in hypertrophied rats and mouse cardiomyopathy model, but it was unknown whether JP-2 expression is affected in accord with T-tubule remodeling during progression of structural heart disease. Our results show that T-tubule remodeling correlates with the loss of JP-2 expression in left ventricular myocytes of pressure overload model and that JP-2 knockdown (by 50%) reduces T-tubule integrity in cultured myocytes, suggesting that reduced JP-2 expression may be important in mediating T-tubule remodeling in pressure overload cardiomyopathy. However, it is recognized that there is no report about the heterozygous phenotype of JP-2 mutants. Careful examination of JP-2 heterozygous mice (at rest and under stress) and, more ideally, future studies with inducible JP-2 knockout/knockdown mice should provide further critical information on JP-2 functions in cardiac health and disease.

In summary, for the first time, we identified T-tubule adverse remodeling in intact hearts during compensated hypertrophy stage and mapped the T-tubule change to the progression of myocardial disease in a temporally and functionally defined disease model. T-tubule disruptive remodeling is a critical event during the development of hypertrophy that is tightly associated with the declining of myocardial function and transition from compensated hypertrophy to HF.

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**Disclosures**

None.

**References**


Novelty and Significance

What Is Known?
- Transverse (T)-tubule system is an organized membrane network important for normal cardiac excitation–contraction coupling.
- End-stage heart failure of animal models or human patients is associated with T-tubule remodeling/disorganization.
- T-tubule remodeling causes Ca$^{2+}$/H$^{11001}$ release and cell signaling dysfunction in isolated cardiomyocytes.

What New Information Does This Article Contribute?
- The first detailed methods for in situ T-tubule imaging on intact heart and quantitative analysis of T-tubule structure.
- T-tubule alteration starts early in compensated hypertrophy stage, featuring discrete local loss and global reorganization of the affected myocardium.
- T-tubule remodeling gradually deteriorates over the progression of heart disease, correlates with the severity of cardiac hypertrophy and predicts heart function.
- T-tubule remodeling represents a key mechanism underlying the transition from compensated hypertrophy to heart failure.
- Junctophilin-2 downregulation is likely responsible for T-tubule remodeling in cardiomyopathy.

T-tubule system is a highly organized membrane network in working mammalian ventricular myocytes. The maintenance of this specialized membrane structure is critical for normal contractile function and the pumping capability of the beating heart. Reduction in T-tubule density and organization has been linked to abnormal Ca$^{2+}$/H$^{11001}$ function and decreased contractility in end-stage heart failure. However, it is unknown whether T-tubule alterations are an early or late event in heart failure. The lack of studies of T-tubule changes before heart failure onset is a critical knowledge gap in understanding heart failure. It is also not clear whether changes in T-tubule structure are related to cardiac function during progression from hypertrophy to heart failure. In this study, we provided evidence showing that T-tubule remodeling is an important early event during heart failure progression, starting at compensated hypertrophy stage.

Our data support the hypothesis that T-tubule remodeling may constitute an important mechanism underlying the transition from compensated hypertrophy to decoupled heart failure, indicating that preventing T-tubule remodeling during the hypertrophy stage may be clinically important for delaying progression to heart failure. Moreover, we present evidence linking for the first time a single molecule (junctophilin-2) to reduced T-tubule integrity during heart failure. These findings suggest new therapeutic strategies for the treatment of heart failure.
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T-tubule remodeling during transition from hypertrophy to heart failure

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Online Extended Methods

Animal Model
Animal experiments were performed according to the protocol approved by the
University of Iowa Institutional Animal Care and Use Committee. Male SD rats (~60
gram) were subjected to pressure overload by TAB surgery as described 1. Briefly, three
and half week old male SD rat pups (~60 gram) were anesthetized with
ketamine/xylazine (40 / 5 mg/kg respectively) by intraperitoneal injection. The rats were
then intubated with a 16 gauge tube, and ventilated with a small rodent ventilator
(Harvard Apparatus, USA). A thoracotomy was created between the second and third
intercostal space, and the aortic arch visualized. A titanium clip was placed on the aortic
arch between the brachiocephalic and left common carotid arteries, causing a pressure
gradient of ~80 mmHg (81±18 mmHg, N=12). The chest wall was then closed, and the
pneumothorax was evacuated. In sham operated animals, the aortic arch was
visualized but not banded. The rats were then allowed to recover and then returned to
their cages.

Echocardiography of cardiac function
Transthoracic echocardiograms were performed in the University of Iowa
Cardiology Animal Phenotyping Core Laboratory, using a Sonos 5500 Imager (Phillips
Medical Systems, Andover, MA) 2. Ketamine HCl (25 mg/kg i.p.) was used to induce a
semiconscious state. 2D images were acquired in LV short- and long-axis planes with an 8-MHz sector-array probe, yielding 100 frames per second. LV mass, volumes, and ejection fractions were calculated with the area-length method. Regions demonstrating akinesis or dyskinesis were visually identified, planimetered, and expressed as percentages of total LV end-diastolic silhouette.

In situ confocal imaging of epicardial myocyte t-tubule structure on intact heart

Intact rat hearts were Langendorff-perfused at room temperature with 0 Ca\(^{2+}\) Tyrode's solution (NaCl 137, KCl 5.4, HEPES 10, Glucose 10, MgCl\(_2\) 1, NaH\(_2\)PO\(_4\) 0.33, pH adjusted to 7.4 with NaOH, oxygenated with 95% O\(_2\) and 5% CO\(_2\) during experiments), containing 2.5 \(\mu\)M FM 4-64, a lipophilic fluorescence indicator of membrane structure (Invitrogen Inc., USA) for 20 min. FM 4-64 gave the same in situ t-tubule staining on intact heart as Di-8-ANEPPS (Data not shown). The hearts were placed in the perfusion chamber attached on the stage of a confocal microscope (Figure 2A), and perfused with indicator free / Ca\(^{2+}\) free solution (with oxygenation). The membrane structure of epicardial myocytes was analyzed in situ with confocal microscope (LSM510, Carl Zeiss Microl Imaging Inc., Germany). The microscope was equipped with 63x (NA=1.4) oil immersion lens. The optical pinhole was set to 1 airy disc (<1 \(\mu\)m axial resolution) during confocal imaging. Each t-tubule image frame contains 202 x 202 \(\mu\)m\(^2\) area of myocytes (Figure 2A). Ten to fifteen images from different locations of each ventricular free walls (not including apex and interventricular area) were acquired, and power values from each ventricle were averaged to represent the global t-tubule structure of each ventricle.

Image Processing

T-tubule images were analyzed offline with custom routines composed with IDL image analysis program (ITT VIS Inc., Colorado). Background noise in confocal images was filtered with a threshold value retrieved from image intensity histogram. (Figure 2A, t-tubule image with background noise filtered). FFT routine (coded in IDL) was utilized to perform a Fast Fourier Transformation (FFT) and convert two dimensional images from the spatial domain into the frequency domain (Figure 2C upper panel). This allowed us to determine which pixels contain the most important information, whether repeating patterns occur and how strong (power) the repeating patterns are. The power spectrum obtained from two dimensional FFT of confocal t-tubule images characterized the magnitude of the regular organization of t-tubules, and was used to evaluate t-tubule...
remodeling. The central bright signals (Figure 2C upper panel), corresponding to 0 \( \mu \text{m}^{-1} \) spatial frequency in lower panel represent low frequency noise. The first peak of spatial frequency components (pointed by blue dashed arrow) centered at \( \sim 0.5 \mu \text{m}^{-1} \) corresponded to the \( \sim 2 \mu \text{m} \) spacing interval of t-tubules, representing the regularity of myocyte t-tubule system. The second and third peaks are the harmonics of the first peak (Figure 2C). The peak power (only the first peak in this study) was measured as the absolute value using Gaussian fitting with pClamp 10 data analysis software (Clampfit, Molecular Devices, USA) (as shown in Figure 2C lower panel). Changes in power (we used the magnitude at the frequency of first peak in this study) were analyzed at different stages of disease.

**Western blotting assay of juntophilin-2 (JP-2)**

The rat left ventricles were harvested, quickly rinsed in 0 Ca\(^{2+}\) Tyrode’s solution, immediately frozen in liquid nitrogen and stored at -80 °C. Frozen tissues were homogenized in lysis buffer (50 mM Tris, PH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM Na\(_3\)VO\(_4\), 5 mM EGTA, 5 mM EDTA, 0.5% Triton X-100, 0.5% Na Deoxycholate, 0.1% SDS), containing protease inhibitors (Sigma P8340). Tissue lysates were then centrifuged at 12,000 X g for 3 min to remove insoluble debris. Protein concentrations were determined using the Pierce BCA assay (Pierce, Thermo Scientific). Samples (10 \( \mu \text{g} \)) were separated by SDS-PAGE (10% acrylamide) and transferred to PVDF membranes. Primary antibodies that recognize JP-2 (Santa Cruz, sc-51313) and GAPDH (Cell Signaling, #2118) were used. HRP linked anti-Goat IgG (1:5000) and anti-rabbit IgG (1:10000) were used to visualize bound primary antibodies with the SuperSignal chemiluminescence substrate (Pierce, Thermo Scientific), and imaged with Fuji life science imaging system (LAS-3000, Fujifilm, Japan). The protein bands were quantified using Image J software (version 1.43d).

**JP-2 shRNA knockdown and t-tubule assay in cultured mouse ventricular myocytes**

The shRNA lentiviral particles carrying JP-2 gene silencing sequence were purchased from Santa Cruz Biotechnology Inc. JP-2 shRNA (m) lentiviral particles contain the following 3 constructs

\[
\text{CCAGTGGGAATACCTTTTGATTCAGAGATCAAAGGTATTCCACTGCTGTTTTT;}
\]
\[
\text{CTCAGAATCAGCAGCATTCAAGAGATGATGTGGTCTGATTGAGTTTT;}
\]
CTGACTTGACCCTCATCTATTCAAGAGATAGATGAGGGTCAAGTCAGTTTTT.

Cultured adult mouse ventricular myocytes, as previously described \(^5\), were transfected with JP-2 shRNA or scrambled control lentiviral particles at a concentration of \(1.0 \times 10^5\) infectious units of virus (IFU) per ml. 64 hours after viral infection, proteins were extracted and quantified using western blotting assay. Myocyte t-tubule images were acquired with confocal microscope with the same configurations for different group studies.

**Statistics**

Data were expressed as mean ± SE. Student's t tests were applied when appropriate. \(p<0.05\) was considered statistically significant. The Chi-Square test and regression / correlation analysis were performed with Winks 4.62 statistics software (TexasSoft, Cedar Hill, Texas).
### Online Table I. Echocardiographic results of anesthetized rats

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Hypertrophy</th>
<th>HF</th>
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</thead>
<tbody>
<tr>
<td>N</td>
<td>7</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>Age (weeks after TAB)</td>
<td>10.0 ± 0.8</td>
<td>9.2 ± 0.48</td>
<td>9.9 ± 1.0</td>
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<tr>
<td>BW (g)</td>
<td>373 ± 26</td>
<td>369 ± 18</td>
<td>346 ± 19</td>
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<tr>
<td>HW (g)</td>
<td>1.49 ± 0.06</td>
<td>2.41 ± 0.11</td>
<td>3.25 ± 0.21</td>
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<td>HW/BW (mg/g)</td>
<td>4.01 ± 0.15</td>
<td>5.84 ± 0.18</td>
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<tr>
<td>LVMass (mg)</td>
<td>684 ± 28</td>
<td>981 ± 65</td>
<td>1090 ± 58</td>
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<tr>
<td>LVMass/BW (mg/g)</td>
<td>1.86 ± 0.10</td>
<td>2.81 ± 0.12</td>
<td>3.13 ± 0.20</td>
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<td>EDV (µl)</td>
<td>334 ± 24</td>
<td>374 ± 17</td>
<td>651 ± 59</td>
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<tr>
<td>ESV (µl)</td>
<td>62 ± 9</td>
<td>72 ± 9</td>
<td>341 ± 41</td>
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<td>Vol/Mass (µl/mg)</td>
<td>0.49 ± 0.02</td>
<td>0.38 ± 0.02</td>
<td>0.62 ± 0.05</td>
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<td>EF (%)</td>
<td>81.8 ± 1.2</td>
<td>79.3 ± 2.2</td>
<td>52.8 ± 2.3</td>
</tr>
</tbody>
</table>

**Notes:**

N, heart number;  
Age (weeks), weeks after banding surgery;  
BW (g), body weight;  HW (g), heart weight (wet);  
HW/BW (mg/g), heart weight to body weight ratio;  
LVMass (mg), left ventricular mass;  
LVMass / BW (mg/g): left ventricular mass to body weight ratio;  
EDV (µl): end diastolic volume;  
ESV (µl): end systolic volume;  
Vol/Mass: end diastolic volume to LV mass ratio  
EF (%): ejection fraction.

* p<0.05 vs sham; **, p<0.01 vs sham; I, p<0.05 vs hypertrophy;  
II, p<0.01 vs hypertrophy.
Online Figure I. In situ t-tubule imaging in atrial cells. A, A typical confocal image from right atrial epicardium displaying irregular, sparse t-tubule network in atrial myocytes. B&C, Power spectrum analysis shows no dominant peak, consistent with the poor regularity of t-tubule system in these atrial myocytes.
Online Figure II. In situ mitochondria imaging in left ventricular myocytes of intact heart. Mitochondria membrane potential probe, TMRE (2.5 mM) were loaded through retrograde Langendorff perfusion system. After 20 minutes of loading, the hearts were then placed into a perfusion chamber attached to confocal microscope equipped with a 63x optical lens (NA=1.3). The excitation wavelength is 561 nm, and emission wavelength >575 nm. **A,** A typical confocal image of mitochondria arrays in normal left ventricular myocytes from a sham operated heart. **B,** In situ mitochondria imaging from a failing heart (pericardial effusion, heart weight = 2.92 g, heart weight / body weight ratio = 9.7 mg/g, Lung weight = 2.45 g, lung weight / body weight ratio = 8.1 mg/g, EF=53%). No significant difference was found in the organization and membrane potential of mitochondria between sham and failing hearts.
REFERENCE


