Microtubule-Mediated Defects in Junctophilin-2 Trafficking Contribute to Myocyte Transverse-Tubule Remodeling and Ca$^{2+}$ Handling Dysfunction in Heart Failure

Caimei Zhang, PhD; Biyi Chen, MD, PhD; Ang Guo, PhD; Yanqi Zhu, MD, PhD; Jordan D. Miller, PhD; Shan Gao, MD, PhD; Can Yuan, PhD; William Kutschke, MS; Kathy Zimmerman, MS; Robert M. Weiss, MD; Xander H. T. Wehrens, MD, PhD; Jiang Hong, MD, PhD; Frances L. Johnson, MD; Luis F. Santana, PhD; Mark E. Anderson, MD, PhD; Long-Sheng Song, MD

Background—Cardiac dysfunction in failing hearts of human patients and animal models is associated with both microtubule densification and transverse-tubule (T-tubule) remodeling. Our objective was to investigate whether microtubule densification contributes to T-tubule remodeling and excitation–contraction coupling dysfunction in heart disease.

Methods and Results—In a mouse model of pressure overload–induced cardiomyopathy by transaortic banding, colchicine, a microtubule depolymerizer, significantly ameliorated T-tubule remodeling and cardiac dysfunction. In cultured cardiomyocytes, microtubule depolymerization with nocodazole or colchicine profoundly attenuated T-tubule impairment, whereas microtubule polymerization/stabilization with taxol accelerated T-tubule remodeling. In situ immunofluorescence of heart tissue sections demonstrated significant disorganization of junctophilin-2 (JP2), a protein that bridges the T-tubule and sarcoplasmic reticulum membranes, in transaortic banded hearts as well as in human failing hearts, whereas colchicine injection significantly preserved the distribution of JP2 in transaortic banded hearts. In isolated mouse cardiomyocytes, prolonged culture or treatment with taxol resulted in pronounced redistribution of JP2 from T-tubules to the peripheral plasma membrane, without changing total JP2 expression. Nocodazole treatment antagonized JP2 redistribution. Moreover, overexpression of a dominant-negative mutant of kinesin 1, a microtubule motor protein responsible for anterograde trafficking of proteins, protected against JP2 redistribution and T-tubule remodeling in culture. Finally, nocodazole treatment improved Ca$^{2+}$ handling in cultured myocytes by increasing the amplitude of Ca$^{2+}$ transients and reducing the frequency of Ca$^{2+}$ sparks.

Conclusion—Our data identify a mechanistic link between microtubule densification and T-tubule remodeling and reveal microtubule-mediated JP2 redistribution as a novel mechanism for T-tubule disruption, loss of excitation–contraction coupling, and heart failure. (Circulation. 2014;129:1742-1750.)

Key Words: excitation contraction coupling ■ junctophilin ■ microtubules ■ myocytes, cardiac

Microtubules, which are ubiquitous cytoskeletal fibers formed by polymerization of α- and β-tubulin dimers, regulate a wide range of cellular processes, including maintenance of cell shape, mitosis, and intracellular protein transport. In human hypertrophied and failing myocardium, tubulin expression levels were increased.1–3 In patients and animal models with symptomatic aortic stenosis, an increase in microtubule density, namely, microtubule densification, was found to be inversely related with left ventricular (LV) fractional shortening.4,5 Epidemiological studies demonstrate that the microtubule stabilizing agent taxol (also paclitaxel), an important antineoplastic chemotherapy agent, is associated with cardiotoxicity, including myocardial infarction, ventricular tachycardia, and congestive heart failure.5–7 Conversely, colchicine, a microtubule depolymerizing agent used to treat gout, decreases the incidence of myocardial infarction.8 Microtubule densification is a common observation in multiple animal models of cardiac disease.9,10 Studies performed in canine, feline, guinea pig, rat, and murine models consistently demonstrated that microtubule depolymerization attenuates cardiac dysfunction.11–13 For
example, in a canine model of pressure overload–induced LV hypertrophy, treatment with colchicine normalizes both in vivo and in vitro cardiac contractile function.11 Thus, multiple lines of evidence suggest a clear role for microtubule destabilization in the preservation of cardiac function, yet the mechanism by which microtubule destabilization results in loss of cardiac function remains incompletely understood.16,17

Clinical Perspective on p 1750

Defective excitation–contraction (E–C) coupling is a hallmark of heart failure.18–20 Emerging evidence demonstrates that disruption of the transverse-tubule (T-tubule) network is mechanistically involved in the development and progression of heart failure.21,22 T-tubules are highly organized invaginations of surface membrane that form tightly physical couplings with the terminal cisternae of the sarcoplasmic reticulum (SR), termed cardiac dyads.23 Precise communication between the voltage-gated L-type Ca2+ channels located mainly on the T-tubule membrane and Ca2+ release ryanodine receptor channels on the SR is essential for rapid electric excitation, initiation, and synchronous triggering of SR Ca2+ release and therefore coordinated myocyte contraction.24,25 Junctophilin-2 (JP2), a member of the junctophilin family, spans the T-tubule and SR membranes and thereby plays an important role in the formation and maintenance of the cardiac dyad.26 Loss of JP2 expression results in T-tubule remodeling after pressure overload, as demonstrated by studies in cultured myocytes and a transgenic mouse expressing JP2 short-hairpin RNA.27,28 Despite the substantial association in cardiac dysfunction between T-tubule remodeling and microtubule destabilization, it remains unclear whether the latter 2 processes are mechanistically linked in the development of heart failure. In addition, progressive loss of T-tubule integrity is a long-standing and well-known phenotype in cultured adult cardiomyocytes.29–31 Related unresolved questions are how progressive T-tubule loss occurs in cultured myocytes and whether it shares common mechanisms with T-tubule remodeling in vivo in response to cardiac stress.

Using a transaortic banding (TAB) murine pressure overload cardiomyopathy model, we first established that treatment with the microtubule disrupting agent colchicine preserves cardiac function and T-tubule integrity as demonstrated by in situ confocal imaging of Langendorff-perfused intact hearts. Our studies in cultured cardiomyocytes provided key mechanistic insights into how microtubules regulate T-tubule function and T-tubule organization by rearrangement and stabilization of microtubules. Microtubule polymerization/stabilization with taxol accelerates this T-tubule alteration, whereas microtubule destabilization is protective. Genetic silencing of JP2 using an inducible cardiac-specific JP2 short-hairpin RNA abrogates the protective effect of microtubule disruption. Moreover, pressure overload or microtubule destabilization induces marked redistribution of JP2 to the cell periphery, similar to that observed in failing human hearts. Last, overexpression of a dominant-negative mutant of microtubule motor protein kinesin 1 (also known as Kif5b) protected against JP2 redistribution and T-tubule remodeling. Analysis of Ca2+ handling properties demonstrate that microtubule destabilization rescues E–C coupling, including an increase in the amplitude of Ca2+ transients and a reduction of Ca2+ sparks. These data collectively indicate that microtubule destabilization contributes to T-tubule remodeling in heart failure by altering JP2 distribution within the membrane system.

Methods

Human Heart Samples

LV samples from patients with ischemic or dilated cardiomyopathies were obtained from explanted hearts at the University of Iowa Heart Failure Transplant Program. Nonfailing donor hearts without evidence of overt cardiac dysfunction were obtained through organ donor networks/organ procurement agencies. For immunostaining experiments, a total of 10 LV samples were studied, including 3 rejected healthy donor hearts and 7 end-stage heart failure patients with either ischemic heart disease (4) or dilated cardiomyopathy (3). All human heart tissue samples were obtained under organ research donation protocols approved by the Institutional Review Boards at the University of Iowa and Mayo Clinic.

Animal Studies and Experimental Methods

Animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (publication no. 85–23, revised 1996) and were approved by the Institutional Animal Care and Use Committee at the University of Iowa. All mice used in the study were in the C57BL/6 background. All experiments were performed in male mice aged 2 to 3 months. The numbers of mice and myocytes for each experimental group are provided in the figures or figure legends.

For detailed experimental methods, see the online-only Data Supplement.

Statistics

Data are expressed as mean±SE. One-way ANOVA with Bonferroni post hoc test was applied to multiple-group comparisons of in vivo animal experiments. The Bonferroni procedure after a global test based on a linear mixed-effects model was performed for multiple-group comparisons of in vitro cardiomyocyte experiments. A compound symmetry correlation structure was assumed for linear mixed-effects model tests. Student t test was used for 2-group comparisons. Statistical analyses were performed using SPSS version 15.0 (SPSS). Values of P<0.05 were considered statistically significant.

Results

Microtubule Depolymerization Attenuates In Vivo T-Tubule Remodeling After Pressure Overload–Induced Hypertrophy

Several studies demonstrated that microtubule stabilization and accumulation is associated with the loss of cardiac function after cardiac stress, yet the mechanisms remain incompletely understood. We hypothesized that microtubule stabilization is involved in T-tubule remodeling after pressure overload. In vehicle-treated mice, TAB produced severe T-tubule disorganization and subcellular T-tubule loss as demonstrated by in situ confocal imaging of the LV (Figure 1A). Whereas colchicine injection had no effect on T-tubule organization in the LV of sham mice, it protected T-tubule structure against pressure overload–induced remodeling (Figure 1A). To quantify the integrity of T-tubules, we conducted a power spectrum analysis of the strength of T-tubule structural integrity. The T-tubule power spectrum analysis demonstrated a marked decrease in the T-tubule integrity in the LV of TAB mice compared with sham mice (Figure 1B). Colchicine treatment attenuated the decrease...
Microtubule Depolymerization Protects T-Tubule Structure in Cultured Cardiomyocytes

Studies were next extended to cultured cardiomyocytes to investigate the mechanisms underlying the T-tubule protection by microtubule depolymerization. We first established reorganization and assembly of the microtubule network adjacent to the sarcolemma after 48 to 72 hours in culture (Figure 3A). Microtubule densification, as seen in culture for a longer time (48 and 72 hours), was abrogated by another microtubule depolymerizing agent, nocodazole (Figure 3A). The increase in microtubule stability during culture was accompanied by progressive T-tubule loss and disorganization (Figure 3B and 3C). Similar to our in vivo data, treatment of cultured cardiomyocytes with colchicine or nocodazole pronouncedly attenuated the progressive loss of T-tubule organization (Figure 3B and 3C).

To confirm the role of microtubule stabilization in T-tubule remodeling, cardiomyocytes were treated with taxol, a microtubule polymerizing agent. Taxol treatment exaggerated the densification and aggregation of the microtubule network, especially at 72 hours (Figure 4A). Disorganization of T-tubules was also exacerbated in cardiomyocytes treated with taxol for 72 hours compared with control cardiomyocytes, and this effect was antagonized by nocodazole treatment (Figure 4B and 4C). These data provide additional evidence that microtubule stabilization is associated with...
loss of T-tubule integrity in vivo after TAB and in isolated, cultured myocytes.

**JP2 Silencing Abolishes the T-Tubule Protection by Nocodazole**

We reported recently that JP2, which tethers T-tubules to the SR and is a critical safeguard of the cardiac dyad, is required to maintain T-tubule integrity.22 To examine whether JP2 is involved in the microtubule densification-mediated T-tubule disorganization, we next performed studies in mice that express a cardiac-specific tamoxifen-inducible JP2 short-hairpin RNA.27

Figure 5A demonstrates robust JP2 silencing in hearts from JP2 knockdown (JP2-KD) mice after 10-day tamoxifen injection (40 mg · kg⁻¹ · d⁻¹; for an incomplete KD with lower dosage or shorter injection period, see Figure II in the online-only Data Supplement). Cardiomyocytes freshly isolated from JP2-KD mice demonstrated significant disorganization of T-tubules (Figure 5B). In contrast to wild-type hearts, treatment with nocodazole failed to protect JP2-KD myocytes from T-tubule remodeling associated with culture (Figure 5C and 5D). These data indicate that JP2 is required for mediating the protective effect of microtubule depolymerization on T-tubule architecture.

**Microtubule Stabilization Induces JP2 Redistribution from T-Tubules to Peripheral Plasma Membrane**

We next investigated how microtubule depolymerization affects JP2 expression and subcellular distribution. Nocodazole had no effect on JP2 mRNA or protein levels in cultured cardiomyocytes (see Figure IIIA and IIIB in the online-only Data Supplement). Additional Western blotting assay with cytosolic and membrane fractions indicates that JP2 was primarily located on the...
after cardiac stress, we next performed studies in cultured JP2 undergoes a profound reorganization after cardiac stress. Dilated cardiomyopathies (Figure 4A, middle). Colchicine injection pronouncedly attenuated the TAB-induced redistribution of JP2 (Figure 4A, right). More importantly, the same phenotype was observed in tissue sections of failing human hearts of both ischemic and right. More importantly, the same phenotype was observed in tissue sections of failing human hearts of both ischemic and dilated cardiomyopathies. Nocodazole treatment for 48 hours did not alter its membranous distribution (see Figure IIIC in the online-only Data Supplement). In intact hearts, in situ immunofluorescence staining showed that JP2 was organized in regular striations similar to T-tubules (Figure 4A, right). Ten to 15 confocal images were acquired from each heart sample. C, Representative images of isolated cardiomyocytes from JP2-KD mice in the absence or presence of 10 μmol/L nocodazole (Noc) during culture. Con indicates dimethylsulfoxide control. Nocodazole treatment (Noc), 20 μmol/L taxol, or the combination of both Taxol+Noc in culture for 48 or 72 hours. D, Average data of TTpower from different treatments. n=25 to 30 cells per group. P<0.05 vs 0-hour control. No difference between the Con and Noc groups of the same time point.

Microtubule depolymerization attenuates the TAB-induced redistribution of JP2 (Figure 4A, middle). Colchicine injection pronouncedly attenuated the TAB-induced redistribution of JP2 (Figure 4A, right). More importantly, the same phenotype was observed in tissue sections of failing human hearts of both ischemic and dilated cardiomyopathies. JP2-KD abolishes the protective effect of microtubule depolymerization against transverse-tubule (T-tubule) remodeling in culture. A, Representative immunoblots of JP2 expression in cardiomyocytes isolated from wild-type (WT) and JP2-KD mice after 10-day tamoxifen injection. GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase (loading control). B, Representative images of T-tubule staining with Di-8-ANNEPS (4-[2-[6-(dioctylamino)-2-naphthalenyl]ethenyl]-1-(3-sulfopropyl) pyridinium inner salt) (left) and summarized average data of T-tubule power spectrum analysis (TTpower; right) in freshly isolated cardiomyocytes from WT and JP2-KD mice. n=25 to 30 cells per group. **P<0.01 vs WT (Student t test). C, Representative T-tubule images in cultured cardiomyocytes from JP2-KD mice in the absence or presence of 10 μmol/L nocodazole (Noc) during culture. Con indicates dimethylsulfoxide control. D, Average data of TTpower from different treatments. n=25 to 30 cells per group. **P<0.01 vs Noc at the same time point. *P<0.05 vs Noc at the same time point. ‡P<0.05 vs Taxol at the same time point; †P<0.05 vs Taxol at the same time point; ‡P<0.05 vs Noc at the same time point. P<0.001 among Con, Taxol, and Noc+Taxol groups at 48 and 72 hours (by linear mixed-effects model).

To better understand the nature of JP2 disorganization after cardiac stress, we next performed studies in cultured membrane in cardiomyocytes. Nocodazole treatment for 48 hours did not alter its membranous distribution (see Figure IIIC in the online-only Data Supplement). In intact hearts, in situ immunofluorescence staining showed that JP2 was organized in regular striations similar to T-tubules (Figure 6A, left). This staining pattern did not distinguish cell boundaries, which suggests that JP2 is primarily localized to the T-tubule regions in normal hearts. However, after TAB in mouse hearts, we detected a significant redistribution of JP2 protein, with punctate staining and aggregation (Figure 6A, middle). Colchicine injection pronouncedly attenuated the TAB-induced redistribution of JP2 (Figure 6A, right). More importantly, the same phenotype was observed in tissue sections of failing human hearts of both ischemic and dilated cardiomyopathies (Figure 6B). These data suggest that JP2 undergoes a profound reorganization after cardiac stress.
cardiomyocytes. Compared with freshly isolated myocytes (0 hours), the organized JP2 distribution was altered after 48 hours, with a more pronounced effect after prolonged culture for 72 hours (Figure 6C). As a quantitative index of the regularity of JP2 staining, we calculated the peak JP2 power value at the dominant frequency, using the same power spectrum analysis as for TTpower. Peak JP2 power was reduced after 48 hours in culture and more dramatically at 72 hours (Figure 6D). In addition, at 48 hours in culture, we observed a clear accumulation of JP2 around the edge of myocytes, suggesting a reorganization of JP2 protein during culture. More dramatically, at 72 hours, a significant amount of JP2 was redistributed from the T-tubule membrane to the periphery plasma membrane (Figure 6C and 6E). Microtubule depolymerization with nocodazole prevented the culture-associated disorganization of JP2 within the T-tubule network, as well as the redistribution to the peripheral membrane (Figure 6C through 6E). Treatment with taxol accelerated the culture-induced redistribution of JP2, and this effect was antagonized by nocodazole (Figure 6C through 6E). These data collectively demonstrate that microtubule stabilization results in JP2 redistribution without changes in JP2 expression, suggesting that the T-tubule remodeling associated with microtubule stabilization is mediated by alteration of normal JP2 localization.

Figure 7. Effects of microtubule motor proteins on [unctrophilin-2 (JP2) distribution and transverse-tubule (T-tubule) integrity. A, Representative images of T-tubules stained with Di-8-ANNEPS (4-[2-[(diocetylaminio)-2-naphthalenyl]ethyl]1-(3-sulfopropyl)pyridinium inner salt) (top) and summarized average data (bottom) of T-tubule power spectrum analysis (TTpower) in cells treated with or without 300 μmol/L erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) or 50 μmol/L ciliobrevin D (Cilio D) for 48 hours. n=25 to 30 cells per group. *P<0.05 among the 3 groups (Con, EHNA, and Cilio D; linear mixed-effects model). B, Isolated cardiomyocytes stained with anti-JP2 antibody after treatment with or without EHNA or Cilio D for 48 hours. Top, Representative images; bottom left, average JP2power; bottom right, semiquantitative analysis of JP2 redistribution from T-tubule membrane to periphery sarcolemma. n=15 to 20 cells per group. *P<0.05 vs Kif5b WT (Student t test).

Kinesin 1 Is Involved in Microtubule Polymerization-Mediated Abnormal Trafficking of JP2 Protein

Microtubule motor proteins kinesin 1 and dynein are responsible for the forward and retrograde trafficking of cargos along microtubules, respectively. To test whether inhibition of retrograde trafficking affects T-tubule remodeling in cultured cardiomyocytes, we used the dynein inhibitors erythro-9-(2-hydroxy-3-nonyl) adenine and ciliobrevin D. Neither 300 μmol/L erythro-9-(2-hydroxy-3-nonyl) adenine nor 50 μmol/L ciliobrevin D treatment for 48 hours altered T-tubule structure and JP2 redistribution in cultured cardiomyocytes compared with that of control (Figure 7A and 7B). In contrast, overexpression of a dominant-negative mutant of kinesin (Kif5b) protected against cultured-induced T-tubule remodeling, as well as JP2 redistribution to the cell periphery (Figure 7C and 7D) compared with that of expressing wild-type Kif5b. These data suggest a role for the anterograde trafficking of kinesin 1, but not the retrograde trafficking of dynein, on JP2 in microtubule densification–mediated T-tubule reorganization.

Protected T-Tubule Structure by Microtubule Depolymerization Preserves Ca2+ Handling

A fundamental effect of T-tubule remodeling in cardiomyocytes is defective E–C coupling between T-tubule and SR.
Accordingly, cultured cardiomyocytes paced at 1 Hz displayed depressed amplitude and prolonged time to peak of Ca\(^{2+}\) transients compared with freshly isolated myocytes (Figure 8A and 8B). Nocodazole treatment normalized these alterations. Under resting conditions, the activity of spontaneous Ca\(^{2+}\) sparks reflects the gating of ryanodine receptors on the SR. We detected a dramatic increase in spontaneous Ca\(^{2+}\) sparks in control cells after culture for 48 hours compared with freshly isolated cardiomyocytes (Figure 8C and 8D). Nocodazole treatment markedly reduced the frequency of Ca\(^{2+}\) sparks without affecting other parameters (Figure 8C and 8D). These data suggest that microtubule depolymerization plays an important role in preserving myocyte Ca\(^{2+}\) handling function by maintaining the normal architecture of E–C coupling machinery.

**Discussion**

The relationship between microtubule densification and loss of myocyte contractility in decompensated hypertrophied hearts was established >20 years ago.\(^{1,3-13}\) Our study using a murine model of chronic pressure overload–induced cardiomyopathy provides new insights by mechanistically linking microtubule accumulation and T-tubule remodeling, which leads to impaired E–C coupling and heart failure. The major findings of our study are as follows: (1) depolymerizing microtubules in vivo mitigated TAB-induced T-tubule remodeling and heart failure; (2) densification of microtubules was accompanied by T-tubule impairment, and attenuation of microtubule densification using microtubule depolymerizers mitigated T-tubule alterations; (3) the normal distribution of JP2 was altered in pressure-overloaded mouse hearts, as well as in human diseased hearts; (4) microtubule densification resulted in JP2 redistribution from T-tubules to the cell periphery, which was preventable by microtubule depolymerization; (5) the protective effect of microtubule depolymerization on T-tubules was abolished in cardiomyocytes from JP2-KD mice; (6) overexpressing a dominant-negative mutant of Kif5b prevented the culture-induced JP2 redistribution and T-tubule damage; and (7) microtubule depolymerization preserved cytosolic Ca\(^{2+}\) transients and markedly reduced spontaneous Ca\(^{2+}\) sparks in cultured cardiomyocytes. Together, these data implicate the critical role of microtubule densification in the mechanism of T-tubule remodeling and loss of E–C coupling in pressure overload–induced cardiomyopathy.

Cardiac E–C coupling relies on precise positioning of ion channels, transporters, and related regulatory/structural proteins. Microtubules form an intricate and dynamic network capable of shuttling protein-containing vesicles to proper destinations.\(^{34-37}\) It appears clear that the microtubule is in close approximation with E–C coupling machinery, such as T-tubular–SR junctions,\(^{38}\) whereby microtubules may modulate adjacent ryanodine receptor 2 activities.\(^{39,40}\) It is also suggested that microtubules are critical in maintaining the structural integrity of the SR network in ventricular myocytes.\(^{41}\)

Microtubule accumulation and densification attributable to increased expression of tubulin and formation of stable microtubules have been observed in multiple models of cardiac hypertrophy, at both the compensated and decompensated stages.\(^{1,10,14}\) It was established soundly that the densification of microtubule network contributes to the cardiac dysfunction in decompensated hypertrophy by 2 reported mechanisms. First, accumulation of microtubules increases the stiffness of the cell and mechanically impedes sarcomere motion.\(^{14,31}\) Second, microtubule densification alters E–C coupling, including Ca\(^{2+}\) influx and SR Ca\(^{2+}\) release, but this remains controversial.\(^{16,17}\) Elevation of stable microtubules occurred during both compensated and decompensated stages.\(^{1,10,14}\) It began at the onset of cardiac hypertrophy and was involved in early hypertrophic response of myocardium induced by pressure overload.\(^{10}\) These studies led us to hypothesize that microtubule densification might also be involved in chronic cardiac remodeling, such as T-tubule remodeling.\(^{22,42}\) Like microtubule densification, loss of T-tubule structural integrity is evident in compensated hypertrophy and progressively deteriorates with the development of heart failure.\(^{22,43}\) Loss of T-tubule integrity alters the spatial distribution of L-type Ca\(^{2+}\) channels and increases “orphaned” ryanodine receptors,\(^{44}\) which leads to reduced amplitude and slower rise of Ca\(^{2+}\) release.\(^{29,44}\) We found that, in both intact pressure-overloaded hearts and cultured cardiomyocytes, treatment with microtubule depolymerizing agents attenuated T-tubule impairment. Moreover, microtubule depolymerization preserved the amplitude of Ca\(^{2+}\) transients and reduced the frequency of Ca\(^{2+}\) sparks. Thus, our data provide a clear mechanistic link between microtubule densification, T-tubule remodeling, and E–C coupling dysregulation.
Our study also resolves a long-lasting question in the field: what mediates the progressive loss of T-tubules associated with prolonged culture of isolated primary cardiomyocytes. In the present study, our data not only suggest a link between microtubule densification and T-tubule remodeling but also provides mechanistic insights by implicating JP2 redistribution in this process in cultured myocytes. Moreover, our data also suggest that this is a common mechanism in cultured cardiomyocytes and in vivo after cardiac stress.

We and others reported a critical role for JP2 in T-tubule integrity such that deficiency of JP2 impairs T-tubule maturation during cardiac development and accelerates T-tubule remodeling and loss of E–C coupling under pathological conditions. One mechanism is through microRNA-mediated gene silencing. JP2 is also regulated at the posttranslational level via degradation by the Ca2+-dependent proteolysis. Our data herein complement these studies by providing new evidence that JP2 is required for the protective effect of microtubule depolymerization on T-tubule structure after cardiac stress. As an additional mechanism, we identified altered JP2 localization from the T-tubule membrane to the surface plasma membrane in response to stresses that induce microtubule densification. The mechanism likely involves enhanced anterograde trafficking of JP2 to the cell periphery. Our data indicate that the densification and likely reorganized microtubule network influence the redistribution of JP2 within the membrane system, thereby inducing T-tubule remodeling and E–C coupling dysfunction and contributing to the development of heart failure.

We reported recently that β-adrenergic blockage improves T-tubule remodeling and cardiac function after myocardial infarction through restoration of JP2 protein expression. In the present study, our data suggest that microtubule densification-mediated JP2 redistribution represents another mechanism associated with heart failure. Thus, in addition to maintaining expression of JP2, proper localization of JP2 to the T-tubule system is also critical for normal myocyte function.

In summary, we identify densification of microtubules as a cause of T-tubule remodeling in heart disease and in cultured adult cardiomyocytes. Our data implicate redistribution of JP2, a critical safeguard of the cardiac dyad, as a mechanism by which microtubule densification leads to T-tubule disruption, loss of E–C coupling, and heart failure. Our study also identifies a novel mechanism for JP2 dysregulation, that is, microtubule densification-mediated JP2 mistrafficking, complementary to other mechanisms of JP2 downregulation, such as microRNA-mediated gene silencing and posttranslational degradation. Together, these findings provide novel insights into the roles of microtubules and JP2 in the pathogenesis of heart failure, which may have important implications for future development of new therapeutic strategies to ameliorate cardiac remodeling and the progression of heart failure.

**Acknowledgments**

We thank Dr Guangmao Cheng (Medical University of South Carolina, Charleston, SC) for help on isolation of free and polymerized tubulin.

**Sources of Funding**

This work was supported by National Institutes of Health grants R01 HL099095 (L.S.), R00 HL099235 (J.D.M.), R01 HL079031 (M.E.A.), HL62494 (M.E.A.), HL70250 (M.E.A.), and HL113001 (M.E.A.), and HL085686 (L.F.S.), American Heart Association Scientific Development grant 0635056 N (L.S.), and American Heart Association Midwest Postdoctoral fellowship 13POST14630077 (A.G.).

**Disclosures**

None.

**References**


**Disclosures**

None.
by guest on July 29, 2017 http://circ.ahajournals.org/ Downloaded from

2014 Circulation 115:1750-1760


Clinical Perspective

Evidence from human patients and animal models of heart failure demonstrate that an increase in microtubule density (“densification”) is strongly associated with myocardial dysfunction, and microtubule depolymerization ameliorates cardiac dysfunction. However, until now, the mechanism by which microtubule densification results in loss of cardiac function remained poorly understood. We demonstrated recently that disruption of myocyte transverse-tubule (T-tubule) integrity is a key event in the development and progression of heart failure. T-tubules are orderly membrane invaginations that are essential for cardiac excitation–contraction coupling. Using human heart failure samples, in situ imaging of intact rodent hearts, and studies with cultured murine adult cardiomyocytes, to our knowledge, we provide the first evidence for a mechanistic link between densification of microtubules and T-tubule remodeling in heart failure. Our data implicate redistribution of junctophilin-2, a critical safeguard of the cardiac dyad, as a mechanism by which microtubule densification leads to T-tubule disruption, loss of excitation–contraction coupling, and ultimately heart failure. Our study identifies a novel mechanism for junctophilin-2 dysregulation: microtubule densification-mediated junctophilin-2 mistrafficking. We also resolve the long-standing experimental question of how cultured adult cardiomyocytes undergo progressive loss of T-tubule integrity. Together, our findings provide novel insights into the roles of microtubules and junctophilin-2 in the pathogenesis of heart failure. These data have important implications for future development of new therapeutic strategies to ameliorate cardiac remodeling and the progression of heart failure.
Microtubule-Mediated Defects in Junctophilin-2 Trafficking Contribute to Myocyte Transverse-Tubule Remodeling and Ca\textsuperscript{2+} Handling Dysfunction in Heart Failure


_Circulation_. 2014;129:1742-1750; originally published online February 11, 2014; doi: 10.1161/CIRCULATIONAHA.113.008452

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/129/17/1742

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2014/02/11/CIRCULATIONAHA.113.008452.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/
Supplemental Methods

Thoracic aortic banding (TAB) model and colchicine treatment

Male C57BL/6 mice (9-10 weeks) were subjected to sham or pressure overload by TAB surgery as described. Colchicine (Sigma, St. Louis, MO, USA) was injected intraperitoneally from 2 days after the Sham or TAB procedure once every other day. The injections began with 0.4 mg/kg and progressed to 1 mg/kg (0.4 mg/kg, 0.6 mg/kg, 0.8 mg/kg and 1 mg/kg) to allow the mice to adjust to the drug, as previously described. Then 1 mg/kg colchicine continued until 5 weeks after TAB. Control mice were injected with saline. LV function was examined by echocardiography at the end of 5 weeks after TAB. In situ confocal imaging of T-tubule structure from epicardial myocytes of intact hearts was performed on the next day after echocardiography.

Adult mouse ventricular myocytes isolation, cell culture and viral transfection

Adult ventricular cardiomyocytes were isolated from C57BL/6 mice or JP2 knockdown (JP2-KD) mice (2 ~ 3 months old) using standard enzymatic method described previously, and ≥75% Ca²⁺ tolerant rod-shaped myocytes were used for experiments. After stabilizing in 1.0 mMol/L Ca²⁺ Tyrode’s solution (in mmol/L: NaCl 137, KCl 5.4, HEPES 10, Glucose 10, MgCl₂ 1, NaH₂PO₄ 0.33, pH adjusted to 7.4 with NaOH) for 10 minutes (min), cell pellet was suspended in Minimum Essential Medium (MEM) with 10% fetal bovine serum (FBS) and plated in 35 mm dishes with laminin (10 μg/ml)-precoated glass at the bottom. The myocytes were cultured in 5% CO2 incubator at 37 °C for 2 hours (hrs), then the medium was changed to FBS-free MEM medium and myocytes were cultured at a period of 24 hrs to 72 hrs. For adenoviral-mediated
gene expression, freshly isolated cardiomyocytes were infected with adenoviruses expressing Kif5b (i.e. Kinesin 1) wild type (WT) or a dominant negative (DN) mutant for 48 hrs.

In some experiments, cardiomyocytes were isolated from JP2-KD mice. JP2-KD mice were generated by crossing JP2-shRNA mice (expressing a cardiac-specific, tamoxifen-inducible junctophilin-2 shRNA, C57BL/6 background) with MerCreMer (MCM) mice (Jackson Laboratory). Two to three month old male MCM (used as control) and double transgenic JP2-KD mice (MCM x JP2-shRNA) mice were treated with daily tamoxifen (~40 mg/kg, Sigma-Aldrich Co., St. Louis, MO) by intraperitoneal injection for 10 days. The next day following the last injection, cardiomyocytes were isolated and cultured as above.

**T-tubule imaging and Analysis**

T-tubules of single cardiomyocytes was stained with Di-8-ANEPPS (10 µmol/L, AAT BioQuest, CA) in Ca²⁺ free Tyrode solution at room temperature for 30 mins. T-tubules from intact hearts were stained with MM 4-64 (2.5 µmol/L, AAT BioQuest, CA) in Ca²⁺ free Tyrode solution via Langendorff perfusion at room temperature for 30 min. And the structure of T-tubules was visualized with confocal microscope (LSM510, Carl Zeiss MicroImaging Inc., Germany) on 63× lens. Quantitative analysis of T-tubule integrity was processed with IDL image analysis program as previously reported. The power value (TTpower) reflects the strength of the regularity of T-tubule organization.

**Immunofluorescent staining of LV cryosections and cardiomyocytes**

Hearts from sham and TAB mice were fixed by perfusion with 4% paraformaldehyde (PFA) for 15-20 min and postfixation of the longitudinally-cut heart with 4% PFA at 4°C for 24-48 hrs. Fixed heart tissue was then incubated in 10%, 20% and 30% sucrose sequentially at 4°C for cryoprotection before cryoembedding with optimal cutting temperature (OCT) compound. The heart tissues were longitudinally sectioned (10 µm) in cryostat at -24°C. Sections were
stored at -80°C before immunofluorescent staining. Isolated cardiomyocytes were fixed in 4% PFA at 37°C for 15 min. For immunofluorescent staining, samples were washed 3 times with PBS for 10 min, and followed by permeabilization with 0.3% Triton-X 100 in PBS for 30 min. Following 1% BSA blocking 30 min at room temperature, samples were incubated with anti-β-tubulin antibody (1:100, T4026, Sigma, St. Louis, MO, USA) / anti-α-tubulin antibody (1:300, ab18251, abcam, Cambridge, MA, USA), or anti-JP2 (1:50, sc-51313; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight followed by incubation with fluorescent-labeled secondary antibodies at room temperature for 2 hrs. Staining was visualized by confocal microscopy with a 63X lens. Microtubule density was quantitated using NIH Image J. JP2 organization (JP2_{power}) was processed with IDL image analysis program. Mean JP2 fluorescence around the cell periphery (within ~4 µm from cell edge) and mean JP2 fluorescence inside the cell were quantitated using a home-complied automatic program coded in MatLab.

**Protein preparation**

Single cardiomyocytes was suspended (Whole hearts or LV were homogenized) and sonicated in lysis buffer (in mmol/L: Tris-HCl 50 [pH 7.4]; NaCl 150; NaF 10; Na₃VO₄ 1; EGTA 5; EDTA 5; 0.5% Triton X-100; 0.5% Na deoxycholate and 0.1% SDS), containing protease inhibitors (Sigma, P8340). Then the lysates were centrifuged at 13,000g 4°C for 15 min. The supernatants were kept as whole cell proteins. Cytosol and membrane fraction was obtained from single cardiomyocytes by using modified method as previously reported. Cardiomyocytes were suspended and sonicated in lysis buffer (in mmol/L: Tris-HCl 25; sucrose 250, EDTA 1, EGTA 1, Na₃VO₄ 1, NaF 1 and protease inhibitor). The lysates were centrifuged at 13,000 g for 15 min, producing the supernatants designated as cytosol fraction. After being washed with lysis buffer 3 times, the pellets were re-suspended with the above lysis buffer with 0.5% Triton X-100,
0.5% sodium deoxycholate and 0.1% SDS, and then were centrifuged at 13,000 g for 15 min. The supernatants were saved as membrane fraction.

Free and polymerized tubulin were prepared by using modified method as previously reported. Fresh LV were homogenized in microtubule stabilization buffer (MTSB) (in mmol/L: GTP 1, Na2HPO4 10, EGTA 0.5, MgCl2 0.5, 50% glycerol, 5% DMSO). Protease inhibitor cocktails were included in this buffer, and the lysate was centrifuged at 100,000 x g 25°C for 20 min. The supernatants were saved as free tubulin. After being washed once with MTSB, the pellets were re-suspended with depolymerizing buffer (in mmol/L: PIPES 80, MgCl1, EGTA 1, CaCl2 5, 1% Triton X-100 and protease inhibitors) and kept on ice for 30 min. Then the suspensions were centrifuged at 20,000x g at 4°C for 10 min. The supernatants were saved as polymerized tubulin. The protein concentration was determined by BCA assay.

**Western blotting**

Whole cell proteins, cytosol and membrane fractions, free and polymerized tubulin were electrophoresed on 4-12% Bis-Tris gels. Proteins were transferred to PVDF and probed with primary antibodies recognizing JP2 (1:1000), β-tubulin (1:000) and GAPDH (1:10,000, G8975, Sigma), respectively, overnight at 4°C. After being washed with PBS solution 3 times, the binding of the primary antibodies was detected by horseradish peroxidase (HRP)-conjugated second antibodies (1:5,000-1:10,000 dilution in PBS solution). The immunoreactions were visualized using an enhanced-chemiluminescent detection kit and the protein bands were quantified with Quantity One 1-D Analysis Software (Bio-Rad, USA).

**Confocal Ca²⁺ Imaging**

Cultured cardiomyocytes were loaded with 5 µmol/L Fluo-4 AM (Invitrogen, Grand Island, NY) in 1.8 mmol/L Ca²⁺Tyrode solution for 30 min. Cells were then washed with dye-free Tyrode solution for 20 min for de-esterification before imaging. The Ca²⁺ images were acquired using
confocal microscope in line–scan mode along the longitudinal axis of myocytes. Steady-state Ca$^{2+}$ transients were measured in Tyrode’s solution containing 1.8 mmol/L Ca$^{2+}$ under field stimulation of 1 Hz. Ca$^{2+}$ sparks were recorded ~ 5 sec after halt of field stimulation.

**Statistical Analysis**

Data are expressed as mean ± SE. Bonferroni procedure followed a one-way ANOVA was applied to multiple group comparisons of in vivo animal experiments. Bonferroni procedure after a global test based on a linear mixed-effects model was performed to multiple group comparisons of in vitro cardiomyocyte experiments. A compound symmetry correlation structure was assumed for linear mixed-effects model tests. Student’s $t$-test was used for two group comparison. Statistical analysis were carried out using SPSS V15.0 software. Values of $p < 0.05$ were considered statistically significant.

**Supplemental References**


Figure S1. Pressure overload increases the protein expression of β-tubulin. Representative Western blot (upper) and summary data (lower) of β-tubulin expression in LV of sham mice and TAB mice injected with saline or Colchicine (Colch). n = 4 hearts per group. * p<0.05, compared to sham. #p<0.05, compared to saline TAB.
Figure S2. Microtubule depolymerizing agent does not affect JP2 mRNA and protein levels.

A. JP2 mRNA levels in cultured cardiomyocytes with or without 10 µM nocodazole for 48 hrs (n = 4 hearts per group).

B. Representative Western blot (upper) and summary data (lower) of JP2 protein levels after treatment with 10 µM nocodazole. Data were normalized to GAPDH and quantitated relative to control levels at 0 hr.

C. JP2 protein expression in cytosolic and membrane fractions of cardiomyocytes cultured for 48 hrs in the absence or presence of 10 µM nocodazole. Con: DMSO control; Noc: nocodazole. n = 3 hearts per group.
Figure S3. JP2 protein expression in the myocardium of MCM mice and MCM-shJP2 mice after tamoxifen (TMXF) injection. Upper: representative Western blot; Lower: summary data. TMXF were administered by intraperitoneal injection ~30 mg/kg for 5 consecutive days or ~40 mg/kg for 7 consecutive days. n = 4 hearts per group.