Release characteristics of cardiac proteins after reversible or irreversible myocardial damage

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Chapter 3

Release kinetics of intact and degraded troponin I and T after irreversible cardiomyocyte damage

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Abstract

Background: For the diagnosis of acute myocardial infarction (AMI) activity assays of enzymes like lactate dehydrogenase (LDH) and creatine kinase (CK) have been replaced by mass assays of CK-MB, cardiac troponin I (cTnI) and cardiac troponin T (cTnT). This study characterized whether the release kinetics of cardiac troponin I (cTnI) and T (cTnT) differ from each other in cardiomyocytes before and after the transition from reversible to irreversible cell damage, as assessed by the release of lactate dehydrogenase (LDH).

Methods: Cultures of cardiomyocytes were exposed to metabolic inhibition by treatment with sodium azide (1 mmol/L) to induce a necrotic cell death process that is characterized by a reversible (0-12 h) and irreversible phase (12-30 h). At various time intervals cells and media were collected and analyzed for LDH activity, intact cTnI and cTnT, and their degradation products.

Results: During the first 12 h of metabolic inhibition, cell viability was unchanged with no release of intact cTnI and cTnT nor their degradation products. Between 12 and 30 h of azide treatment, cardiomyocytes showed progressive cell death accompanied by release of intact cTnI (29 kDa), intact cTnT (39 kDa), four cTnI degradation products of 26, 20, 17 and 12 kDa, and three cTnT degradation products of 37, 27 and 14 kDa. Possibly due to degradation, there is progressive loss of cTnI and cTnT protein that is obviously undetected by the antibodies used.

Conclusion: Mild metabolic inhibition of cardiomyocytes induces a parallel release of intact cTnI and cTnT and their degradation products, starting only after onset of irreversible cardiomyocyte damage.

Keywords

necrosis, cardiac troponin I, cardiac troponin T, lactate dehydrogenase, cardiomyocytes, metabolic inhibition

Introduction

Cardiac troponin I and T (cTnI and cTnT) are highly sensitive and specific markers of myocardial injury in patients with acute myocardial infarction (AMI)[1;2]. Within a few hours after onset of AMI, troponins are released from necrotic cardiomyocytes into the circulation. Typically, troponin levels peak at 16-18 h, and remain elevated for several weeks after AMI, long after levels of most other markers have normalized[1;3;4]. Despite the high cardiac specificity, several studies have demonstrated that the appearance of troponins in serum of patients with AMI is delayed compared with the release of CK-$MB_{activity}$ and CK-MB_{mass}[5-8], suggesting that troponins and CK-MB do not share identical release kinetics, and that troponins may not be ideal early markers for ruling out AMI. Previously, we investigated the release kinetics of intact cTnI and lactate dehydrogenase (LDH) from cultured neonatal cardiomyocytes exposed to severe metabolic inhibition using sodium cyanide, which induces necrosis over a time period of 3-3.5 hours[9]. We found that the release of intact cTnI from irreversibly damaged cardiomyocytes was partial and delayed as compared with the release of LDH. This delay can theoretically be explained by (1) slow dissociation of cTnI molecules from the myofilaments as only 3-8% of cTnI is free in the cytoplasmic pool[10], or (2) the early formation of cTnI degradation products that remained undetected by the specific ELISA that is used, as detection of cTnI degradation products is antibody dependent[11;12].

Troponins are susceptible to proteolytic degradation by intracellular proteases, such as calpain-I, caspases, or matrix metalloproteinase-2 (MMP-2)[13-16]. Degradation products of troponins are detected in serum of patients with AMI, and the release pattern of these degradation products changes in the days following onset of AMI[14;17-19]. In addition to troponin degradation during irreversible cell damage, several studies have suggested that proteolysis of troponin may also occur in reversibly damaged cardiomyocytes[20-22].

In the present study we investigated the release kinetics of cardiac troponins from cultured neonatal cardiomyocytes undergoing metabolic inhibition by sodium azide. Azide is an inhibitor of cytochrome c oxidase and induces cell death that, at 1 mmol/L, develops slower compared to cyanide at 5-10 mmol/L. Chen *et al.*[23] have demonstrated that cultured neonatal rat cardiomyocytes exposed to azide showed a transition from reversible to irreversible cell damage after 6-12 hours. The purpose of the present study

was to characterize the release kinetics of intact cTnI, intact cTnT, and degradation products of cTnI and cTnT, in relation to LDH, the golden standard for cell death, from azide-treated cardiomyocytes before and after the transition from reversible to irreversible cell damage.

Materials and Methods

Primary cultures of neonatal cardiomyocytes

Primary cultures of cardiomyocytes were prepared from the ventricles of 2-day old Wistar rats as described previously[24]. Briefly, ventricles were minced and cells were isolated enzymatically using collagenase type I (CLS, Worthington, Lakewood, NJ, USA) at 37°C. The cell suspension was centrifuged and the cell pellet was resuspended in growth medium. Cells were seeded in Primaria®-coated plastic culture dishes (∅ 6.0 cm, Falcon, Becton Dickinson, Etten-Leur, The Netherlands), and after 45 min non-adherent cells, i.e. the cardiomyocytes, were collected and plated in Primaria®-coated plastic culture dishes at a density of ≈$3x10^6$ cells per well. Cardiomyocytes were incubated in a humidified incubator at 37°C and 5% CO_2 and culture medium was refreshed after 20 h and 48 h. After three days, a monolayer of spontaneously beating cardiomyocytes had formed. The investigations had the approval of the Animal Experiments Committee of the LUMC, according to Dutch law.

Metabolic inhibition of cardiomyocytes

Three days after isolation, cardiomyocyte cultures were washed in HEPES-buffered salt solution (HBSS), containing (in mmol/L) NaCl (125), KCl (5), $MgSO_4$ (1), KH_2PO_4 (1), $CaCl_2$ (2.5), $NaHCO_3$ (10), sodium pyruvate (5), and HEPES (20), pH 7.4 at 37˚C. In each experiment, six cultures of neonatal rat ventricular myocytes were exposed to metabolic inhibition by incubation in 3 mL HBSS containing sodium azide (1 mmol/L) for 0, 6, 12, 18, 24 and 30 h at 37˚C .

At the end of a period of metabolic inhibition, medium was separated from the cells, cells were collected in 1.5 ml ice-cold lysis buffer containing (in mmol/L) Tris-HCl (20), NaCl (100), EDTA (5), 1% (v/v) Triton X-100, and 10% (v/v) glycerol, pH 7.5, and both cell

and medium samples were kept on ice awaiting assay of LDH activity at the same day. Aliquots of cell and medium samples were stored at -20˚C until cTnI and cTnT assays.

LDH activity assay

Necrotic cell death was quantified by assay of LDH activity released from cells into medium, according to Wroblewski and Ladue[25]. After various periods of metabolic inhibition (0 to 30 h), LDH activities in medium and cell samples were determined using a spectrophotometer (Ultrospec 3000, Pharmacia, Roosendaal, The Netherlands) at 25°C. Per culture tested, the LDH activities in medium and cell extract were calculated after volume correction. The percentage activity of LDH released from each culture was $[LDH_{medium}/(LHD_{medium}+ LDH_{cells})]x$ 100%. The detection limit of the LDH assay was 5 U/L. Inter-assay variability was 12% at LDH activity of 110 U/L. The intra-assay variability was 3% and 6% at LDH activity of 110 U/L and 48 U/L, respectively.

Troponin-I ELISA

Purified human heart cTnI (Calbiochem, LaJolla, CA, USA) was dissolved in urea/Tris buffer according to the manufacturer's instructions. Monoclonal mouse anti-cTnI clone 19C7 (recognizing amino acid residues (a.a.r.) 40-50) and monoclonal mouse anti-cTnI clone 6F9 (recognizing a.a.r. 189-195) were purchased from HyTest (Turku, Finland). The ELISA was based on the sandwich principle as described previously[9]. The percentage cTnI released from each culture equalled $[TnI_{medium}/(TnI_{medium} + TnI_{cells})]*100\%$. The detection limit of the assay was 6 µg/L. Inter-assay variability was 6% and 10%, at cTnI concentrations of 200 µg/L and 25 µg/L, respectively. The intra-assay variability was 6% at TnI concentration of 100 µg/L.

Detection of troponin-I degradation products in autolytic myocardium and medium

Testing the antibody. The detection of intact cTnI and cTnI degradation products is antibody dependent[11;12;26]. Therefore we tested whether cTnI degradation products in rat heart tissue undergoing ischemic autolysis could be detected by a specific anti-cTnI antibody (clone K83085G, Biodesign, Saco, Maine, USA) recognizing a.a.r. 27-40.

Hearts from anesthetized 2-day old Wistar male rats were rapidly dissected, and washed in a solution containing (in mmol/L) NaCl (137), KCl (5.4), Na_2HPO_4 (0.34), KH_2PO_4 (0.44), D-glucose (5.6), HEPES (20), and 0.02% Phenol Red, pH 7.4. Subsequently, ventricular myocardium (46 ± 7 mg tissue) was incubated for 0, 2, 4, 6, 8 and 10 h in a petri dish (∅ 1.5 cm) that was placed into another petri dish (∅ 3.5 cm) containing water at 37°C. After incubation, cardiac tissue was snap-frozen in liquid nitrogen, freeze-dried for at least 24 h, and homogenized in a buffer containing 0.1 mol/L Tris-HCl and 0.1% (v/v) Tween-20 pH 7.5, using a Potter tube (Kimble/Kontes, Vineland, NJ, USA). Homogenates were stored in aliquots at -80°C. Protein concentrations were determined by bicinchonic acid (BCA) protein assay (Pierce, Etten-Leur, the Netherlands). In autolysing myocardium intact cTnI was detected by ELISA and Western blotting, and cTnI degradation products were detected by Western blotting.

Western blotting. Homogenates of heart tissue and medium samples of control and azide-treated cardiomyocyte cultures (10x concentrated by freeze-drying for 18 h) were size-fractionated on NuPage Novex 12% Bis-Tris gels (Invitrogen) and transferred to Hybond PVDF membranes (Amersham Biosciences, Roosendaal, The Netherlands). After blocking non-specific binding sites, membranes were incubated for 1 h with the anti-TnI antibody (K83085G, Biodesign), recognizing a.a.r. 27-40, and for 1 h with the horseradish peroxidase-labelled secondary antibody (rabbit anti-goat IgG, Santa Cruz Biotechnology, Heidelberg, Germany). Chemiluminescence was induced by ECL Advance Detection reagent (Amersham Biosciences) and detected by exposure to Hyperfilm ECL (Amersham Biosciences).

Troponin-T assay

cTnT concentrations in cell and medium samples of control and azide-treated cardiomyocytes were measured using the Elecsys 2010 (fourth-generation troponin-T test, Roche, Mannheim, Germany). Monoclonal human anti-cTnT clone M11.7 (recognizing a.a.r. 136-147) and monoclonal human anti-cTnT clone M7 (recognizing a.a.r. 125-131) purchased from Hytest were used as capture and detection antibody, respectively. The detection limit for human cTnI in serum is 0.01 µg/L. The inter-assay

variability was 8% and 7% at concentrations of 0.134 µg/L and 2.85 µg/L, respectively (n=89).

Detection of troponin-T degradation products

Intact cTnT and its degradation products in medium samples of control and azide-treated cardiomyocytes were detected by a novel immunoprecipitation assay[27;28]. Briefly, a mixture of four anti-cTnT antibodies (clones 9G6, 7F4, 7A9, and 1C11, a kind gift from Hytest) recognizing specific epitopes throughout the cTnT molecule were used to detect as many degradation products as possible. These antibodies were covalently immobilized on protein-A sepharose (GE Healthcare Europe, Diegem, Belgium) using dimethylpimelimidate (DMP, Sigma, Zwijndrecht, The Netherlands) as a cross-linking agent. Media of cardiomyocyte cultures were incubated with the sepharose beads to extract cTnT and its degradation products. Next, the beads were washed and incubated in sample buffer (40 mmol/L Tris, 33 g/L sodium dodecyl sulphate (SDS), 500 mL/L glycerol, and bromophenol blue). Samples were separated by SDS-PAGE on a Tris-HCl 4-15% polyacrylamide gradient gel (BioRad, Veenendaal, the Netherlands) and transferred to nitrocellulose (0.45 µm, BioRad) before incubation with a mixture of the same monoclonal mouse anti-cTnT antibodies. A peroxidase-conjugated goat anti-mouse antibody (Dako, Glostrup, Denmark) was used for visualization by chemoluminescence (Perkin-Elmer Life Sciences, Waltham, MA, USA). Blots were captured on Kodak X-Omat blue film (Perkin-Elmer Life Sciences).

Statistics

Results are expressed as means ± SD. Statistical analysis was performed by Student´s t-test. Differences were regarded as statistically significant if $p<0.05$. SPSS14 for Windows (SPSS Inc, Chicago, IL, USA) was used for statistical analysis.

Results

Detection of cardiac TnI degradation products in autolytic myocardium

At baseline, the mean cTnI content in neonatal rat heart tissue was 0.72 µg/mg tissue (100%). After 10 h of incubation, intact cTnI (29 kDa) was progressively reduced to ≈ 40% (Fig. 1a and b), and three cTnI degradation products of 26, 24, 20 kDa were formed (Fig. 1b). At baseline (t=0 h), a weak 26 kDa band was observed, being roughly 10 times smaller than the 29 kDa band (intact cTnI). This 26 kDa band represents a degradation product of cTnI. The intensity of this band increased during the first 4 h, followed by a decrease in the next 6 h of incubation, compatible with the formation and subsequent further degradation of a cTnI degradation product.

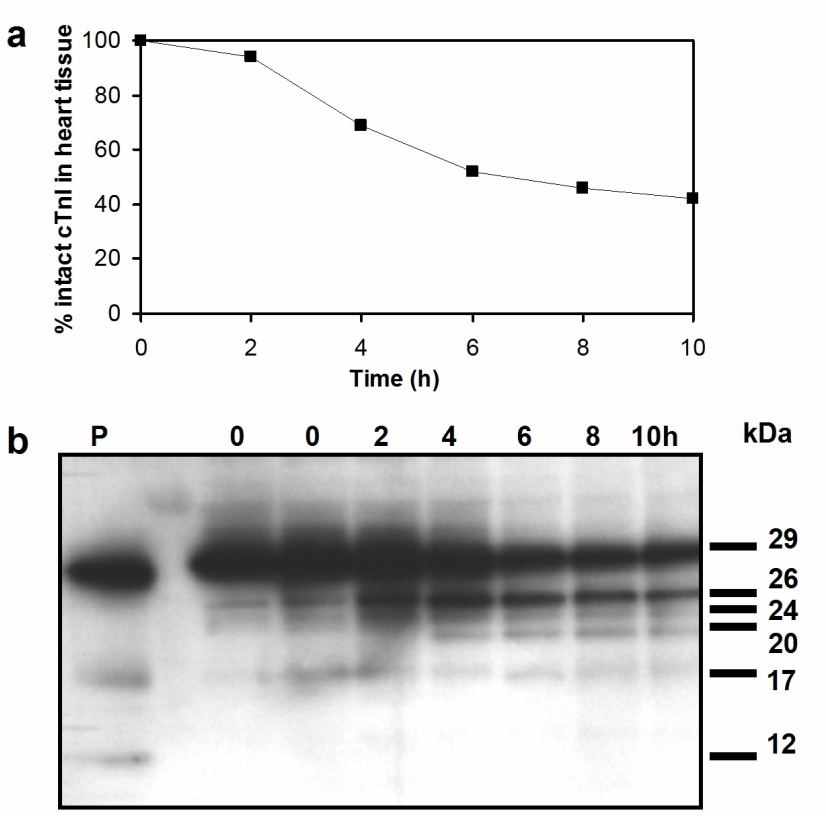

Figure 1. (a) Degradation of intact cTnI in neonatal rat heart tissue undergoing autolysis for 0-10 h at 37°C, detected by ELISA. The mean cTnI content at t=0 (100%) was 0.72 µg/mg tissue. **(b)** Formation of cTnI degradation products in neonatal rat heart tissue undergoing autolysis for 0-10 h at 37°C, detected by Western blotting. Purified human heart cTnI was used as a positive control (P).

LDH release during metabolic inhibition

The mean LDH activity of 10 cultures at t=0 was 2.00 ± 0.43 U per culture. When incubated in HBSS, cardiomyocytes released ≈ 15% of total LDH in 30 h (Fig. 2a), which confirms previous findings of cell death of cultured cardiomyocytes in serum-free medium[29]. At times 0, 6 and 12 h, release of LDH from azide-treated cardiomyocytes equalled the LDH release of control cardiomyocytes, indicating no cardiomyocyte necrosis in the first 12 h. However, from 12 to 30 h, a prominent LDH release from azide-treated cardiomyocytes (up to 90% of total LDH) was demonstrated, indicating that necrotic cell death started after 12 h and was nearly complete at 30 h (Fig. 2a).

Release of cTnI during metabolic inhibition

Intact cTnI assessed by ELISA. The mean content of intact cTnI in 10 cultures at t=0 was 1.2 ± 0.7 µg per culture. Control cardiomyocytes had released ≈ 9% of total cTnI after 30 h, which is most likely the result of cell death (≈15%) when incubated for 30 h in serum-free medium. The release of intact cTnI from azide-treated cardiomyocytes equalled that of control cardiomyocytes at time 0, 6 and 12 h, not exceeding 3%. At 18 h of azide treatment, cTnI release started to exceed control values (Fig. 2b). At 30 h, cTnI release had increased to 51±16%, indicating a less abundant release of intact cTnI compared with the release of LDH. Secondly, during 12-30 h of azide treatment, the total amount of intact cTnI per culture was significantly reduced compared to that at t=0 (100%) (Table).

cTnI degradation products assessed by western blotting. To investigate whether a less abundant release of intact cTnI compared with that of LDH can be explained by the early formation of undetected cTnI degradation products, cTnI degradation products from control and azide-treated cardiomyocytes were studied by Western blotting (Fig. 3a). In the first 12 h, only a relatively low quantity of intact cTnI was released that is consistent with the ELISA results. This intact cTnI is probably derived from the small proportion of necrotic cells (6-7%) in serum-free medium, as this band is also observed in medium of control cardiomyocytes (Fig. 3a).

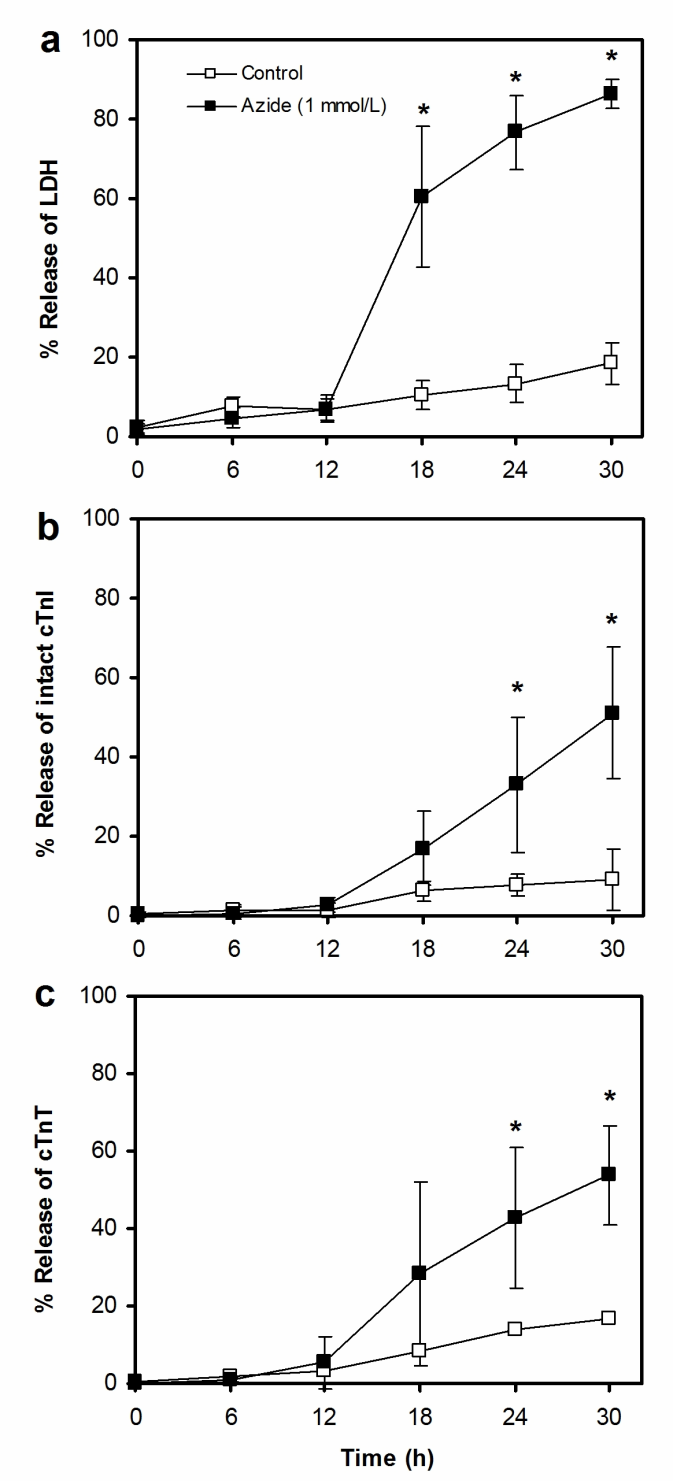

Figure 2. (a) Release of LDH (n≥12), **(b)** release of intact cTnI (n≥9), and **(c)** release of cTnT (n=3) from control and azide-treated (1 mmol/L) cardiomyocytes between 0 and 30 h. At t=0 (100%), the mean content of LDH, cTnI and cTnT was 2.00±0.34 U/culture, 1.2±0.7 µg/culture, and 0.23±0.08 µg/culture, respectively. Control cardiomyocytes are indicated by □ and azide-treated cardiomyocytes by ■.* $p<0.05$ versus control.

Table. Total LDH, intact cTnI and cTnT per cardiomyocyte culture

Time (h)	Total LDH (n≥12)	Total cTnI (n≥9)	Total cTnT (n=3)
0	100	100	100
6	95 ± 24	92 ± 21	105 ± 19
12	96 ± 17	74 ± 12*	70 ± 6*
18	97 ± 22	40 ± 26*	45 ± 19*
24	86 ± 17*	34 ± 21*	39 ± 25*
30	89 ± 19*	21 ± 7*	39 ± 28*

Total LDH, intact cTnI and cTnT per cardiomyocyte culture (cells+medium) treated with azide determined at t=0, 6, 12, 18, 24 and 30 h and expressed as percent-tage of activity or concentration present at t=0. * $p<0.05$ versus t=0

At 18 h, the intensity of intact cTnI (29 kDa) released from azide-treated cardiomyocytes had increased progressively and was accompanied by release of a 26 kDa band, indicating cTnI degradation (Fig. 3a). Enhanced degradation of cTnI was demonstrated at 24 and 30 h. In total four degradation products of 26, 20, 17 and 12 kDa were detected in medium of azide-treated cardiomyocytes, and only after extensive cell death. In cell extracts of azide-treated cardiomyocytes, no degradation products of cTnI were detected during metabolic inhibition (data not shown).

Release of cTnT during metabolic inhibition

cTnT assessed by immunoassay. The mean content of cTnT in five cultures at t=0 was 0.23 ± 0.08 µg per culture. Control cardiomyocytes had released ≈ 16% of cTnT after 30 h in serum free-medium. The release of cTnT from azide-treated cardiomyocytes equalled that of control cardiomyocytes at 0, 6, and 12 h, not exceeding 5%. At 18 h of azide treatment, cTnT release exceeded control values (Fig. 2c). At 30 h, cTnT release had increased to 54±13%, which is comparable with the release of cTnI and less abundant compared to that of LDH. During 12-30 h of azide treatment also the total amount of cTnT per culture was significantly reduced compared with that at t=0 (100%)(Table).

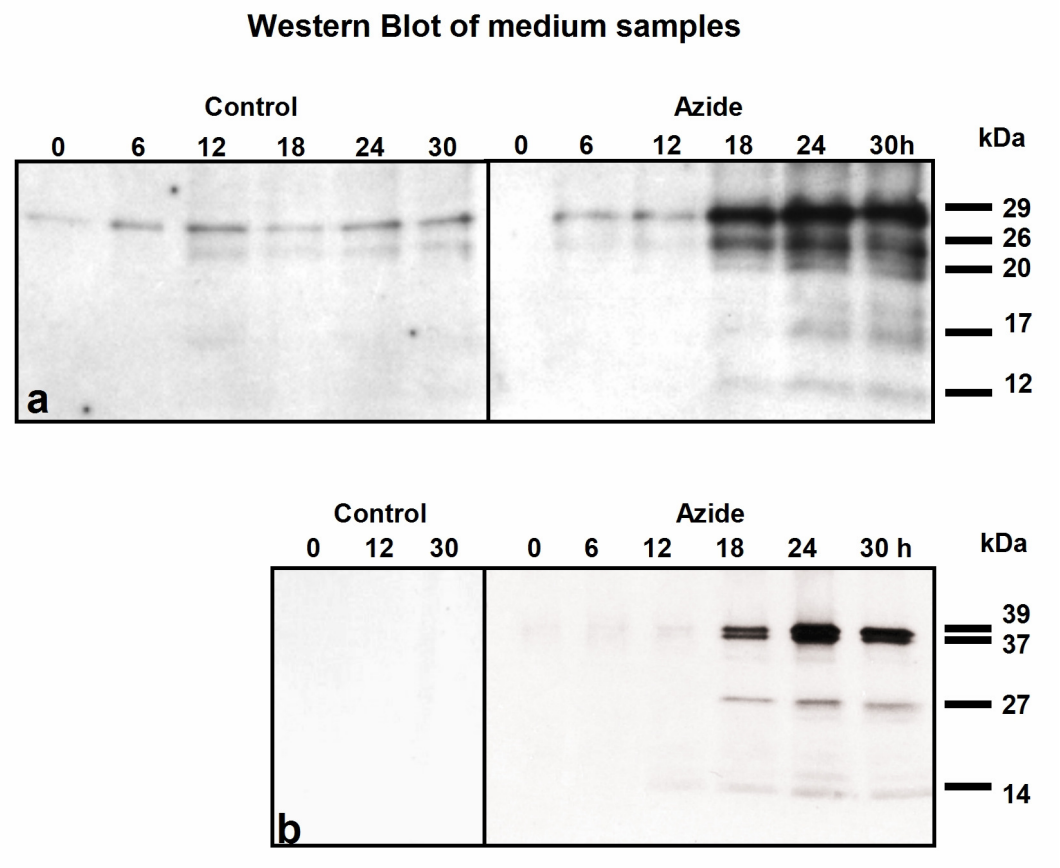

Figure 3. (a) Western blot of intact cTnI (29 kDa) and its degradation products in medium of control (LEFT) and azide-treated cardio-myocytes (RIGHT). **(b)** Western blot of intact cTnT (39 kDa) and its degradation products in medium of control (LEFT) and azide-treated cardiomyocytes (RIGHT).

cTnT degradation products assessed by western blotting.

In medium of control cardiomyocytes no intact or degraded cTnT was detected between 0 and 30 h (Fig. 3b). In medium of azide-treated cardiomyocytes, no intact or degraded cTnT was detected in the first 12 h, but at 18 h and later, release of intact cTnT (39 kDa) was observed and the intensity of this band increased after 24 and 30 h (Fig. 3b). In total three cTnT degradation products of 37, 27, and 14 kDa were detected and only after extensive cell death. In cell extracts of azide-treated cardiomyocytes, no degradation products of cTnT were observed during metabolic inhibition (data not shown).

Discussion

The main findings of the present study are (1) release of cTnI or cTnT from energy-deprived cardiomyocytes does not occur before necrosis sets in, (2) cTnI and cTnT from necrotic cardiomyocytes are both released as intact protein and as degradation products, (3) the release kinetics of cTnI and cTnT from necrotic cardiomyocytes are comparable, and (4) the percentage release of troponins from necrotic cardiomyocytes remained lower than the percentage release of LDH.

Myocardial ischemia causes extensive proteolytic damage to the myocardium and the plasma concentrations of cardiac proteins are used as biochemical markers of cardiac injury. Proteins released upon cardiac necrosis include LDH[25], α-hydroxybutyrate dehydrogenase (αHBDH)[30], CK[31], the MB-isoenzyme of CK (CK-MB)[32], cTnI[2] and cTnT[1], and these biochemical markers differ in sensitivity and specificity and vary in release kinetics[33;34]. LDH, αHBDH and CK are predominantly cytosolic proteins and not cardiac specific. Both troponins are highly cardiac specific and are bound, via tropomyosin, to actin filaments of sarcomeres, whereas only a small proportion of cTnT (6-8%) and cTnI (3-8%) is found in the soluble cytoplasmic pool[10].

Several studies have reported that the release of cTnI and cTnT in plasma of patients with AMI starts later than the release of CK-MB[5-8], suggesting that troponins are not ideal early markers for ruling out AMI. Vice versa, other studies have suggested that troponin release may also occur from reversibly damaged cardiomyocytes before necrosis sets in[20-22]. Elevated troponin levels have been observed in patients without coronary syndromes who had normal CK-MB_{mass} or CK-$MB_{activity}$ levels[35;36], indicating that under particular conditions circulating troponins may be present in the absence of necrotic cardiomyocyte death.

The present study demonstrates that the first 12 h of mild metabolic inhibition did not cause necrosis. A sharp decline in cell viability was observed between 12 and 18 h and extensive cell death was demonstrated at 24 and 30 h. These results are consistent with a previous study of Chen *et al.*[23], who defined the first 6-12 h of azide treatment (1 mmol/L) as a reversible phase, because wash-off experiments revealed that cardiomyocytes could regain full viability if azide was removed within the first 12 h. The

reversible phase was followed by an irreversible phase (12-30 h) with progressive cell death.

Release of both intact and degraded cTnI or cTnT did not occur from reversibly damaged cardiomyocytes (0-12 h), but was associated with irreversible cell damage at t ≥ 18 h (Figs. 2 and 3). The release of intact cTnI and cTnT from necrotic cardiomyocytes was accompanied by the release of several degradation products (Fig. 3). Four degradation products of cTnI (26, 20, 17 and 12kDa) and three degradation products of cTnT (37, 27 and 14 kDa) were observed when cardiomyocytes became irreversibly damaged. These results are in contrast with results reported by Kositprapa *et al*.[37], who showed that metabolic inhibition of neonatal cardiomyocytes by azide treatment (1 mmol/L) caused degradation of cTnI in irreversibly damaged cardiomyocytes, whereas cTnT remained unaffected. The discrepancy between Kositprapa´s results and ours may be explained by the use of different cTnT antibodies. In the present study we used an immunoprecipitation method and western blotting with several cTnT antibodies that recognized specified epitopes throughout the cTnT molecule to detect as many degradation products as possible, whereas Kositprapa *et al*. detected cTnT with only one cTnT antibody[37].

In the present study, release of intact cTnI and cTnT from irreversibly damaged cardiomyocytes showed similar kinetics (Figs. 2b and 2c). Nevertheless, the release of LDH from cardiomyocytes that become necrotic is more pronounced than the release of both troponins during 12-30 h azide-treatment (Fig. 2). The quantity of LDH released at 18 h was ≈60%, whereas the quantity of intact cTnI and cTnT was 18-28%. After 30 h, a maximal LDH release of ≈90% was associated with a cTnI and cTnT release of 50-55%, and these results confirmed our previous study[9]. Although azide-treatment (1 mmol/L) is less severe than metabolic inhibition induced by cyanide (5-10 mmol/L), 3 h of cyanide treatment resulted in a maximal LDH release (80-90%)[9;38] that was also associated with a cTnI release of ≈ 50%[9].

The mean content of total cTnI at t=0 was 1.2 ± 0.7 µg per culture, whereas the mean content of cTnT at t=0 was 0.23 ± 0.08 µg per culture. This difference might be explained by immunoassay characteristics. The fourth-generation cTnT assay has been developed and optimized for measurement of human cTnT and utilizes anti-human cTnT

antibodies. Although rat cTnT is highly homologous to human cTnT, this might result in lower recoveries. In the cTnI ELISA, developed by our group[9], we used human anti-cTnI antibodies for the capture and detection of rat intact cTnI, but these antibodies demonstrated a comparable cross reactivity for rat cTnI as compared to human cTnI.

The cTnI ELISA that was used in the present study as well as in the previous study[9] only detects full length cTnI (211 a.a.r.). Thus any cTnI degradation product that does not contain the two antigenic sites (a.a.r. 40-50 and a.a.r 198-195) will not be detected. Metabolic inhibition of cardiomyocytes induces the release of several degradation products of both cTnI and cTnT (Fig. 3), which remained undetected by the immunoassays (Fig. 2). During 12-30 h of azide treatment, total quantity of both intact cTnI and cTnT per culture (cells+medium) was significantly reduced compared with the total quantity at t=0 (100%), whereas total LDH activity was hardly reduced during azide treatment (Table). These results indicate a loss of both intact cTnI and cTnT during metabolic inhibition. Degradation of intact troponins may be responsible for this reduction in total troponin per culture, as the immunoassay only detects intact troponin. However, troponin degradation products in medium of azide-treated cardiomyocytes detected by western blotting (Fig. 3) may partially explain this reduction but do not explain a reduction by 60-80% (Table). In addition, there is no evidence for intracellular degradation of intact troponins, as degradation products were not observed in cell extracts of azide-treated cardiomyocytes (data not shown). Therefore, these results suggest that also the antibodies used for western blotting may not be able to detect all troponin degradation products formed during metabolic inhibition.

Undetected quantities of total intact cTnI and cTnT during azide treatment may be the result of post-translational modifications of intact troponin. Post-translational modifications such as phosphorylation, oxidation or nitrosylation may result in an impaired binding capacity of the capture and/or detection antibody, leading to a reduced detection of intact troponin. The present study demonstrates that the detection of cardiac troponins depends on the formation of troponin degradation products and the immunoreactivity of the currently available antibodies for these degradation products. Released troponin degradation products may influence the result of the diagnostic assay. Whether the extent of irreversible cell damage after AMI is underestimated by the

presence of troponin degradation products remains to be studied. Therefore further research is needed to identify troponin degradation products *in vivo* and to develop methods to detect these fragments quantitatively.

Conclusions

Mild metabolic inhibition of neonatal rat cardiomyocytes induces parallel release of intact cTnI and cTnT and their degradation products, starting after onset of irreversible cardiomyocyte damage.

Limitations of the study

In this study release kinetics of intact and degraded cTnI and cTnT were assessed by immunoassays and western blotting using currently available antibodies. If troponin degradation products do not contain the epitope for specific antibodies, they remain undetected. These limitations even apply to commercially available plasma troponin assays to be used for diagnosis of AMI.

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