Impaired Diastolic Function After Exchange of Endogenous Troponin I With C-Terminal Truncated Troponin I in Human Cardiac Muscle

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Abstract—The specific and selective proteolysis of cardiac troponin I (cTnI) has been proposed to play a key role in human ischemic myocardial disease, including stunning and acute pressure overload. In this study, the functional implications of cTnI proteolysis were investigated in human cardiac tissue for the first time. The predominant human cTnI degradation product (cTnI1–192) and full-length cTnI were expressed in *Escherichia coli*, purified, reconstituted with the other cardiac troponin subunits, troponin T and C, and subsequently exchanged into human cardiac myofibrils and permeabilized cardiomyocytes isolated from healthy donor hearts. Maximal isometric force and kinetic parameters were measured in myofibrils, using rapid solution switching, whereas force development was measured in single cardiomyocytes at various calcium concentrations, at sarcomere lengths of 1.9 and 2.2 μm, and after treatment with the catalytic subunit of protein kinase A (PKA) to mimic β-adrenergic stimulation. One-dimensional gel electrophoresis, Western immunoblotting, and 3D imaging revealed that approximately 50% of endogenous cTnI had been homogeneously replaced by cTnI1–192 in both myofibrils and cardiomyocytes. Maximal tension was not affected, whereas the rates of force activation and redevelopment as well as relaxation kinetics were slowed down. Ca2+ sensitivity of the contractile apparatus was increased in preparations containing cTnI1–192 (pCa0.5: 5.73±0.03 versus 5.52±0.03 for cTnI1–192 and full-length cTnI, respectively). The sarcomere length dependency of force development and the desensitizing effect of PKA were preserved in cTnI1–192-exchanged cardiomyocytes. These results indicate that degradation of cTnI in human myocardium may impair diastolic function, whereas systolic function is largely preserved. (Circ Res. 2006;99:1012-1020.)

Key Words: cardiac function • contractility • cardiomyocytes • cardiomyopathy • ischemia

Ischemic myocardial disease is manifested in a mild form of reversible contractile dysfunction and a very serious, irreversible form (necrosis). The latter may be prevented only by timely restoration of coronary perfusion, but, even then, reversible contractile dysfunction is frequently apparent. The specific and selective proteolysis of the myofilament protein cardiac troponin I (cTnI) has been proposed to play a key role in human myocardial ischemia/reperfusion injury, including stunning1–3 and in acute pressure overload.4 cTnI is part of the cardiac troponin (cTn) complex that, in concert with tropomyosin (TM), regulates muscle contraction in response to a rise in intracellular Ca2+. The troponin complex consists of 3 subunits: troponin C (cTnC), the Ca2+ binding protein; cTnI, which inhibits the actin/myosin interaction; and troponin T (cTnT), which transduces the Ca2+ binding signal to TM. On Ca2+ binding to the regulatory site of cTnC, the C-terminal regulatory segment of cTnI (residues 137 to 210) moves away from the actin filament,5,6 thereby altering the orientation and/or flexibility of cTn and TM relative to the actin filament.5 Hence, cTnI plays a pivotal role in activation of cardiac myofilaments, and truncation of the cTnI C terminus, as observed in human ischemic cardiac disease, might alter the Ca2+-induced force development as well as the inhibition of force development at low Ca2+ concentrations.

In rodents, McDonough et al7 showed that with moderate ischemia/reperfusion, cTnI is cleaved at its C terminus,
resulting in a degraded subunit cTnI_{1–193}. Ischemia-induced cTnI degradation has been proposed to be attributable to activation of the Ca^{2+}-dependent proteolytic enzyme calpain-1 during Ca^{2+} overload that occurs during reperfusion. Increased preload in the absence of ischemia has also been shown to induce cTnI degradation. In larger mammalian models, such as a postinfarct pig and dog model, minimal degradation of cTnI (<4%) was observed in the viable remodeled left ventricle. Whereas in the dog, cTnI degradation did not correlate with the in vivo cardiac dysfunction in the whole heart, in the pig, the maximal force generating capacity of the individual myofilaments was reduced compared with myocardium of sham-operated animals. In advanced human heart failure, serum cTnI elevations have been reported. In addition, cTnI degradation has been found in human cardiac tissue from coronary artery disease patients with different degrees of heart failure and a history of myocardial infarction. The primary cTnI degradation product found in these hearts was identified as cTnI_{1–192} (equivalent to cTnI_{1–193} in rodent myocardium). Hence, also in humans, C-terminal truncation of cTnI may underlie impaired cardiomyopathy and heart failure. However, the direct effects of cTnI proteolysis on contractile function of human cardiac myofilaments and isolated cardiac myocytes have not been investigated.

The contractile apparatus responds quickly to increases in hemodynamic load (via the Frank–Starling mechanism) and to β-adrenergic stimulation, resulting in an increase and decrease in myofilament Ca^{2+} sensitivity, respectively. In failing hearts, both processes are blunted. Evidence suggests that cTnI plays an important role in these regulatory processes, by modulating length-dependent activation and by its phosphorylation through protein kinase A (PKA) of Ser23/24 in the N terminus. For instance, it has been shown that transduction of the PKA-induced phosphorylation signal from cTnI to the regulatory site of cTnC involves a global change in cTnI structure. Hence it is of interest to study whether or not the changes in Ca^{2+} sensitivity associated with the Frank–Starling mechanism and β-adrenergic stimulation are perturbed by C-terminal truncation of cTnI in human isolated myocytes and cardiomyocytes.

In this study, protein engineering was combined with functional measurements to assess the direct effects of degradation of the cTnI C terminus in human myocardium. Force measurements were performed in human cardiac myofibrils and cardiomyocytes, in which endogenous troponin complexes were partly replaced by complexes, denoted by cTnI_{192} and cTnI_{1–192}, containing human full-length cTnI or truncated cTnI_{1–192}, respectively. The advantage of this technique is that it allows to investigate the direct effect of cTnI degradation on contractility of human cardiac preparations without degredation of other contractile proteins (as eg, follows calpain-1 treatment) or other compensatory protein changes, which may develop in transgenic animals.

Force development at maximal and submaximal [Ca^{2+}] and kinetic parameters of force activation and relaxation in preparations containing cTnI_{192} were compared with those containing cTnI_{1–192}. In the presence of cTnI_{1–192}, maximal isometric force, length-dependent activation and the effect of PKA were preserved, whereas Ca^{2+} sensitivity was increased and the kinetics of activation and relaxation of human cardiac preparations were slowed. These results indicate that C-terminal truncation of cTnI mainly impairs diastolic properties of human myocardium, whereas the maximum force generating capacity, the Frank–Starling effect and the β-adrenergic responsiveness are preserved.

### Materials and Methods

An expanded Materials and Methods section can be found in the online data supplement, available at http://circres.ahajournals.org.

### Preparation of Recombinant Troponin Complexes

The human cardiac full-length cTnI and truncated cTnI (cTnI_{1–192}) were prepared as described previously.

### Isolation of Human Myofibrils and Cardiomyocytes

Left ventricular tissue was obtained from healthy donor hearts (n=4). Samples were obtained with approval of the local ethical committees and after informed written consent. Single myofibrils, bundles of a few myofibrils or single cardiac myocytes were prepared from these biopsies, as described previously.

### Exchange of Human Cardiac Troponin Complex

Exchange of human cTnI complex into human myofibrils and myocytes was performed according previously published methods. Evaluation of both cTnI_{192} and cTnI_{1–192} exchange into human cardiac preparations was based on the amount of cTnI_{1–192} exchange.

### Force Measurements in Exchanged Human Myofibrils and Cardiomyocytes

The protocols used for rapid solution changes and to record force in single myofibrils and cardiomyocytes were as described previously.

### Microscopy

To determine whether the cTn complex was homogeneously incorporated into cardiomyocytes, cTnI_{192} exchanged cardiomyocytes were studied using a Marianas digital imaging microscopy workstation.

### Data Analysis

Values are given as means ± SEM of n myofibrils or myocytes. Differences between preparations with cTnI_{192} and cTnI_{1–192} were tested by means of unpaired 2-tailed Student’s t tests at a level of significance of 0.05. The effects of sarcomere length and PKA treatment were tested with paired Student’s t tests.

### Results

#### Amount and Distribution of Troponin Complex Exchange

Figure 1A shows an example of a gel used for the calculation of the amount of exchange in cardiac myofibrils. Replacement of the endogenous troponin complex with cTnI_{192} amounted to 54±6% (n=4) in human cardiac myofibrils and to 50±2% (n=5) in the isolated human cardiomyocytes. These results confirmed by anti-cTnI Western blot analysis (Figure 1B for myofibrils and Figure 1C for cardiomyocytes).

To monitor the cTn distribution in cardiomyocytes following the exchange, a novel method was used in which human cardiomyocytes exchanged with cTnI_{1–192} were stained with a specific antibody P45-3 and studied using 3D digital imaging microscopy. P45-3 reacts only with full-length cTnI and not
with truncated cTnI1–192. Thus, staining with this antibody will reveal any inhomogeneity of cTn 1–192 exchange into the cardiomyocytes. A summation in the z-axis of the x-y images of a representative cardiomyocyte is shown in Figure 1D. Fluorescence intensity was averaged per xy plane and plotted against plane number in Figure 1E. This panel illustrates that with overnight incubation, the penetration of cTn into the cardiomyocyte is not limited by diffusion, because in that case, a parabolic intensity profile would be expected with a minimum in the core of the preparation. The fluorescence intensity distribution in the y-axis direction (Figure 1F) in the central part of the myocyte also reveals a rather homogeneous distribution of cTn complex within the exchanged cardiomyocyte. Three-dimensional image reconstruction performed in 3 typical cTn1–192-exchanged cardiomyocytes showed similar results. This indicates that the protocol used ensures a uniform exchange of cTn1–192 inside the cardiomyocytes (and in the much thinner myofibrils, where diffusion would be even less critical).

**Force Measurements in Exchanged Human Myofibrils**

Representative recordings of force production in myofibrils at saturating [Ca^{2+}] are shown in Figure 2. Maximal isometric tension (ie, force at pCa 3.5 divided by cross-sectional area) was not significantly different between cTnFL- and cTn1–192-exchanged myofibrils (Figure 3A). However, the rate of tension rise (k_{ACT}) and the rate of force redevelopment (k_{TR}) were both significantly decreased in cTn1–192-exchanged myofibrils compared with cTnFL-exchanged myofibrils by 24% and 31%, respectively (P<0.05) (Figure 3B).

After a rapid switch from activating (pCa 3.5) to relaxing (pCa 9) solution, isometric force started to decline (Figure 2). Force relaxation takes place in 2 phases: a slow initial linear phase, with a rate constant k_{REL,S} calculated from its slope and duration t_{SLOW}; and a rapid exponential phase characterized by the rate constant k_{REL,F} (Figure 3C).22 The maximum force and kinetic parameters in cTnFL-exchanged myofibrils did not differ significantly from the values obtained in untreated myofibrils. However, compared with cTnFL-exchanged myofibrils, the duration of the slow phase t_{SLOW} was significantly increased in cTn1–192-exchanged myofibrils (Figure 3D), whereas k_{REL,F} remained unchanged (Figure 3C). The k_{REL,F} significantly decreased in cTn1–192-exchanged myofibrils (Figure 3C). An overview of averaged data are presented in Table 1.
Force Measurements in Exchanged Human Cardiomyocytes

The maximal isometric tension and its Ca\(^{2+}\) sensitivity could be determined more accurately in cardiomyocytes than in myofibrils, because the cross-sectional area could be determined with a higher precision in cardiomyocytes than in myofibrils (Figure 4A). Representative force recordings of cTnFL- and cTn1–192-exchanged myocytes are presented in Figure 4B. Maximal isometric tension and mean passive tension were not significantly different between cTnFL- and cTn1–192-exchanged myofibrils (Figure 5A). However, cTn1–192 cardiomyocytes had a significantly higher Ca\(^{2+}\) sensitivity than cTnFL-exchanged cardiomyocytes (pCa\(_{50}\): 5.79 ± 0.02 and 5.54 ± 0.02 for cTn1–192 and cTnFL, respectively; P < 0.05) (Figure 5B). Moreover, the cooperativity of force development (nH) was significantly decreased in cTn1–192-exchanged cardiomyocytes (1.95 ± 0.06 and 2.95 ± 0.14 for cTn1–192 and cTnFL, respectively; P < 0.05).

Sarcomere Length Dependence

To investigate the effect of cTnI proteolysis on the Frank–Starling mechanism, force development and its Ca\(^{2+}\) sensitivity was studied in cardiomyocytes after exchange. Measurements were performed near slack length, at a sarcomere length of 1.9 μm and after adjustment of sarcomere length to 2.2 μm. As expected on the basis of the sarcomere length/tension relation, maximum force increased for both cTnFL and cTn1–192 cardiomyocytes. Passive tension increased as well. Ca\(^{2+}\) sensitivity also increased with sarcomere length (Figure 3).

### Table 1. Functional Data of Human Myofibrils

<table>
<thead>
<tr>
<th></th>
<th>Untreated (n=22)</th>
<th>cTnFL (n=22)</th>
<th>cTn1–192 (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximal tension (kN·m(^{-2}))</td>
<td>71 ± 12</td>
<td>54 ± 12</td>
<td>52 ± 9</td>
</tr>
<tr>
<td>(k_{\text{ACT}}) (sec(^{-1}))</td>
<td>0.84 ± 0.07</td>
<td>0.80 ± 0.07</td>
<td>0.61 ± 0.05*</td>
</tr>
<tr>
<td>(k_{\text{TR}}) (sec(^{-1}))</td>
<td>0.76 ± 0.05†</td>
<td>0.77 ± 0.07</td>
<td>0.53 ± 0.05*</td>
</tr>
<tr>
<td>Relaxation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(t_{\text{SLOW}}) (sec)</td>
<td>0.22 ± 0.01</td>
<td>0.21 ± 0.02</td>
<td>0.30 ± 0.02*</td>
</tr>
<tr>
<td>(k_{\text{REL,S}}) (sec(^{-1}))</td>
<td>0.21 ± 0.03</td>
<td>0.31 ± 0.05</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td>(k_{\text{REL,F}}) (sec(^{-1}))</td>
<td>2.09 ± 0.28</td>
<td>3.75 ± 0.37</td>
<td>2.54 ± 0.25*</td>
</tr>
</tbody>
</table>

Maximal tension: \(k_{\text{ACT}}\) and \(k_{\text{TR}}\) were obtained at maximal [Ca\(^{2+}\)] (pCa 3.5). \(t_{\text{SLOW}}, k_{\text{REL,S}}, k_{\text{REL,F}}\) were obtained in relaxing solution (pCa 9). Values are given as mean ± SEM of n myofibrils (n=21). There were no significant differences between untreated and cTnFL myofibrils. *Significant at P < 0.05, cTn1–192 vs cTnFL.
6A), but the steepness of the force/pCa relation (nH) remained the same. The mean shifts in Ca\(^{2+}\)/H11001sensitivity (\(\Delta\)pCa50) were very similar and amounted to 0.10 \(\pm\) 0.02 and 0.14 \(\pm\) 0.02 for cTnI–192 and cTnFL, respectively (\(P=0.2\)). An overview of averaged data are presented in Table 2.

Effects of PKA
To assess the effect of PKA phosphorylation of cTnI and its truncated fragment, cardiomyocytes after cTn exchange were incubated with the catalytic subunit of protein kinase A. Addition of PKA resulted in a decrease in Ca\(^{2+}\)/H11001sensitivity and, for cTnI–192 cardiomyocytes, in a small but significant increase in steepness of the force/pCa relation (Figure 6B). The mean shifts in Ca\(^{2+}\)/H11001sensitivity (\(\Delta\)pCa50) were very similar and amounted to 0.08 \(\pm\) 0.01 and 0.09 \(\pm\) 0.01 for cTnI–192 and cTnFL, respectively (\(P=0.5\)). Maximum forces were not influenced by PKA. Mean passive forces were slightly reduced after PKA treatment, but the differences were not significant. An overview of the averaged data are included in Table 2.

Comparison Between Properties of cTnI–192-Exchanged Cardiomyocytes and Untreated Controls
Measurements were also performed in untreated cardiomyocytes from the same donors as used for the exchange experiments. The maximum force in untreated cardiomyocytes 26.9 \(\pm\) 3.2 kN/m\(^2\) (n=4) did not differ significantly from the value in cTnFL-exchanged cardiomyocytes: 31.5 \(\pm\) 3.2 kN/m\(^2\) (n=12; Figure 5B), whereas the pCa50 values observed were consistent with the observed difference in cTnI phosphorylation (online data supplement), i.e., Ca\(^{2+}\)/H11001 responsiveness was significantly higher in exchanged preparations containing dephosphorylated full-length cTnI (pCa50=5.54 \(\pm\) 0.02) than in untreated cardiomyocytes (pCa50=5.48 \(\pm\) 0.01) in which the amount of dephosphorylated cTnI was lower.

Discussion
This study describes the first exchange experiments in human cardiac preparations, in which the direct effects of cTnI degradation are assessed. The results obtained indicate that (1) cTnI degradation at the C terminus enhances myofilament Ca\(^{2+}\)/H11001 sensitivity and slows force development as well as its relaxation, whereas (2) maximal isometric force development, passive force, length-dependent activation (the Frank–Starling effect), and the responsiveness to \(\beta\)-adrenergic stimulation appear to be preserved.

Comparison With Previous Studies on cTnI1–192 Function
The decrease in maximal force generating capacity of cardiac muscle is among the main features of reversible ischemia/reperfusion injury (stunning) in rodents and has been attributed to degradation of cTnI, primarily at its C terminus.\(^{7,8,23}\) In a transgenic mouse line expressing 9% to 17% of truncated human cTnI,\(^{1,24}\) maximal force development of intact trabeculae was approximately 40% depressed compared with non-transgenic mice. In view of these previous findings, it is surprising that in our study, the maximum force was not affected in human preparations containing truncated cTnI. However, it should be noted that the processes involved in stunning differ in small and large animals.\(^{25}\) The reduction in force in animal models could, at least in part, also be attributable to an additional degradation or modification of other, ultrastructural proteins\(^{15}\) or to secondary reactive oxy-
gen species–related effects. Moreover, other compensatory protein changes, which may develop in transgenic animals, and species differences may be involved as well.

An in vitro motility assay, with reconstituted actin filaments attached to anchoring α-actinin, showed a 24% decrease in an index of force development of filaments consisting of 100% human cTnI1–192, compared with filaments with full-length cTnI. This reduction probably would have been less with partial replacement of human cTnI1–192, as was the case in our experiments. Exchange of the human cTnI1–192 complex into rabbit psoas myofibrils under the same experimental conditions as used in this study, revealed a 26% decrease in maximal force development in comparison with cTnI1 preparations. A similar reduction of maximal tension was observed by Tachampa and coworkers, where the endogenous rat cTn complexes were partly exchanged with truncated human or mice cTn. The main difference in these exchange experiments is that chimerical thin filaments were formed, whereas in the present experiments human cardiac proteins were exchanged into human cardiac tissue. It is conceivable that, despite 95% identity of the human, mice, rat, and rabbit cTn amino acid sequences, signal transduction and transmission between troponin subunits and other contractile proteins is isoform and species dependent. This might result in diverse functional changes on cTnI1–192 exchange.

### Table 2. Functional Data of Exchanged Human Cardiomyocytes

<table>
<thead>
<tr>
<th>Group and Force Parameter</th>
<th>Sarcomere Length</th>
<th>PKA Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.9 μm</td>
<td>2.2 μm</td>
</tr>
<tr>
<td>cTnFL, n=10/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximal tension (kN·m⁻²)</td>
<td>13±2</td>
<td>28±3*</td>
</tr>
<tr>
<td>Passive tension (kN·m⁻²)</td>
<td>0.87±0.27</td>
<td>3.04±0.51*</td>
</tr>
<tr>
<td>pCa₅₀</td>
<td>5.44±0.02</td>
<td>5.54±0.02*</td>
</tr>
<tr>
<td>nH</td>
<td>3.14±0.09</td>
<td>2.88±0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cTnI₁–192, n=12/13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximal tension (kN·m⁻²)</td>
<td>15±2</td>
<td>30±4*</td>
</tr>
<tr>
<td>Passive tension (kN·m⁻²)</td>
<td>1.01±0.12</td>
<td>3.87±0.41*</td>
</tr>
<tr>
<td>pCa₅₀</td>
<td>5.68±0.021</td>
<td>5.81±0.02*</td>
</tr>
<tr>
<td>nH</td>
<td>2.07±0.08</td>
<td>1.88±0.06†</td>
</tr>
</tbody>
</table>

n denotes no. of cardiomyocytes in the sarcomere length/PKA-treated group, respectively. Values are given as mean±SEM. *Significant at P<0.05, within groups 1.9 vs 2.2 μm and before vs after PKA; †significant at P<0.05, cTnI₁–192 vs cTnFL.
in matching or nonmatching species, but further experiments are required to test this hypothesis.

In viable remote remodeled tissue after infarction in pigs, minor degradation of cTnI (<4%) was observed, whereas the maximal force-generating capacity was reduced. If our findings in humans are characteristic for the situation in large animal models, this would suggest that other factors than cTnI degradation would be responsible for this loss in force.

The results on kinetics and Ca^{2+} sensitivity observed in the present study are in line with those found in exchange experiments using rabbit psoas myofibrils and rat cardiac trabeculae. This indicates that the impact of cTnI degradation on regulatory and kinetic aspects of contractile function in rodent and human tissue are shared. However, caution should be exerted with the interpretation of these findings within the context of stunning. For instance Gao and coworkers have observed an increase within the context of stunning. For instance Gao and coworkers have observed an increase in ACT and also provided evidence suggesting that the rate-limiting step of the transition from force-generating to non–force-generating states accelerated in stunned myocardium. Moreover, the direction of the change in the maximum shortening velocity in stunned myocardium is unclear.

**Structural and Biochemical Considerations on cTnI_{1-192} Function**

Previous studies indicate that the presence of cTn and TM enhance force development and also influence kinetic parameters of cross-bridge interaction. This suggests that cTnI degradation may affect the maximum force-generating capacity as well as the kinetics of activation and relaxation of force. However, if steric hindrance of cross-bridge interaction by the C-terminal end of cTnI would be important, as suggested previously to explain the increase in ATPase activity, its truncation would tend to increase the number of interacting cross-bridges. This would result in an increase in force rather than in a decrease.

The increase in Ca^{2+} sensitivity observed can be caused by (1) an increase in Ca^{2+} affinity of cTnC, which promotes the transition from blocked to closed state; and/or (2) enhancement of strong myosin binding to actin, the transition from closed to open state. It has been shown using various truncated forms of cTnI that progressive deletion of parts of the C terminus impairs the ability of cTnI to bind to actin/tropomyosin at low [Ca^{2+}], promoting the availability of actin for binding with myosin. This is consistent with the increase in Ca^{2+} sensitivity observed in our experiments. The nH of cardiomycocytes exchanged with cTnI_{1-190} was significantly lower than that of cTnT-exchanged cardiomycocytes. This may reflect a decrease in cooperativity of thin filament activation, in agreement with the destabilization of TM on actin, but it could also be caused by the increase in Ca^{2+} sensitivity because Ca^{2+} activation of force becomes more dependent on Ca^{2+} binding to troponin C, which is a relatively noncooperative process.

Both k_{ACT} and k_{TR} of the cTnI_{1-192}-exchanged cardiomycocytes were significantly decreased. Evidence suggests that k_{ACT} and k_{TR} are mainly determined by the kinetics of cross-bridge attachment. The parallel changes in k_{ACT} and k_{TR} observed in these experiments are most likely attributable to cooperative changes within the thin filament that lead to the transition to the open state. Thus, the decreased cooperativity of thin filament regulation may play a role in the slowing of the activation kinetics.

It has been shown that during the first slow linear phase of relaxation, all sarcomeres remain isometric, whereas the fast relaxation phase starts when the first mechanically weakest sarcomere “gives.” The slow k_{REL,S} is determined largely by the isoform of myosin heavy chain. This may explain the lack of a significant effect of cTnI degradation on this parameter in our experiments. The duration of the slow phase f_{SLOW} was, however, increased in cTnI_{1-192}-exchanged preparations, ie, sarcomeres remain isometric for a longer time during relaxation. This might be a consequence of persistent enhanced activation caused by the increased Ca^{2+} sensitivity.

In a simple 2-state model for cross-bridge interaction the maximum isometric force is proportional to the number of cross-bridges in the force generating state (N_m), ie, f/f+g and k_{TR}=f+g, where f equals the rate of cross-bridge attachment and g equals the rate of cross-bridge detachment. If we assume that at saturating Ca^{2+} concentrations g_{TR}=k_{REL,S}, N_{att} amounts to 0.6, both for cTnI_{1-192} and cTnH_{2}. Hence, the kinetic values obtained are consistent with the preservation of the maximum force-generating capacity observed.

The reduction in the kinetic parameters cannot be reconciled easily with the increase in ATPase rate observed earlier. However, it should be noted that the ATPase rate reported characterizes the activity during unloaded shortening, whereas our observations characterize the isometric steady state.

**Modulation of Contractile Function**

Evidence suggests that cTnI, the N-terminal region in particular, is involved in length-dependent activation (the Frank–Starling mechanism). Our results indicate that the sarcocerele length dependency of maximum force development and its Ca^{2+} sensitivity in cTnI_{1-192}-containing cardiomycocytes were very similar to those containing full-length cTnI. The location of the putative length sensor, the mechanism underlying the Frank–Starling mechanism as well as the involvement of other proteins such as titin, myosin-binding protein C, and myosin light chain 2 still need to be addressed in human myocardium. However, our data suggest that the C-terminal truncated part of cTnI is not essential for signal transmission of length-dependent activation.

The effects of β-adrenergic stimulation were mimicked using PKA. In a previous study, it has been shown that the PKA sites of the endogenous cTnI in donor tissue were almost completely phosphorylated and that cTnT was partially monophosphorylated (~60%). These endogenous complexes were partly exchanged in our experiments with dephosphorylated cTnI (and cTnT). Consistent with these
earlier results, the shift in pCao, on PKA treatment was intermediate between the shift observed in donor and end-stage failing heart tissue.41 However, because treatment of both complexes was identical, the overall phosphorylation levels will be the same, as was confirmed by the Western blots shown in the online data supplement. Hence, our experiments revealed that the desensitizing effect of PKA was preserved in cTnI1–192-containing cardiomyocytes. This indicates that the transduction of the PKA-induced phosphorylation signal from cTnI to the regulatory site of cTnC is preserved.

Recently, Metzger and colleagues42 provided evidence that replacement of alanine with histidine at position 163 in human cTnI may represent a therapeutic avenue to improve myocardial performance in the ischemic and failing heart. Because alanine 163 is within the region retained after cTnI proteolysis, the engineering of the histidine button may be protective also in the presence of degraded cTnI.

Implications for Cardiac Function In Vivo

The amount of cTnI exchange into cardiac preparations in our experiments was approximately 50%, whereas the degree of cTnI degradation observed in failing human myocardium was less (up to 26%).43 Thus, we expect that the slowing of the kinetics and the increase in Ca2⁺ sensitivity in failing human myocardium will be less pronounced than observed in our experiments.

The slowing of relaxation after cTnI1–192 exchange observed in the present study suggests that cTnI degradation in vivo might impair cardiac relaxation and contribute to diastolic dysfunction, whereas the increased myofilament Ca2⁺ sensitivity with preserved maximal force generating capacity implies that at low Ca2⁺ concentrations present during systole, more force will be developed. However in vivo, during a twitch, this effect will be counteracted by the slowing of force development in preparations with cTnI1–192. The net effect during systole might thus be rather small. Hence, our data indicate that diastolic dysfunction in ischemic and failing human myocardium44,45 might, at least in part, be attributable to cTnI proteolysis.

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Disclosures

None.

References


Impaired Diastolic Function After Exchange of Endogenous Troponin I With C-Terminal Truncated Troponin I in Human Cardiac Muscle

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Materials and Methods

Preparation of recombinant troponin complex

All human troponin subunits (human full-length cTnI and C-terminal truncated cTnI, cTnT and cTnC) were expressed separately in E. coli and purified using ion-exchange chromatography. The purified troponin subunits were reconstituted into a full troponin complex by mixing the subunits in a 1:1:1 molar ratio. After 2 hours incubation at 23°C the concentration of urea (6M) and KCl (1M) were reduced by step-wise dialysis. Purity and stoichiometry of both reconstituted complexes, containing either full-length cTnI (cTnFL) or the primary cTnI degradation product (cTn1-192), was verified by 1D-gel electrophoresis (described below). Protein complexes were stored at -80°C prior to the experiments.

Isolation of human myofibrils and cardiomyocytes

Left ventricular tissue was obtained from healthy donor hearts (n=4), frozen in liquid nitrogen and stored in liquid nitrogen or at -80°C. Samples were obtained with approval of the local ethical committees and after written informed consent.

Single myofibrils or bundles of two to three myofibrils were prepared from these biopsies, as described previously. Shortly, small pieces of frozen ventricular tissue (70-100 mg wet weight) were defrosted in rigor solution (10 mmol/L Tris, 132 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl2, 5 mmol/L EGTA, 2 mmol/L DTT, 10 mmol/L NaN3, pH 7.1), dissected in a few thin muscle strips (diameter 0.5-1.0 mm) and permeabilized in rigor solution containing 1% Triton X-100 for 2 hours on ice. To remove Triton, myofibrils were washed in rigor solution 3 times. Thereafter, muscle strips were processed in a tissue homogenizer (Biospec Products, OK, USA) (2-3 cycles of 10 s each) to produce
a suspension of human cardiac myofibrils (final protein concentration: ca. 0.5 mg/ml).

Exchange of troponin complexes was started immediately after myofibril isolation. All solutions to which the myofibrils were exposed contained a protease inhibitor cocktail (0.2 mmol/L PMSF, 0.01 mmol/L leupeptin, 0.01 mmol/L E-64 and 0.005 mmol/L pepstatin, Sigma).

Single cardiac myocytes were mechanically isolated using the protocol described previously except that the tissue was handled in cold rigor solution rather than in relaxing solution, to minimize the number of washing steps that would inevitably result in loss of cardiomyocytes. The cells were permeabilized in rigor solution supplemented with 0.5 % Triton X-100 for 5 min on ice, subsequently washed and used for exchange.

Exchange of human cardiac cTn complex

Exchange of human cardiac troponin complex into human myofibrils and myocytes was performed according the method of Brenner et al. Myofibrils and cardiomyocytes were incubated overnight at 4°C in an exchange solution containing 0.5 mg/ml (ca 6 μM) of human troponin complex (either cTnFL or cTn1-192). Composition of the exchange solution was in mmol/L: imidazole 10, NaCl 170, MgCl2 5, CaCl2 3, EGTA 2.5, DTT 0.5, NaN3 0.5, pH: 6.9 and the protease inhibitor cocktail described above. After exchange the myofibril and cardiomyocyte suspensions were washed twice by centrifugation (at 4000g for 10 min at 10°C and 900g for 1 min at 4°C, for myofibrils and cardiomyocytes, respectively) to replace the exchange solution with rigor solution. Half of the suspension was used for mechanical experiments, and the remaining half was used for analysis of the amount of cTn exchange by 1D-gel electrophoresis.
**Force measurements in exchanged human myofibrils and cardiomyocytes**

The set-up used for rapid solution changes and to record force in single myofibrils was as described previously. Briefly, myofibrils were mounted horizontally between a calibrated cantilever force probe and a motor. Sarcomere length and myofibril diameter were measured from video images (magnification 1800X, phase-contrast optics). The initial sarcomere length of attached myofibrils between the attachments (mean ± S.E.M.) amounted to 2.20±0.04 µm (n=22) in the cTnFL exchanged myofibrils and to 2.19±0.04 µm (n=25) in cTn1-192 exchanged myofibrils.

Myofibrils were activated and relaxed by rapid (10 ms) switching between two continuous streams of relaxing (pCa 9; pCa = -log\(_{10}[Ca^{2+}]\)) and activating (pCa 3.5) solutions flowing by gravity from a double-barreled glass pipette placed at right angles to, and within 1 mm of, the preparation. A release–restretch protocol was used to measure the rate of force redevelopment (k\(_{TR}\)). The rate of rise of force following the step decrease in pCa by fast solution switching (k\(_{ACT}\)) was estimated from the time required to reach 50% of the maximum isometric force.

Isometric force in single human cardiomyocytes was measured at different [Ca\(^{2+}\)] (pCa ranging from 4.5 to 6.0) as described previously. Maximal activation at pCa 4.5 and a sarcomere length of 2.2 µm was used to calculate F\(_{max}\), the maximal calcium-activated isometric force/cross-sectional area. The cross-sectional area of the preparations was calculated assuming an elliptical shape, i.e. cross-sectional area = (width × depth × π) / 4. Accurate determination of myocyte depth was performed at the end of the experiments at a sarcomere length of 2.2 µm, by projecting the myocyte via a small mirror onto the objective of the inverted microscope. Mean dimensions (± S.E.M.) of the cTnFL (n=12) and cTn1-192 (n=15) exchanged cardiomyocytes amounted to 48.5±2.6 and 58.3±5.1 µm in length, 18.2±1.6 and 16.7±1.5 µm in width, and 19.7±2.1
and 17.0±1.6 µm in depth, respectively. After maximal activation 4 to 5 measurements were carried out at submaximal [Ca$^{2+}$] (pCa>4.5) followed by a maximal activation. Force values obtained in solutions with submaximal [Ca$^{2+}$] were normalized to the interpolated maximal force values (pCa 4.5). Maximum force at 1.9 µm sarcomere length was normalized to the cross-sectional area determined at 2.2 µm sarcomere length. On average force decline between the first and the last maximal control activation amounted to 10±3% and 14±1% for cTnFL and cTnI-192 exchanged cardiomyocytes, respectively. Experiments where force decline exceeded 25% were discarded. All mechanical experiments were performed at 15°C.

The solutions used for the myofibrils were pretreated with a P$_i$ scavenging solution to reduce P$_i$ concentration to less than 5 µmol/L. In cardiomyocytes the P$_i$ concentration will be higher (~0.2 mmol/L) because of a small P$_i$-contamination of the chemical compounds used and because some P$_i$ will be produced during activation. In view of this P$_i$-production it was also not useful to use the P$_i$ scavenging solution for cardiomyocyte experiments. The depressant effect of P$_i$ on force and the increase in diameter due to the inter-myofibrillar space in cardiomyocytes may explain why maximum tension in cardiomyocytes was lower than in myofibrils.

**Effects of PKA**

After measuring the force–pCa relation at 2.2 µm sarcomere length, myocytes were incubated in relaxing solution containing the catalytic subunit of protein kinase A (3 mg/L [100 U/mL]; Sigma, batch 35H9522) and 6 mmol/L dithiothreitol for 40 minutes at 20°C. Thereafter the force–pCa series was repeated.

**Protein analysis**
The amount of cTnI exchange was analyzed in cardiac samples in which endogenous cTnI was replaced by cTnI1-192. It has been shown previously for rabbit psoas myofibrils that the amount of human full length cTnI exchange was the same as for cTnI1-192, and amounted to 47.0±4.3 and 52.6±4.8, respectively (n=5 in both groups). Hence, in the present study evaluation of both cTnFL and cTn1-192 exchange into human cardiac preparations was based on the amount of cTnI1-192 exchange. This analysis was performed using Coomassie stained 1D-SDS-polyacrylamide gel electrophoresis (1D-SDS-PAGE) and was based on the reduction in the ratio between endogenous cTnI and α-actinin in cTn1-192 preparations compared to cardiac samples, which were incubated in exchange solution without exogenous cTnI. The α-actinin band was chosen as a measure of myofibrillar protein loading because its intensity was within the linear range and not affected by the exchange procedure.

The separating gel of 1D-SDS-PAGE used for the extent of cTn1-192 exchange determination contained 15% total acrylamide (acrylamide to bis-acrylamide ratio 200:1; pH 9.3), while the stacking gel contained 3.5% total acrylamide (acrylamide to bis-acrylamide ratio 20:1; pH 6.8). Cardiomyocytes and myofibrils were dissolved in standard Laemmli sample buffer. Gels were stained with 0.1% Coomassie solution and analyzed by laser densitometry (LKB Produkter AB, Bromma, Sweden) using the GelScan XL software package (Pharmacia, Uppsala, Sweden). To check for linearity, different amounts of tissue were loaded and the intensity of the cTnI and α-actinin bands was analyzed. Protein content of the cardiomyocyte suspension was determined using the RC-DC protein concentration assay kit (BioRad). Based on these determinations protein loads <90 µg were found to be within the linear range.

The cTnI, cTnI1-192 and α-actinin bands on gel were identified and the amount of troponin complex exchange was confirmed by Western Immunoblotting. Semi-dry
blotting was performed at 45 Volt for 90 min, using the discontinue system. Staining with the specific antibodies directed against troponin I (8I-7, Research Diagnostics Inc, dilution 1:10 000) and α-actinin (clone EA53, Sigma, dilution 1:200) was done using the Vectastain ABC-Amp protocol (Vector laboratories, Burlingame, USA).

To check if myosin light chain-1 or -2 (MLC1, MLC2) were extracted during the exchange protocol, their intensity values normalized to α-actinin content after exchange were compared to the values obtained in untreated tissue. The values obtained were: MLC1/α-actinin: 1.28±0.04 (n=4; untreated) en 1.51±0.23 (n=3; after exchange); MLC2/α-actinin: 1.94±0.21 (n=4; untreated) en 1.89±0.40 (n=3; after exchange). There were no significant differences in the untreated vs. after exchange values.

Microscopy

To check if the cTn complex was homogeneously incorporated into cardiomyocytes, cTnI1-192 exchanged cardiomyocytes were studied using a Marianas™ digital imaging microscopy workstation equipped with a nanostepper motor (Z-axis increments: 10 nm) and a cooled CCD camera (Cooke Sensicam, Cooke Co., Tonawanda, NY, USA). The microscope and camera were controlled by SlideBook™ software (SlideBook 4.1.0.9, Intelligent Imaging Innovations, Denver, CO, USA). After the exchange protocol cardiomyocytes were fixed with 4% paraformaldehyde. The cTnI1-192 exchange into cardiomyocytes was examined using a specific antibody directed against amino acid residues 195-209 in the C-terminus region of cTnI (clone P45-3, Research Diagnostics Inc, dilution 1:50) and a secondary fluorescent antibody Alexa Fluor 488 (Molecular Probes, dilution 1:40). Images were acquired in the Z-axis direction with a step size of 0.5 µm. Image processing and data analysis was performed using SlideBook™ and ImageJ (public domain software available at http://rsb.info.nih.gov/ij/). Images were
deconvoluted using the constrained interactive mode, with interpolation of 1 plane between each acquired plane and mirror edge padding at the Z-axis (20 planes).

**Data analysis**

Force-pCa relations were fit to a Hill equation\textsuperscript{11}:

\[
F(\text{Ca}^{2+})/F_0 = [\text{Ca}^{2+}]^{n_H}/(\text{Ca}_{50}^{n_H} + [\text{Ca}^{2+}]^{n_H}),
\]

where \( F \) is steady state force, \( F_0 \) denotes the steady force at saturating \( \text{Ca}^{2+} \) concentration at 1.9 or 2.2 \( \mu \text{m} \) sarcomere length, \( n_H \) reflects the steepness of the relation, and \( \text{Ca}_{50} \) (or \( p\text{Ca}_{50} \)) represents the midpoint of the relation.

Values are given as means ± S.E.M. of \( n \) myofibrils of myocytes. Differences between preparations with cTnI\textsubscript{1-192} and full length cTnI were tested by means of unpaired two tailed Student’s \( t \)-tests at a level of significance of 0.05. The effects of sarcomere length and PKA treatment were tested with paired Student’s \( t \)-tests.

**Protein phosphorylation**

The supplemental Figure 1A shows the results from Western immunoblotting using an antibody (clone 22B11; Research Diagnostics) against dephosphorylated PKA-sites on cTnI (Ser 23/24). In donor tissue, treated with TCA to preserve basal phosphorylation, no band was discernible, indicative for the low amount of dephosphorylated cTnI.\textsuperscript{12} In a myocyte suspension from the same donor kept in exchange solution overnight, in the absence of the cTn complex (CTR), a faint band at the level of full length cTnI in tissue can be seen, indicating that cTnI was partly dephosphorylated during the exchange procedure. In tissue exchanged with cTn complex both full length cTnI and truncated cTnI were recognized illustrative for the dephosphorylated nature of these proteins.
The supplemental Figure 1B illustrates the composition of troponin T separated by 2D-gel electrophoresis. The basal level of cTnT phosphorylation in TCA-treated donor tissue is approximately 60%.\textsuperscript{12} Overnight incubation of donor myocytes in exchange solution reduced phosphorylation status of cTnT to ~40% as illustrated in the upper panel of the supplemental Figure 1B. Similar 2D gels showed that MLC-2 phosphorylation decreased from ~ 33% in donor to ~15% in CTR.

References

1. Foster DB, Noguchi T, VanBuren P, Murphy AM, Van Eyk JE. C-terminal truncation of cardiac troponin I causes divergent effects on ATPase and force: implications for the pathophysiology of myocardial stunning. Circ Res. 2003;93:917-924.


Supplemental Figure 1

A: Western Immunoblot of a 1D-gradient gel (4-15%) using an antibody against human cTnI with dephosphorylated PKA sites (Ser 23/24). Donor: donor tissue, treated with TCA to preserve basal phosphorylation; cTnFL and cTn1-192 denote a myocyte suspension with exchanged full length cTnI (cTnFL) and truncated cTnI, respectively; CTR denotes myofibrils treated similarly, but without cTn complexes.

B: Sypro Ruby stained 2D-gel of the CTR suspension. The cTnT and cTnTP spots denote the unphosphorylated and mono-phosphorylated forms of cardiac TnT.

Legend supplemental Figure 1

A: Western Immunoblot of a 1D-gradient gel (4-15%) using an antibody against human cTnI with dephosphorylated PKA sites (Ser 23/24). Donor: donor tissue, treated with TCA to preserve basal phosphorylation; cTnFL and cTn1-192 denote a myocyte suspension with exchanged full length cTnI (cTnFL) and truncated cTnI, respectively; CTR denotes myofibrils treated similarly, but without cTn complexes. B: Sypro Ruby stained 2D-gel of the CTR suspension. The cTnT and cTnTP spots denote the unphosphorylated and mono-phosphorylated forms of cardiac TnT.