ALTERATIONS OF TENSION-DEPENDENT ATP-UTILIZATION IN A TRANSGENIC RAT MODEL OF HYPERTROPHIC CARDIOMYOPATHY

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While it is established that familial hypertrophic cardiomyopathy (FHC) is caused by mutations in several sarcomeric proteins, including cardiac troponin T (TnT), its pathogenesis is still not completely understood. Previously, we established a transgenic rat model of FHC expressing a human TnT molecule with a truncation mutation (DEL-TnT). The present study now investigated, whether contractile dysfunction and electrical vulnerability observed in DEL-TnT hearts might be due to alterations of intracellular Ca2+-homeostasis, myofibrillar Ca2+-sensitivity and/or myofibrillar ATP-utilization. Simultaneous measurements of force of contraction and intracellular Ca2+-transients were performed in right ventricular trabeculae of DEL-TnT hearts at 0.25 and 1.0 Hz. Rats expressing wild-type human troponin T (TnT) as well as non-transgenic rats (NT) served as controls. In addition, calcium-dependent ATPase activity and tension development were investigated in skinned cardiac muscle fibers. Force of contraction was significantly decreased in DEL-TnT compared to NT and TnT. Time parameters of Ca2+-transients were unchanged at 0.25 Hz, but prolonged at 1.0 Hz in DEL-TnT. The amplitude of the fura-2 transient was similar in all groups investigated, while diastolic and systolic Fura-2 ratios were found elevated in rats expressing non-truncated human troponin T. In DEL-TnT rats, myofibrillar Ca2+-dependent tension development as well as Ca2+-sensitivity of tension were significantly decreased, while tension-dependent ATP-consumption (“tension cost”) was markedly increased. Thus, a C-terminal truncation of the cardiac TnT-molecule impairs the force-generating capacity of the cycling cross-bridges resulting in increased tension-dependent ATP-utilization. Taken together, our data support the hypothesis of energy compromise as a contributing factor in the pathogenesis of FHC.

Introduction

Familial hypertrophic cardiomyopathy (FHC) is an autosomal-dominant inherited disease characterized by ventricular hypertrophy, arrhythmias and sudden death. The phenotype of affected individuals may vary from early sudden death, marked hypertrophy with contractile dysfunction to an asymptomatic carrier status (reviewed in (1-3)). In the past ten years, several genes could be linked to FHC, almost uniformly encoding contractile proteins such as ß-myosin heavy chain and cardiac Troponin T (4). FHC has thus been labeled a “disease of the sarcomere” (5). Mutations of the cardiac troponin T (cTnT) gene are of particular interest since they are associated with a high incidence of arrhythmias and sudden death even in the absence of significant myocardial hypertrophy (6). Yet, the pathogenesis of FHC is still poorly understood and there is no clear understanding how these mutations lead to the development of FHC with diastolic dysfunction and sudden cardiac death. Biochemical and biophysical analyses have failed to identify a common
mechanism underlying the alterations of cardiac contractility resulting from these mutations. For example, missense mutations in β-myosin heavy chain have been shown to both depress (7) as well as enhance (8) contractile function, whereas mutations in alpha-tropomyosin increase calcium sensitivity and force development of the sarcomere (9). Likewise, it is still unclear, how FHC-associated cTnT-mutations trigger the initiation of FHC (10). The exact molecular function of troponin T is also still a matter of debate; however, it is believed that cTnT stabilizes the troponin complex, consisting of tropinin T, I and C. In addition, cTnT may affect and potentially regulate the Ca²⁺-sensitivity of myofibrillar ATPase activity, the level of ATPase activation and/or force development (11-15).

Recently, it has been observed that FHC-patients with cardiac Troponin T- as well as β-myosin heavy chain- and myosin binding protein C- mutations display a markedly altered phosphocreatine to ATP-ratio, irrespective of the presence of myocardial hypertrophy (16). This finding led to the new hypothesis of myocardial energy depletion being a critical factor in the pathogenesis of FHC (17).

To get a closer understanding of the pathophysiology underlying FHC, we have previously (18) generated a transgenic rat model of the disease by overexpressing a C-terminal cTnT truncation (DEL-TnT), resulting from an intron 15 splice donor site mutation observed in FHC patients. Wildtype rats as well as rats overexpressing non-mutated human TnT served as controls. In a working heart model, DEL-TnT-transgenic rats exhibited significant systolic and diastolic dysfunction in the absence of cardiac hypertrophy. Of note, relatively small amounts of DEL-TnT (~5% of endogenous TnT) were sufficient to induce this phenotype, similar to observations in other transgenic models of HCM (19, 20). In contrast, transgenic rats overexpressing a non-mutated human TnT molecule (~30% of endogenous TnT) displayed improved contractile performance. Moreover, after exercise training, myocardial disarray and ventricular arrhythmias such as ventricular tachycardia and ventricular fibrillation, were observed in DEL-TnT-transgenic rats (18). Immunofluorescence analyses of transgenic cardiomyocytes demonstrated that the mutant TnT is incorporated into the sarcomere, suggesting a dominant-negative mode of action rather than haploinsufficiency.

To further investigate the mechanisms underlying contractile dysfunction in DEL-TnT transgenic rats and specifically, if alterations of Ca²⁺-homeostasis contribute to the phenotype, we now performed simultaneous measurements of force and intracellular Ca²⁺-transients in isolated electrically stimulated papillary muscle strips. Furthermore, to test whether ATP-utilization might be impaired in DEL-TnT transgenic rats, calcium-dependent tension development and myofibrillar ATPase activity were measured in triton-X100 skinned cardiac fiber preparations. Rats overexpressing the normal, i.e. non-truncated, human TNT-molecule as well as non-transgenic controls were studied for comparison.

The current findings confirm and extend our previous data that a C-terminal truncation of the cardiac TnT-molecule impairs the force-generating capacity of the cycling cross-bridges. Moreover, this effect is accompanied by increased tension-dependent ATP-utilization in DEL-TnT rats, thus supporting the hypothesis of energy compromise as a contributing factor in the pathogenesis of FHC.

**Experimental procedures**

**Animals**

Transgenic rats overexpressing the predominant adult isoform of human cTnT (288 amino acids) were generated as described previously (18). Cardiac skinned fiber preparations as well as right ventricular papillary muscles were investigated from transgenic rats overexpressing human wild-type cTnT (TnT-rats, n=10) or a truncated human cTnT molecule resulting from an intron 15 splice donor site mutation, lacking the most C-terminal 14 amino acids (DEL-TnT rats, n=10), in comparison to non-transgenic controls (NT, Sprague Dawley rats, n=10). The age of the rats ranged from 14-16 months. The animals were killed by cervical dislocation and hearts were rapidly removed.

Control and transgenic animals did not differ in their body weight (NT: 414±42 g, DEL-TnT: 427±33 g, hTnT: 415±22 g) or their heart wet weight (NT: 1.6±0.1 g, DEL-TnT: 1.9±0.1 g, hTnT: 1.9±0.1 g). The investigation conformed with institutional guidelines as well as the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

**Simultaneous measurements of force and intracellular Ca²⁺-transients**
The intracellular Ca\(^{2+}\)-transient was measured at room temperature in isolated, electrically driven right ventricular papillary muscles by the fluorescence indicator fura-2 (21). To facilitate cell loading fura-2 was used as acetoxymethyl (AM) ester. These AM esters passively cross the plasma membrane and, once inside the cell, are cleaved to cell-impermeant products by intracellular esterases. After an initial control measurement of the force of contraction (FOC), the muscle strips were incubated for 4 hours in darkness to avoid photobleaching of the dye in an oxygenated (95% O\(_2\); 5% CO\(_2\)) Ringer’s solution containing 5 µmol/l of fura-2-AM. The external calcium concentration was 1.8 mmol/l. The experimental setup was purchased from Scientific Instruments, Heidelberg, Germany and has been described previously (22). Since the right papillary muscles are more suitable for fura-2 measurements due to their smaller diameter, Ca\(^{2+}\)-transients were studied in these preparations. The cross sectional areas of papillary muscle preparations were comparable in all experimental groups: NT-rats: 0.58±0.05 mm\(^2\), TnT-rats: 0.45±0.03 mm\(^2\), DEL-TnT rats: 0.58±0.06 mm\(^2\). Left ventricular myocardium was used for skinned fiber experiments (see below).

Preparation of Triton X100-skinned fibers

Skinning of the heart muscle fibers was performed as described previously (23). Briefly, the fiber bundles (diameter <0.2 mm) were dissected from left ventricular papillary muscles and permeabilized at 4°C for 20 h in a solution containing 50% (v/v) glycerol, 1% triton X, and in mmol/l NaN\(_3\) 10, ATP 5, MgCl\(_2\) 5, EGTA 4, 1,4-dithioerythritol (DTE) 2, and imidazole 20 (pH 7.0). Subsequently, the fibers were stored in the same buffer without triton X100 at -20°C. The experiments were performed within 5 days.

Force and ATPase activity measurements

Triton X100-skinned fiber bundles were prepared under the microscope and then mounted isometrically and connected to a force transducer (Scientific Instruments, Heidelberg). Mean sarcomere length was 1.9 ± 0.03 µm. ATPase activity was simultaneously measured with tension development using a linked NADH fluorescence assay (0.6 mM NADH, 140 U lactate dehydrogenase) as described previously (23-25) (experimental setup: Scientific Instruments, Heidelberg, Germany). Relaxation solution contained 20 mM imidazole, 10 mM ATP, 5 mM NaN\(_3\), 5 mM EGTA, 12.5 mM MgCl\(_2\), and 0.2 mM P\(_i\)P\(_\gamma\)-di(adenosine 5')pentaphosphate. The contraction solution contained calcium EGTA (5 mM) instead of EGTA. The ATP-concentration was stabilized with an ATP-regenerating system, including phosphoenolpyruvate (12.5 mM) and pyruvate kinase (100 U/ml). Both solutions were mixed by a gradient mixer such that the Ca\(^{2+}\)-concentration was incrementally increased every 15 s. Fibers were fixed in ‘slack’ position in relaxation solution (composition see above) and fiber length was adjusted to an extent where resting tension was just threshold. Measurement of developed tension and myofibrillar ATPase activity started 3 s after the solution was exchanged when a stable plateau was reached. At the end of the experiment, the NADH decline was determined by perfusion with a defined solution not containing NADH (in mmol/l: imidazol 20, NaN\(_3\) 5, EGTA 5, MgCl\(_2\) 12.5, phosphoenolpyruvate 5, calcium 5) as well as with the use of a the same solution plus 600 µmol/l NADH. These solutions did not contain additional calcium. Calibration of the signal was performed under conditions of a continuous perfusion of the respective calibration solutions until a stable signal had been reached. Normalization between muscle samples was achieved by multiplication of the ATPase signal with the ratio of cuvette diameter and fiber diameter. Free Ca\(^{2+}\)-concentration was determined by calculator programs designed for experiments in skinned muscle cells (27). Experiments were performed at 25°C. The ratio of ATPase activity and force was assumed as a measure for ‘tension cost’. Table 1 summarizes the parameters measured to characterize myofibrillar function.

Materials

Salts used were high analytical grade, purchased from Merck, Darmstadt, Germany. All other chemicals were of analytical grade or the best grade commercially available.

Statistics

All values are means ± S.E.M. unless otherwise noted. Student’s t-test, or paired t-test were used to test for significance. P-values of <0.05 were accepted as significant. pCa-force as well as pCa myofibrillar ATPase activity relationships were fitted by a modified Hill equation (28) as follows:

\[ Y = \frac{[Ca^{2+}]^{nH}}{[pCa_{50}]^{nH} + [Ca^{2+}]^{nH}} \]

where Y is the fractional force, or acto-myosin-ATPase activity, pCa\(_{50}\) is the Ca\(^{2+}\)-concentration...
giving half maximal activation (inhibition), and H is an index of cooperativity (Hill-coefficient). The pCa_{50} for tension development or myofibrillar ATPase activity, all Hill-coefficients, and the tension cost were analyzed by Graph Pad Prism (San Diego, USA).

Results

Frequency-dependent alterations of force and Ca^{2+}-transients in right ventricular muscle strips of TnT-transgenic rats

It has been proposed that the impaired cardiac relaxation observed in working heart models of transgenic mice expressing a truncated TNT-molecule might involve altered intracellular Ca^{2+}-homeostasis (20). To investigate whether dysregulation of intracellular Ca^{2+}-homeostasis is present in DEL-TnT-transgenic rats, simultaneous measurements of the intracellular Ca^{2+}-transient and force of contraction were performed at 0.25 Hz and 1.0 Hz. Table 2 and figure 1 summarize the results obtained for the frequency-dependent changes of the isometric force of contraction as well as intracellular Ca^{2+}-transients.

**Force.** At a stimulation frequency of 0.25 Hz, force of contraction (FOC), as well as contraction (+dP/dt) and relaxation (-dP/dt) velocities were markedly decreased in rats expressing the truncated human cardiac TnT-molecule (DEL-TnT) both compared to TnT-transgenic and NT-rats (Fig. 1A). Conversely, FOC, +dP/dt and –dP/dt were significantly increased in rats expressing the non-mutated human cardiac TnT-molecule (TnT) compared to non-transgenic controls (NT). The same pattern was observed at the higher stimulation frequency of 1.0 Hz: Again, DEL-TnT rats displayed impaired contractile function, whereas TnT-rats showed “supranormal” contractile behaviour (Table 2, Fig. 1A). In all three experimental groups, a comparable frequency-dependent decline in force of contraction was observed (NT: -24.2±5.2%, hTnT: -18.4±3.8%, DEL-TnT: -19.3±2.6%).

**Ca^{2+}-transients.** At 0.25 Hz, Fura-2 amplitudes were similar in all three groups (Table 2). Systolic and diastolic Fura-2 ratios were again significantly increased in TNT-rats compared to NT rats. At higher stimulation frequencies, the amplitude of the intracellular Ca^{2+}-transient declined to a similar degree in all three experimental groups investigated. Time to peak Ca^{2+}-transient and time to half peak Ca^{2+}-transient decay were not significantly different in DEL-TnT, TnT- and NT-rats at 0.25 Hz. However, at a higher stimulation frequency (1.0 Hz), both time to peak Ca^{2+}-transients as well as time to half peak Ca^{2+}-transient decay were prolonged in the DEL-TnT group compared to TnT-rats (Fig. 1).

Tension development and myofibrillar ATPase activity in DEL-TnT transgenic rat hearts

To examine a possible influence of the cardiac troponin T truncation mutation (DEL-TnT) on tension-dependent ATP-utilization in the transgenic model, simultaneous measurements of tension and myofibrillar ATPase activity were performed in skinned fibres at increasing extracellular Ca^{2+}-concentrations. Figure 2 shows representative original tracings of the experiments. Fig. 3 and table 3 summarize the results. In DEL-TnT rats, maximal Ca^{2+}-activated tension and Ca^{2+}-sensitivity of tension development were significantly decreased compared to rats expressing the non-truncated human TnT-molecule (Table 3). The steepness (nHill) of the Ca^{2+}-tension relationship was increased to a similar degree in DEL-TnT and TnT- compared to NT-fibres (Table 3). The expression of a truncated TnT-molecule in transgenic rat hearts did not significantly alter basal and maximal myofilament ATPase activity nor the Ca^{2+}-dependent increase in acto-myosin ATPase activity compared to non-transgenic controls (NT). However, Ca^{2+}-sensitivity of the myofibrillar ATPase activity was significantly shifted to the right in DEL-TnT compared to NT-rats (p<0.05; Table 3).

Tension development and myofibrillar ATPase activity in TnT-transgenic rat hearts

To investigate whether myofibrillar function might also be altered by expressing the non-truncated, human TnT-molecule in rat myocardium, simultaneous measurements of tension and myofibrillar ATPase activity were performed in skinned fibres from these animals. Figure 2 shows representative original tracings of the experiments. Fig. 3 and table 3 summarize
the results. In TnT-rats, maximal Ca\textsuperscript{2+}-activated tension was significantly increased compared to both DEL-TnT- and NT-fibres, while Ca\textsuperscript{2+}-sensitivity of tension development was not different (Table 3). Moreover, basal and maximal ATPase activity, as well as the Hill-coefficient of the Ca\textsuperscript{2+}/tension relation, which characterizes the cooperativity of the myofilaments, were significantly increased compared to NT-rats (Fig. 3, table 3). In contrast, myofibrillar Ca\textsuperscript{2+}-sensitivity of tension was not significantly influenced by the expression of a human TnT-molecule in rat cardiac tissue.

**Tension cost in TnT- and TnT-DEL rats**

Tension-dependent ATP-utilization, a parameter reflecting myofibrillar economy, was analyzed by the ratio of myofibrillar ATPase activity over tension (‘tension cost’). While TnT-transgenic rats showed a tension cost ratio (0.71) comparable to non-transgenic rats (0.74), the slope of tension-dependent ATP-utilization was significantly decreased in the DEL-TnT-group (0.31; \(p<0.05\) (table 3, fig. 4)). Myofibrillar ATP-consumption was thus markedly increased in rats expressing a truncated TnT-molecule compared to controls.

**Discussion**

While multiple mutations in sarcomeric genes have been found in patients with familial hypertrophic cardiomyopathy (FHC), the pathogenesis of the disease remains poorly understood. We have previously established a transgenic rat model for FHC that displays typical FHC-features, such as diastolic dysfunction and a propensity towards arrhythmias (18). In order to gain additional insight into the pathophysiology of this animal model, the present study investigated myofibrillar tension development, intracellular Ca\textsuperscript{2+}-homeostasis, and acto-myosin ATPase activity in hearts of DEL-TnT-transgenic rats. Our findings demonstrate that a C-terminal truncation of the cardiac TnT-molecule markedly impairs the force-generating capacity of the cycling cross-bridges. In addition, calcium transients were altered in transgenic rats expressing mutant TnT, but not in animals carrying a non-mutated human TnT transgene. Finally, significantly increased tension-dependent ATP-utilization was observed in DEL-TnT rats, suggesting that compromised cardiac energy homeostasis contributes to the pathogenesis of FHC.

**Reduced myofibrillar tension development due to a truncation of the human TNT-molecule**

In isolated working heart preparations from transgenic rats we have previously shown that DEL-TnT hearts display contractile dysfunction, whereas hearts expressing non-mutated human TnT actually reveal enhanced contractility compared to wildtype controls. We could now corroborate and extend these findings by measuring force of contraction (FOC) in isolated papillary muscle strips as well as tension development in skinned fibre preparations. Again, DEL-TnT resulted in significant depression of FOC, whereas a non-mutated human TnT improved FOC and tension development, respectively. While it is now widely accepted that a dominant-negative mechanism accounts for the deleterious effects of sarcomeric protein mutations in FHC (3,4), it is still unclear, how precisely these mutations modulate myofilament function and eventually lead to the clinical phenotype of hypertrophy, contractile dysfunction and lethal arrhythmias.

The TnT-molecule is critical for binding of the regulatory proteins troponin C and troponin I to tropomyosin on the thin filament (29-31). The association of TnT and tropomyosin facilitates tropomyosin assembly on the actin filament (32) and thus the cooperative activation of muscle contraction (33, 34). Given that the TnT C-terminus also binds troponin C and I, it seems possible that alterations of this region due to a mutation could directly influence myofibrillar cooperativity. Moreover, it has recently been shown that PKC-dependent phosphorylation of TnT at Thr\textsuperscript{195}, Thr\textsuperscript{204} and Thr\textsuperscript{285} (35) and Thr\textsuperscript{206} (36), all located at the carboxyl terminus of cardiac TnT, results in a decrease in maximum tension in murine fiber preparations. It has been suggested that the degree of TnT phosphorylation affects myofilament activation by modulating the interaction with troponin I (15), which may account for the observed functional impairment. The present study provides evidence that a truncation of the TnT-molecule modulates both Ca\textsuperscript{2+}-dependent tension and myofibrillar ATPase activity, consistent with the notion that conformational changes of the C-terminus can affect TnT function. These alterations could contribute to the contractile dysfunction previously described in isolated working heart preparations of DEL-TnT rats (18). The critical role of the TnT-C-terminus in this regard is further supported by a recent study.
demonstrating that not only PKC- but also Rho-dependent cTnT-phosphorylation at Ser$^{278}$ and Thr$^{287}$ is able to regulate tension and ATPase activity (37).

In contrast, transgenic rats overexpressing the wild-type, non-truncated human TnT-molecule display unchanged Ca$^{2+}$-sensitivity of myofibrillar tension development, yet a significant increase in maximal Ca$^{2+}$-activated tension compared to controls. These findings imply that functional differences must exist between rat and human cTnT. Interestingly, the same principal finding, i.e. improved performance of human wildtype TnT, has also been observed in a transgenic mouse model of FHC (38), further suggesting distinct properties of human vs. rodent TnT. While human and rat TnT display 89% of amino acid identity, the N-termini show significant sequence variations. Of note, this region is also critical in the regulation of cross-bridge kinetics and calcium sensitivity (39), possibly accounting for the observed species-specific functions. In line with this, our current results indicate that expression of a wild-type human TnT-molecule in rat myocardium may alter the co-operativity between thick and thin filaments resulting in improved Ca$^{2+}$-dependent tension development without altering the tension-dependent energy demand (s. below). However, we cannot exclude that other molecular adaptations such as myosin isoform switching (40) or altered expression of calcium handling proteins (s. below) contribute to the differential effects of the transgenes on force of contraction as well.

**Intracellular Ca$^{2+}$-homeostasis in TnT-transgenic rats**

From functional studies in murine models of TnT-associated human FHC, it has been proposed that diastolic dysfunction observed in these animals might involve a dysregulation of intracellular Ca$^{2+}$-homeostasis (29, 41). The present study provides evidence that at least at higher stimulation frequencies the regulation of intracellular Ca$^{2+}$-transients is in fact altered in rats expressing the truncated human TnT-molecule: At a stimulation frequency of 1 Hz, both time to peak Ca$^{2+}$-transient and time to peak Ca$^{2+}$-decay were found to be prolonged (Fig. 1). Intracellular Ca$^{2+}$-transients are subject to tight control and depend in part on the activity of SERCA2a as well as the Na$^{+}$/Ca$^{2+}$-exchanger (42, 43). In this regard, it has recently been shown that isolated cardiomyocytes from transgenic mice expressing a I79N TnT-mutation also display a slowed Ca$^{2+}$-transient decay. This was associated with differential activation of the Ca$^{2+}$-dependent Na$^{+}$/Ca$^{2+}$-exchanger and the increased occurrence of ventricular arrhythmias (41). In line with this, ventricular arrhythmias have also been observed in our DEL-TnT-rat model (18), raising the possibility that Na$^{+}$/Ca$^{2+}$-exchanger activity might also be altered in transgenic rats expressing a truncated human TnT-molecule.

In contrast, in rats expressing a non-truncated human TnT molecule, we did not observe a prolongation of calcium transients, yet diastolic and systolic Fura-2 ratios were found to be significantly elevated. This finding may be due to a complex adaptation to altered myofilament calcium sensitivity in TnT transgenic animals. Alterations in calcium cycling proteins have been observed in other transgenic models of HCM. Semsarian et al. (44) found that mice carrying an α-myosin heavy chain mutation (Arg403Gln) display a marked downregulation of the ryanodine receptor, calsequestrin, as well as triadin, while SERCA2a levels were unchanged. Remarkably, these changes could be reversed upon treatment with the calcium channel inhibitor diltiazem. It thus remains to be seen, if dysregulated expression of calcium handling proteins also contributes to the phenotype of DEL-TnT rats.

**Myofilament ATP-utilization in TnT-transgenic rats**

FHC is considered to be a “disease of the sarcomere” (5), as multiple different mutant alleles have been described in at least nine genes encoding cardiac contractile proteins (for review see (4)). Yet it has been difficult to devise a “unifying hypothesis” of its pathogenesis, since FHC-associated mutations have vastly variable (or even opposing) effects i.e. on sarcomeric calcium sensitivity, force development and ATPase activity. Therefore, it has recently been proposed that a common feature shared by many (if not all) different classes of FHC-causing mutations might be inefficient sarcomeric ATP-utilization (17, 39, 45). The high risk of sudden death in FHC-patients with TnT mutations could thus be attributed to alterations in cardiac energy consumption resulting from an increase in the energy cost of force production (39). This concept is also supported by the fact that inherited disorders of mitochondrial function, i.e. CD36 deficiency (46) or Friedreich’s ataxia (47), result in a phenocopy of hypertrophic cardiomyopathy. Moreover, carriers of FHC-
associated mutations that do not (yet) display cardiac hypertrophy, already reveal clearly abnormal phosphocreatine/ATP-ratios (16), suggesting that altered energy metabolism is not merely secondary to hypertrophic growth of the myocardium, but rather an early feature in disease progression. The present study provides additional evidence that dysregulation of myofibrillar energy utilization could contribute to the functional alterations associated with FHC: The “tension cost”, reflecting the ratio of myofibrillar ATPase activity and tension was markedly altered in DEL-TnT transgenic rats, such that myofibrillar energy consumption for a given tension was significantly increased (Fig. 4). While enhanced and inefficient ATP-consumption in DEL-TnT mutant hearts might be negligible under basal conditions, it may become limiting in situations of increased cardiac work load, such as physical exercise, and cardiac arrhythmias or even sudden cardiac death might ensue. Findings in other transgenic models of FHC further support this notion: Javadpour and coworkers reported that in (R92Q)-TnT-transgenic mouse cardiac ATP-utilization is increased as well, and resulted in the impaired ability of the heart to recruit its contractile reserve (48). Similarly, Montgomery et al. (49) reported an increase in tension cost (and unchanged Ca²⁺-activated maximal ATPase activity) in two independent transgenic mouse models expressing TNT-mutations. Moreover, Stelzer et al. (50) investigated a mouse model with overexpression of C-terminally truncated TnT and observed enhanced thin filament activation at submaximal calcium concentrations. However, in contrast to our work, an increase in calcium activated force was found, which may be due to the different truncation mutation analyzed in this study.

Finally, in transgenic rat hearts expressing non-mutated human TnT we observed a significantly increased force production without an increase in tension cost, suggesting a “supranormal” or even potentially beneficial effect on contractility. However, we believe that these data need to be interpreted with caution, since it is not known what the effects of human TnT were at the physiological rat heart rate of 500-600/min. Yet it is conceivable that subtle differences in TnT function must exist between humans and rodents in order to adapt the sarcomere to an about 10-fold difference in heart rate.

Limitations of the study

While our findings support previous in vivo data of the DEL-TnT-transgenic rat model (18), the current measurements have been performed in vitro and thus cannot necessarily be generalized. In addition, calcium transients and force measurements in right ventricular papillary muscle were performed under conditions not identical to the physiological situation (stimulation frequency of 0.25-1Hz, temperature of 25°C) in order to obtain stable and reproducible signals. However, it appears unlikely that these limitations introduced systematic errors, since the conclusion of the study largely rely on the relative differences between transgenic rats and controls.

Conclusion

In conclusion, the present study provides evidence that overexpression of a cardiac TnT molecule lacking the 14 C-terminal amino acids impairs myofibrillar tension development and increases tension-dependent myofibrillar ATPase activity. Taken together, our data support the hypothesis of energy compromise as a contributing factor in the pathogenesis of FHC.

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References

1. Maron, B.J. (2002) *JAMA* **287**, 1308-20.
2. Franz, W.M., Muller, O.J., and Katus, H.A. (2001) *Lancet* **358**, 1627-1637.
3. Arad, M., Seidman, J.G., and Seidman, C.E. (2002) *Hum. Mol. Genet.* **11**, 2499-506.
4. Marian, A.J., and Roberts, R. (2001) *J. Mol. Cell. Cardiol.* **33**, 655-70.
5. Thierfelder, L., Watkins, H., MacRae, C., Lamas, R., McKenna, W., Vosberg, H.P., Seidman J.G., and Seidman, C.E. (1994) *Cell* **77**, 701-712.
6. Moolman, J.C., Corfield, V.A., Posen, B., Ngumbela, K., Seidman, C.E., Brink, P., and Watkins, H. (1997) *J. Am. Coll. Cardiol.* **29**, 549-55.
7. Cuda, G., Fananapazir, L., Epstein, N.D., and Sellers, J.R. (1997) *J. Muscle Res. Cell. Motil.* **18**, 275-283.
8. Tyska, M.J., Hayes, E., Giewat, M., Seidman, C.E., Seidman, J.G., and Warshaw, D.M. (2000) *Circ. Res.* **86**, 737-744.
9. Moolman, J.C., Corfield, V.A., Posen, B., Ngumbela, K., Seidman, C.E., Brink, P., and Watkins, H. (1997) *J. Am. Coll. Cardiol.* **29**, 549-55.
10. Cuda, G., Fananapazir, L., Epstein, N.D., and Sellers, J.R. (1997) *J. Muscle Res. Cell. Motil.* **18**, 275-283.
11. Maron, B.J. (2002) *JAMA* **287**, 1308-20.
12. Franz, W.M., Muller, O.J., and Katus, H.A. (2001) *Lancet* **358**, 1627-1637.
13. Arad, M., Seidman, J.G., and Seidman, C.E. (2002) *Hum. Mol. Genet.* **11**, 2499-506.
14. Marian, A.J., and Roberts, R. (2001) *J. Mol. Cell. Cardiol.* **33**, 655-70.
15. Thierfelder, L., Watkins, H., MacRae, C., Lamas, R., McKenna, W., Vosberg, H.P., Seidman J.G., and Seidman, C.E. (1994) *Cell* **77**, 701-712.
16. Moolman, J.C., Corfield, V.A., Posen, B., Ngumbela, K., Seidman, C.E., Brink, P., and Watkins, H. (1997) *J. Am. Coll. Cardiol.* **29**, 549-55.
17. Cuda, G., Fananapazir, L., Epstein, N.D., and Sellers, J.R. (1997) *J. Muscle Res. Cell. Motil.* **18**, 275-283.
18. Tyska, M.J., Hayes, E., Giewat, M., Seidman, C.E., Seidman, J.G., and Warshaw, D.M. (2000) *Circ. Res.* **86**, 737-744.
19. Moolman, J.C., Corfield, V.A., Posen, B., Ngumbela, K., Seidman, C.E., Brink, P., and Watkins, H. (1997) *J. Am. Coll. Cardiol.* **29**, 549-55.
36. Sumandea, M.P., Pyle, W.G., Kobayashi, T., de Tombe, P.P., and Solaro, R.J. (2003) *J. Biol. Chem.* **278**, 35135-44.
37. Vahebi, S., Kobayashi, T., Warren, C.M., de Tombe P.P., and Solaro, R.J. (2005) *Circ. Res.* **96**, 740-7.
38. Miller, T., Szczesna, D., Housmans, P.R., Zhao, J., de Freitas, F., Gomes, A.V., Culbreath, L., McCue, J., Wang, Y., Xu, Y., Kerrick, W.G., and Potter, J.D. (2001) *J. Biol. Chem.* **276**, 3743-3755.
39. Sweeney, H.L., Feng, H.S., Yang, Z., and Watkins, H. (1998) *Proc. Natl. Acad. Sci. U S A* **95**, 14406-14410.
40. Alpert, N.R., Brosseau, C., Federico, A., Krenz, M., Robbins, J., and Warshaw, D.M. (2002) *Am. J. Physiol. Heart Circ. Physiol.* **283**, H1446-54.
41. Knollmann, B.C., Kirchhof, P., Sirenko, S.G., Degen, H., Greene, A.E., Schober, T., Mackow, J.C., Fabritz, L., Potter, J.D., and Morad, M. (2003) *Circ. Res.* **92**, 428-436.
42. Frey, N., McKinsey, T.A., and Olson, E.N. (2000) *Nat. Med.* **6**, 1221-7.
43. Bassani, J.W., Bassani, R.A., and Bers, D.M. (1994) *J. Physiol.* **476**, 279-293.
44. Semsarian, C., Ahmad, I., Giewat, M., Georgakopoulos, D., Schmitt, J.P., McConnell, B.K., Reiken, S., Mende, U., Marks, A.R., Kass, D.A., Seidman, C.E., and Seidman, J.G. (2002) *J. Clin. Invest.* **109**, 1013-20.
45. Spindler, M., Saaue, K.W., Christe, M.E., Sweeney, H.L., Seidman, C.E., Seidman, J.G., and Ingwall, J.S. (1997) *J. Mol. Cell. Cardiol.* **29**, 121-7.
46. Tanaka, T., Sohmiya, K., and Kawamura, K. (1997) *J. Mol. Cell. Cardiol.* **29**, 121-7.
47. Lodi, R., Cooper, J.M., Bradley, J.L., Manners, D., Styles, P., Taylor, D.J., and Schapira, A.H. (1999) *Proc. Natl. Acad. Sci. U S A* **96**, 11492-5.
48. Javadpour, M.M., Tardiff, J.C., Pinz, I., and Ingwall, J.S. (2003) *J. Clin. Invest.* **112**, 768-75.
49. Montgomery, D.E., Tardiff, J.C., and Chandra, M. (2001) *J. Physiol.* **536**, 583-92.
50. Stelzer, J.E., Patel, J.R., Olsson, M.C., Fitzsimons, D.P., Leinwand, L.A., and Moss, R.L. (2004) *Am. J. Physiol. Heart Circ. Physiol.* **287**, H1756-61.
Figures legends

**Figure 1:** Frequency-dependent alterations in isometric force of contraction (A) and the intracellular Ca$^{2+}$-transient (B-D) measured in right ventricular papillary muscle strips of TnT transgenic rats.

(A) **Isometric force of contraction:** In all three groups a frequency-dependent decline in force was observed. At low (0.25 Hz) and elevated stimulation frequency (1 Hz), contractility was decreased in rats expressing the human TnT-molecule (DEL-TnT, n=7) as compared to rats expressing the wild-type human TnT-molecule (TnT, n=9) and non-transgenic animals (NT, n=10).

(B) **Amplitude of the fura-2 transient:** The amplitude of the Ca$^{2+}$-transient was similar in all three groups at low and elevated stimulation frequencies. At increased stimulation frequencies the fura-2 amplitude declined.

(C) **Time to peak Ca$^{2+}$-transient:** At elevated stimulation frequencies, time to peak Ca$^{2+}$-transient was significantly prolonged in transgenic rats expressing the truncated human TnT-molecule (DEL-TnT) vs. rats expressing non-truncated human TnT.

(D) **Time to half peak Ca decay:** At elevated stimulation frequencies, time to half peak Ca decay was significantly prolonged in DEL-TnT transgenic rats compared to rats expressing non-truncated human TnT.

+: p<0.05 vs. 0.25 Hz, *: p<0.05 vs.NT, #: p<0.05 vs. TnT

**Figure 2:** Original recordings of the simultaneous measurements of myofibrillar ATPase activity (top) and developed tension (bottom) of ventricular skinned fiber preparations of non-transgenic rats (NT), rats expressing a human TnT molecule with a truncation mutation (DEL-TnT) and rats expressing the non-mutated human TnT-molecule (TnT). Ca$^{2+}$-dependent tension and myofibrillar ATPase activity were found to be significantly impaired in DEL-TnT rats.

**Figure 3:** Concentration-response curves for Ca$^{2+}$ on isometric tension development and myofibrillar ATPase activity in ventricular skinned fiber preparations of non-transgenic rats (NT, n=13), rats expressing a human TnT molecule with a truncation mutation (DEL-TnT, n=9) and rats expressing the human TnT-molecule (TnT, n=6).

**Figure 4:** Tension-dependent ATP utilization of TnT-transgenic rats. In DEL-TnT rats tension-dependent ATP-utilization was significantly decreased (i.e. tension cost was increased), while TnT-transgenic rat hearts are comparable to non-transgenic rats (NT).
### Table 1: Parameters characterizing tension development, myofibrillar ATPase activity and energy consumption

#### Ca\textsuperscript{2+}-activated tension development

| Parameter                                | Definition                                                                 | Abbreviation |
|------------------------------------------|---------------------------------------------------------------------------|--------------|
| Maximally Ca\textsuperscript{2+}-activated tension | Tension development at pCa 4.5                                             | DT\textsubscript{max} |
| Ca\textsuperscript{2+}-sensitivity of tension | Concentration of free Ca\textsuperscript{2+} at which a 50% increase in tension is achieved | pCa\textsubscript{50} DT |
| Hill-coefficient                         | Steepness of the Ca\textsuperscript{2+}-tension relationship              | n\textsubscript{Hill} |

#### Ca\textsuperscript{2+}-activated myofibrillar ATPase activity

| Parameter                                | Definition                                                                 | Abbreviation |
|------------------------------------------|---------------------------------------------------------------------------|--------------|
| Basal myofibrillar ATPase activity      | Myosin ATPase activity measured at pCa 8                                  | MYO\textsubscript{bas} |
| Maximal myofibrillar ATPase activity    | Myosin ATPase activity measured at pCa 4.5                                 | MYO\textsubscript{max} |
| Ca\textsuperscript{2+}-activated myofibrillar ATPase activity | MYO max-MYO bas                                                               | Δ MYO |
| Ca\textsuperscript{2+}-sensitivity of myofibrillar ATPase | Concentration of free Ca\textsuperscript{2+} at which a 50% increase in myosin ATPase activity is achieved | pCa\textsubscript{50} MYO |

#### Energy utilization

| Parameter                                | Definition                                                                 | |
|------------------------------------------|---------------------------------------------------------------------------|---|
| Slope of the ATPase/tension relation     |                                                                           | α  |
| X-intercept                              |                                                                           | X-intercept |
Table 2: Frequency-dependent alterations of force in TNT-transgenic rats

| FORCE | Ca²⁺-TRANSIENT |
|-------|----------------|
|       | Force mN/mm² | TPT ms | T1/2T ms | +dP/dt mN/(mm²s) | -dP/dt mN/(mm²s) | Fura_{sys} | Fura_{dia} |
| NT    | 19.3±1.1     | 222±8  | 141±7    | 87.1±5.2        | 137.7±9.3       | 2.02±0.03 | 1.77±0.04 |
| TnT   | 24.0±5.0*    | 241±36 | 150±43   | 109.2±33.5*     | 201.8±30.0*     | 2.18±0.05*| 1.97±0.06*|
| DEL-TnT | 9.9±1.7*,#  | 213±8  | 131±4    | 46.3±7.5*,#     | 76.6±13.4*,#    | 2.20±0.15| 1.90±0.19 |

| FORCE | Ca²⁺-TRANSIENT |
|-------|----------------|
|       | Force mN/mm² | TPT ms | T1/2T ms | +dP/dt mN/(mm²s) | -dP/dt mN/(mm²s) | Fura_{sys} | Fura_{dia} |
| NT    | 16.0±0.9+    | 174±6+ | 112±4+   | 94.0±7.8        | 146.0±13.1       | 2.05±0.02+| 1.89±0.03+|
| TnT   | 21.6±2.6 +,* | 186±17+| 113±12+  | 123.7±20.7*     | 201.5±76.3*      | 2.21±0.06*| 2.06±0.06*,*|
| DEL-TnT | 7.1±0.9+,*,# | 176±6+ | 111±5+   | 41.0±5.8*,#     | 65.9±10.1*,#     | 2.21±0.15| 2.04±0.18+|

FOC: force of contraction, TPT: time to peak tension, T1/2T: time to half peak relaxation, +dP/dt: maximal contraction velocity, -dP/dt: maximal relaxation velocity, Fura_{sys}: systolic fura-2 ratio, Fura_{dia}: diastolic fura-2 ratio, NT: non-transgenic, wild-type rats, TnT: rats expressing the non-mutated human TnT-molecule, DEL-TnT: rats expressing the truncated human TnT-molecule.

+: p<0.05 vs 0.25 Hz, *: p<0.05 vs. NT, #:p<0.05 vs. TnT
Table 3: Tension development, myofibrillar ATPase activity and tension cost in TnT-transgenic rats

| Parameter                        | NT (n=13) | DEL-TnT (n=9) | TnT (n=6) |
|----------------------------------|-----------|---------------|-----------|
| **Tension Development**          |           |               |           |
| Maximum (mN/mm²)                 | 11.3±1.4  | 7.2±1.4 #     | 24.3±4.3 *|
| pCa_{50} (µM)                    | 1.47±0.16 | 2.84±0.43 *#  | 1.77±0.49 |
| DT_{Hill}                        | 3.8±0.2   | 4.9±0.4 *     | 5.4±0.8 * |
| **Myofibrillar-ATPase Activity**|           |               |           |
| Basal (µmol ADP/s)               | 31±7      | 31±6#         | 90±15 *   |
| Maximum (µmol ADP/s)             | 60±10     | 64±15#        | 142±18 *  |
| Ca^{2+}-act. increase (µmol ADP/s)| 29±5     | 33±9#        | 52±8*     |
| pCa_{50} (µM)                    | 1.16±0.22 | 2.65±0.76 *   | 2.50±1.25 |
| **Tension Cost**                 |           |               |           |
| α((mN*mm²)/(µmol ADP*s^{-1}))    | 0.74±0.15 | 0.31±0.04 *#  | 0.73±0.11 |
| X-intercept (µmol ADP/s)         | 29±5      | 34±7#        | 95±24 *   |

*: p<0.05 vs. NT  
#:p<0.05 vs. TnT

NT: non-transgenic rats, DEL-TnT: rats overexpressing the truncated TnT-molecule, TnT: rats overexpressing non-mutated human TnT; α: slope of the myofibrillar ATPase/tension relationship, pCa_{50} : Ca^{2+}-concentration at which a 50% increase of tension or myofibrillar ATPase activity was achieved.
TROPNIN T-TRANSGENIC RATS

Force/Ca²⁺-Frequency Relation

0.25 Hz

A. Force of Contraction

B. Fura-2 Amplitude

C. Time to peak Ca-transient

D. Time to half peak Ca decay

Fig. 1
Fig. 2
Tension-dependent ATP-utilization

Fig. 4
Alterations of tension-dependent ATP-utilization in a transgenic rat model of hypertrophic cardiomyopathy
Norbert Frey, Klara Brixius, Robert H.G. Schwinger, Thomas Benis, Alex Karpowsky, Hans P. Lorenzen, Mark Luedde, Hugo A. Katus and Wolfgang M. Franz

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