Revision of the Troponin T Release Mechanism from Damaged Human Myocardium

Karin Starnberg,1 Anders Jeppsson,2,3 Bertil Lindahl,4 and Ola Hammarsten1*

BACKGROUND: Cardiac troponin T (cTnT) is released from damaged heart tissue in patients with acute myocardial infarction. It is presumed that most cTnT is tightly bound and released following the degradation of myofibrils in necrotic cardiomyocytes, resulting in sustained increases in circulating cTnT. Evidence of a large irreversibly bound fraction is based on the inability to extract most cTnT from cardiac tissue in cold low-salt extraction buffers.

METHODS: Here we examined in vitro extraction of cTnT from human cardiac tissue in serum at 37 °C.

RESULTS: We found that over 80% of the cTnT can be extracted from human cardiac tissue in 90 min using large volumes of human serum at 37 °C. The release ratio was highly dependent on the extraction volume and was only 3% if an equal volume of serum and heart tissue was used. In contrast, extraction of the cytoplasmic cardiac damage markers myoglobin and creatinine kinase was much less affected by changing these conditions. Purified cTnT was poorly soluble in a low-salt extraction buffer at 0 °C, previously used to define the free cTnT fraction.

CONCLUSIONS: Our data indicate that the diffusible fraction of cTnT is likely substantially larger in vivo than previously reported and likely is not fixed but dependent on local plasma flow. It is therefore possible that the sustained increase in circulating cTnT after myocardial infarction is at least in part due to a slow washout of cTnT that interacts reversibly with tropomyosin in myofibrils.

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Materials and Methods

HUMAN HEART TISSUE
A biopsy sample (5 × 5 × 4 mm) from the auricle of the right atrium was obtained from a patient undergoing cardiac surgery. In addition, a left-ventricle transmural biopsy sample (20 × 20 × 20 mm) was obtained from the explanted heart from a patient with end-stage heart failure undergoing heart transplantation. All tissue samples were obtained at the Department of Cardiothoracic Surgery, Sahlgrenska University Hospital. The human cardiac tissue was cleared from epicardial fat of Gothenburg, SE-413 45 Gothenburg, Sweden. Fax +46-31-82-84-58; e-mail ola.hammarsten@clinchem.gu.se.

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and cut into small pieces with a scalpel and frozen at −80 °C within 1 h. The collection of the biopsy samples was approved by the regional Research Ethics Committee.

EXTRACTION PROCEDURES
Frozen human cardiac muscle was weighed and homogenized using a small glass Dounce homogenizer (D8938, 2 mL; Sigma Aldrich) at room temperature in different volumes of pooled human serum with a free Ca²⁺ concentration of 1.25 mmol/L at the ratios specified in Fig. 1 and Table 1. The resulting homogenate was incubated for 30 min with gentle agitation at 37 °C before centrifugation at 20 000 RCF in a temperature-controlled centrifuge at 25 °C for 5–10 min. The extent of centrifugation, even for 30 min, did not significantly affect the extent of extraction of cTnT or other heart damage markers. The serum supernatants were recovered and the tissue pellets were rehomogenized in >200 volumes of a high-salt buffer optimized for cardiac troponin extraction [40 mmol/L NaPO₄ (pH 7.0), 1 mol/L NaCl, 5 mmol/L EDTA, 0.1% TritonX100] (4) at room temperature for 2 h and then at +4 to −8 °C overnight with gentle agitation before centrifugation as above. After dilution of the extracts in PBS supplemented with 1% BSA, cTnT and other heart damage markers were measured using routine clinical methods with CV <5% (Roche Cobas). The cTnT concentration in these dilutions was from 24.5 to 2729 ng/L, well within the linear range of the assay (limit of quantification, 13 ng/L; maximum of the master curve, 10 000 ng/L). The performance of the high-sensitivity cTnT (hs-cTnT) method has been summarized (15, 16). The performance of the hs-cTnT method in different solutions is summarized in Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol60/issue8. The CVs of the cTnT measurements were 3.2% after 1:10 dilutions, 5.3% after 1:100 dilutions, and 6.6% after 1:1000 dilutions. The total amount of cTnT recovered from the serum and high-salt extractions and the fraction of the total cTnT in the serum extraction was calculated: fractional extraction = cTnT from serum extraction (ng)/[cTnT in serum extraction (ng) + cTnT in high-salt extraction (ng)]. Similar calculations of fractional extraction were used for aspartate aminotransferase (AST) (μkat), lactate dehydrogenase (LD) (μkat), CK (μkat), CKMB (μg/L), and Myo (μg/L).

Control experiments showed that repeated high-salt buffer extractions resulted in <1% of the total cTnT, LD, AST, CK, CKMB, and Myo recovered from the human heart tissue in subsequent extractions. A single high-salt extraction was therefore used in the experimental procedure. The total recovery of cTnT from human cardiac muscle was 0.405 mg/g human

![Fig. 1. Volume-dependent extraction of cTnT from human cardiac muscle.](image)

(A), Fraction of total cTnT, CK, CKMB, LD, AST and, Myo in human cardiac muscle extracted in a single serum extraction at 37 °C for 30 min at different relationships between serum volume (μL) and human cardiac muscle (mg) from 1:1 to 1:1000. (B), Fraction of total cTnT, CKMB, and Myo in human cardiac muscle extracted in 3 repeated serum extractions, each at 37 °C for 30 min at a relationship between serum volume (μL) and human cardiac muscle (mg) of 1:434. The fractional extraction after 3 extractions was 80% for cTnT, 99% for CKMB, and 99% for Myo. (C), Fraction of total cTnT, CKMB, and Myo in human cardiac muscle extracted in 3 repeated serum extractions each at 37 °C for 30 min at a relationship between serum volume (μL) and human cardiac muscle (mg) of 1:1. Fractional extraction after 3 extractions was 9% for cTnT, 92% for CKMB, and 93% for Myo. Error bars from double samples are shown.
cardiac muscle (SD, 0.077 mg/g), which is in good agreement with previous studies (4). We did not observe any difference in recovery using fresh or frozen human cardiac muscle (see online Supplemental Fig. 1). The data in Fig. 1 and Table 1 were from 2 separate human cardiac biopsy samples. Repetitions of these experiments on 2 additional human cardiac biopsy samples and pig cardiac muscle from the left ventricle gave similar results (see online Supplemental Fig. 2).

The extent of cTnT extraction was also examined under conditions previously used to define a free pool of cTnT in cardiac muscle (Table 1) (4). In these experiments, frozen human cardiac muscle was homogenized as described above in low-salt buffer (0.05 mol/L Tris hydrochloride, 2 mmol/L ethylene dinitrilotetraacetic acid, 0.5 mmol/L dithiothreitol) at 0 – 4 °C and incubated at 0 °C for 30 min in a rocking cradle. The samples were then centrifuged in 0 °C for 5 min at 20 000 RCF. Supernatants were collected. The tissue pellets were extracted in the high-salt buffer and fractional extraction was determined as described above.

### Table 1. Fractional extraction of cTnT in different solutions.

<table>
<thead>
<tr>
<th>Buffer conditions</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum 37 °C&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.3%</td>
<td>62.1%</td>
</tr>
<tr>
<td>EDTA-plasma 37 °C&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44.8%</td>
<td>43.5%</td>
</tr>
<tr>
<td>PBS 37 °C</td>
<td>33.8%</td>
<td>25.1%</td>
</tr>
<tr>
<td>Low-salt 0 °C</td>
<td>12.9%</td>
<td>13.9%</td>
</tr>
</tbody>
</table>

<sup>a</sup> Using a 1:1000 relationship between the microliter solution per milligram human heart tissue and an extraction time of 30 min at the specified temperatures.

<sup>b</sup> Free Ca<sup>2+</sup> concentrations: serum, 1.25 mmol/L; EDTA-plasma, <0.2 mmol/L.

### Fig. 2. cTnT is poorly soluble in the cold low-salt buffer used to define a free early releasable fraction of cTnT.

The low-salt buffer (see Materials and Methods) and human serum were used to dissolve dry purified human cTnT at the temperatures and times specified. Error bars from double samples are too small to be visible at this scale.

### Results

Human heart tissue was homogenized in different volumes of human serum at 37 °C. After centrifugation the tissue pellet was homogenized in a high-salt buffer optimized for troponin extraction (4, 14). The extent of the cTnT extraction and extraction of other heart damage markers was analyzed. The volume of serum per milligram of human heart tissue marginally affected the extraction of the cytoplasmic heart damage markers CK, CKMB, LD, AST, and Myo (Fig. 1A). In contrast, the extent of the cTnT extraction was highly dependent on the serum volume used. When equal volumes of serum and heart tissue were used, only 3.3% of the total amount of cTnT in the heart tissue was extracted (Fig. 1, A and C). In contrast, when the volume of serum was increased the extraction of cTnT reached over 60% (Fig. 1A and Table 1). Using repeated extractions in excess serum, it was possible to remove over 80% of the cTnT from the cardiac tissue in 90 min (Fig. 1B). As expected from previous studies (4, 17), when extraction was performed using a low-salt buffer at 0 °C, the conditions used to define a free early releasable fraction, only 13% of total cTnT was extracted (Table 1). With pig heart muscle tissue, the fractional recovery was based on the cTnT concentration measured with the COBAS hs-cTnT method using 50 µL of the original cTnT solution diluted in 450 µL of either a low-salt buffer or serum to compensate for matrix effects. Coating of the plastic tubes with BSA to prevent nonspecific retention of cTnT to the plastic surfaces did not increase the fractional recovery in the low-salt buffer (data not shown).
 extraction using the low-salt buffer at 0 °C was 8% when we used a standard tissue homogenizer, whereas the fractional extraction of cTnT was 12%–13% with a Dounce homogenizer. Only 2% of dried purified human cTnT dissolved in the low-salt buffer at 0 °C compared to 65% in human serum at 37 °C (Fig. 2).

Discussion

Previous in vitro studies of cTnT release have defined a fixed minor free early releasable fraction and a major bound fraction of cTnT in cardiomyocytes (4, 7, 17, 18). According to the current model, release of the 5%–10% of cTnT that is free is responsible for cTnT increases early after an AMI. The subsequent sustained increases are thought to be due to the slow release of irreversibly bound cTnT by proteolytic degradation of myofibrils analogous to the release of structural muscle proteins like the myosin light chain (8). In studies that examine the free and bound fraction of cTnT, the free fraction was defined as the limited amount of cTnT that can be extracted from cardiac tissue using a cold low-salt buffer (4). We found that purified human cTnT has low solubility under these conditions. In fact, this extraction procedure resembles the first steps in cTnT purification protocols, in which ground up heart muscle is first washed extensively with cold low-salt buffers and then conditions are optimized to retain most of the cTnT:I/C complex in the tissue pellets while allowing removal of unwanted highly soluble proteins like lysosome enzymes and Myo (14). This extraction procedure has little resemblance to the in vivo situation following cardiac damage. In addition, the extent of extraction in this buffer was, in our hands, dependent on the volume-to-tissue weight ratio and the extent of homogenization.

A previous study of cTnT turnover failed to provide any evidence of an intracellular free pool of cTnT, indicating that all cTnT is bound to myofibrils in rat cardiomyocytes (19). In addition, in vitro experiments have shown that over 80% of all cTnT:I/C complexes in myofibrils can be exchanged in near-physiological buffers within 60 min (2). This could indicate that the fraction of cTnT bound to myofibrils is not fixed because it is part of an equilibrium based on the cTnT: tropomyosin interaction and limited local plasma flow could allow most cTnT to prevail in the central necrotic area (6) until tissue degradation became the prominent cTnT release mechanism. In contrast, the extraction of Myo, CK, CKMB, AST, and LD was almost as efficient when limited serum volumes were used. The extensive difference in extraction using limited volumes of serum could explain why Myo, CKMB, and other cytoplasmic cardiac damage biomarkers are cleared faster than cTnT in patients with AMI.

Our data therefore indicate that the sustained increase in cTnT after an AMI is a combination of slow washout and local tissue degradation. According to this model, release of cTnT after an AMI not only results in the degradation of myofibrils and release of irreversibly bound cTnT, but also reflects the fact that large volumes of plasma must pass through the damaged area to
washout the cTnT that reversibly interacts with tropomyosin in necrotic cardiomyocytes.

In contrast, cytoplasmic cardiac damage biomarkers such as Myo and CKMB are all highly soluble and lack strong binding partners in cardiac tissue and are therefore extracted and cleared with faster kinetics.

The slow-washout model is consistent with several observations that are not as easy to explain if the cTnT release, and therefore also the cTn release, is based only on a fixed minor free and a larger irreversibly bound pool of cTnT.

First, serum cTnT and cTnI concentrations return to baseline levels faster, often within 24 h, after diffuse infarctions that occur in the presence of unrestricted blood flow such as after an isoproterenol injection (22–24). After isoproterenol-induced AMI in rats, necrotic myocytes are diffusely distributed in the heart and exposed to an unrestricted plasma flow, resulting in fast extraction and clearance of cTnT and cTnI, compared with a classical thrombotic AMI when the damage is focal and there is only a limited plasma flow. Animal studies in which diffuse AMIs of variable size were induced by different doses of isoproterenol showed a similar time to the first increases in cTnT and cTnI but faster cTnT and cTnI clearance following a small AMI. On histological examination at different times following a small AMI, damaged heart muscle cells, although fewer in number, show a more rapid loss of cTnI staining compared with damaged muscle cells in animals with a larger AMI (22). Although this could be a result of fast degradation of myofibrils when the damaged area is small and subjected to large plasma flow, our data indicate that washout of cTnT and therefore also cTnI can occur without extensive myofibrillar degradation, because cTnT extraction can be almost complete after 90 min. Over 1500 µL of plasma per mg of tissue per day perfuse the human heart (25), conditions that according to our data would allow extraction of the majority of cTnT in 24 h without the need for extensive myofibrillar degradation given an unrestricted plasma flow in the damaged area. It is possible that this high flow rate, in addition to cTnT release from viable cardiomyocytes, explains the fast cTnT clearance observed after exercise-induced increases in cTnT in human study participants (26).

Second, the slow-washout model potentially explains why CK and CKMB reach their maximum concentrations in the circulation at an earlier time point than with cTnT following an AMI (7). The molecular weight of CK and CKMB is over 80 kDa, with a hydrodynamic diameter of 3.8 nm. CKMB is therefore expected to have a more restricted uptake through blood vessels (27) and lymph vessels (28) than do cTnI complexes (29) that are between 23 and 40 kDa and cTnT that during the first hours exists for the most part as a 37-kDa monomer (20) in serum from AMI patients (29, 30) and has an elongated structure with a hydrodynamic diameter of 3.37 nm (31). For this reason, it is not clear why CK and CKMB often peak faster than cTnT if the early cTnT release originates from a fixed fraction of free cTnT. However, according to our data, washout of CK and CKMB is similar when the serum volume is limited, whereas washout of cTnT is an order of magnitude less efficient under the same conditions. Therefore, highly soluble CK and CKMB enter the circulation and reach maximum levels faster than less soluble cTnT, given a restricted plasma flow through the damaged myocardium.

Third, in contrast to Myo and CKMB, cTnT often does not show a clear rise-and-fall pattern during the first days following a nonreperfused AMI. Studies in which cTnT concentrations have been followed for a week in patients with a developing AMI without active reperfusion show a rise in serum cTnT during the first 10–15 h and relatively stable cTnT concentrations for 1–2 days followed by a slow decline (4, 5), in sharp contrast to the kinetics seen after successful reperfusion (4, 32). This is also in contrast to the rise-and-fall pattern of Myo or CKMB, irrespective of active reperfusion (32) or not (4–6). The rise followed by stable cTnT concentrations is compatible with a constant extraction rate from the damaged area, once dead cardiomyocytes have accumulated to saturate the limited local plasma flow in the ischemic area (21) with cTnT, as we observed when cTnT extraction was performed using small volumes of serum (Fig. 1C). As only a small fraction of the available cTnT is extracted from the damaged area per unit of time (6), little depletion of available cTnT occurs during the first days, resulting in a steady-state situation in which cTnT delivery to the circulation and clearance is balanced. This steady state will prevail until the available cTnT in the damaged myocardium is depleted to the point at which local plasma flow no longer becomes saturated with cTnT, resulting in a declining delivery of cTnT to the circulation. In combination, this potentially explains why cTnT concentrations in nonreperfused AMI often reach a plateau for a few days (33), followed by a slow decline. The resulting cTnT concentration in the circulation therefore follows the slow-washout model, a reflection of both the damage volume and the local plasma flow through the damaged myocardium. Further, in the washout model the prominent peak followed by stable cTnT concentrations seen in reperfused AMI could be further enhanced by reflow, reactive hyperemia, and reperfusion injury, as well as by a washout of cTnT dissolved in the extensive local edema that develops in the ischemic area (21).

A direct clinical implication of the slow-washout model is that cTnT and hence circulating concentra-
tions of cTnT will respond to changes in the plasma flow through the damaged area. Therefore, further increases in cTnT that occur during the infarction process could be due to increased regional plasma flow as well as to reinfarction and slow local degradation of cTnT in regions with permanently occluded arteries. For instance, after mechanical occlusion of coronary arteries in dogs there is substantial restoration of the microcirculation in the ischemic area (34) and an increase in circulating cTnT 2–4 days after the occlusion (35). Therefore, in addition to slow local degradation of cTnT in regions with permanently occluded arteries, it is possible that local partial reflow might explain the second cTnT peak observed in several studies 2–4 days after AMI that is not reperfused acutely (4–6). Unfortunately, this means that the slow-washout model adds a level of complexity to interpretations of changing cTnT concentrations.

This study has several limitations. Because all experiments were performed in vitro, our findings do not replicate the intricate processes known to occur in the ischemic region that result in contraction band necrosis and edema (21). In addition, cTnT uptake and clearance from the circulation, as well as the role of local inflammation and tissue degradation in the release of cTnT, must be addressed using animal studies. A complete understanding of how cTnT is released from intact myofibrils can be addressed only in biochemical studies using purified proteins. Finally, the slow-washout model does not explain stable increases in cTnT often observed in old age, heart failure, and renal failure and after anthracycline treatment.

In conclusion, we find that the diffusible fraction of cTnT is likely to be substantially larger in vivo than previously reported and likely affected by the local plasma flow.

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