I did my B.Sc. degree in Pharmaceutical Sciences at Alexandria University, Egypt. In 2002, I was invited to work as an instructor in the Department of Pharmacology, Alexandria University where I completed my M.Sc. studies.

In the fall of 2006 I joined the Department of Pharmacology, University of Alberta as a PhD graduate student under the supervision of Dr. Richard Schulz. The Schulz lab studies ischemic and inflammatory heart diseases and investigates the role of specific molecules contributing to oxidative stress injury of the heart. Being in the first laboratory to discover that matrix metalloproteinase (MMP)-2 proteolyses specific proteins inside cardiomyocytes, my main project is to investigate further novel intracellular targets of MMP-2 in myocardial cell injury and/or death.

My paper in Circulation showed the unique role of MMP-2 in the degradation of titin, the largest known protein (3000-4000 kD) inside the heart responsible for heart muscle contraction. This effect particularly explains the poor contractile function of the heart following ischemia and reperfusion injury and suggests that increased MMP-2 activity inside heart muscle may be a fundamental problem in ischemic heart disease. Accordingly, the results from this research could form the basis for the development of novel therapies (e.g. specifically targeted MMP-2 inhibitors) which may ultimately prevent or reduce ischemic heart disease including reperfusion injury.

Finally I would like to acknowledge all the co-authors in this paper and members of the Schulz lab and Granzier lab, University of Arizona. I would also like to thank my supervisor, Dr. Richard Schulz, for the continuous positive mentoring I have received during my program.
Titin is a Target of Matrix Metalloproteinase-2: Implications in Myocardial Ischemia/Reperfusion Injury


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Molecular Cardiology

Titin is a Target of Matrix Metalloproteinase-2
Implications in Myocardial Ischemia/Reperfusion Injury

Mohammad A.M. Ali, MSc; Woo Jung Cho, MSc; Bryan Hudson, PhD; Zamaneh Kassiri, PhD; Henk Granzier, PhD; Richard Schulz, PhD

Background—Titin is the largest mammalian (~3000 to 4000 kDa) and myofilament protein that acts as a molecular spring in the cardiac sarcomere and determines systolic and diastolic function. Loss of titin in ischemic hearts has been reported, but the mechanism of titin degradation is not well understood. Matrix metalloproteinase-2 (MMP-2) is localized to the cardiac sarcomere and, on activation in ischemia/reperfusion injury, proteolyzes specific myofilament proteins. Here we determine whether titin is an intracellular substrate for MMP-2 and if its degradation during ischemia/reperfusion contributes to cardiac contractile dysfunction.

Methods and Results—Immunohistochemistry and confocal microscopy in rat and human hearts showed discrete colocalization between MMP-2 and titin in the Z-disk region of titin and that MMP-2 is localized mainly to titin near the Z disk of the cardiac sarcomere. Both purified titin and titin in skinned cardiomyocytes were proteolyzed when incubated with MMP-2 in a concentration-dependent manner, and this was prevented by MMP inhibitors. Isolated rat hearts subjected to ischemia/reperfusion injury showed cleavage of titin in ventricular extracts by gel electrophoresis, which was confirmed by reduced titin immunostaining in tissue sections. Inhibition of MMP activity with ONO-4817 prevented ischemia/reperfusion-induced titin degradation and improved the recovery of myocardial contractile function. Titin degradation was also reduced in hearts from MMP-2 knockout mice subjected to ischemia/reperfusion in vivo compared with wild-type controls.

Conclusion—MMP-2 localizes to titin at the Z-disk region of the cardiac sarcomere and contributes to titin degradation in myocardial ischemia/reperfusion injury. (Circulation. 2010;122:2039-2047.)

Key Words: contractile dysfunction ▪ ischemia ▪ matrix metalloproteinase-2 ▪ sarcomere ▪ titin

Matrix metalloproteinase-2 (MMP-2) is a zinc-dependent protease that is best known for its ability to degrade the extracellular matrix in both physiological and pathological conditions. MMP-2 is synthesized as a zymogen by a variety of cells, including cardiac myocytes, and is activated either by proteases1 (such as by action of MMP-14) or by posttranslational modifications to the full-length enzyme caused by enhanced oxidative stress. For example, peroxynitrite, which is generated in early reperfusion after ischemia,2 directly activates several MMPs,3 including MMP-2,4 via a nonproteolytic mechanism involving the S-glutathiolation of a critical propeptide cysteine in its autoinhibitory domain.

MMPs are best recognized for their role in tissue remodeling by proteolyzing various components of the extracellular matrix in both health and disease, ie, in angiogenesis, embryogenesis, wound healing,5 atherosclerosis,6 aortic aneurysm,7 and myocardial infarction.8 More recent studies, however, show that MMP-2 is involved in several acute biological processes independently of its actions on extracellular matrix proteins. This includes platelet activation,9 regulation of vascular tone,10 and myocardial stunning injury immediately after reperfusion of the ischemic heart.11 Indeed, several reports indicate that MMP-2 does not exclusively degrade extracellular matrix components.12,13

In normal cardiac myocytes, MMP-2 is found in discrete subcellular compartments, including the thin and thick myofilaments of the cardiac sarcomere,14,15 cytoskeleton,16,17 nuclei,18 mitochondria,14 and caveolae19 (see Schulz20). MMP-2 is activated in rat hearts subjected to myocardial oxidative stress injury and is responsible for the degradation of specific sarcomeric and cytoskeletal proteins, including troponin I,14,21 myosin light chain-1,15 and α-actinin.17 Inhibition of MMP activity reduced both the loss of contractile...
function and the degradation of these substrates, to which MMP-2 was colocalized. Furthermore, transgenic mice with myocardium-specific expression of a mutant, constitutively active MMP-2, in the absence of additional injury, show significantly impaired cardiac contractile function, disrupted sarcomeres, profound myofilament lysis, breakdown of Z-band registration, and reduced troponin I level.22

Titin, the largest known mammalian protein (3000 to 4000 kDa), forms an intrasarcomeric elastic filament that is thought to serve as a framework for the ordered assembly of other myofilament proteins.23 In the sarcomere, the titin molecule spans the distance from the Z-disk to the M-line region (half the length of the sarcomere). Moreover, the I-band region of titin comprises distinct elastic segments that allow titin to behave as a molecular spring, contributing to the passive tension of myofibrils and maintaining the structural and functional stability of the sarcomere. Titin is an important determinant of both systolic and diastolic function and the Frank-Starling mechanism of the heart.24 Although loss and/or disorganization of titin in ischemic and failing human hearts has been reported,25,26 the mechanism of titin degradation has not been extensively studied in hearts subjected to ischemia/reperfusion (I/R) injury. Because MMP-2 is localized to sarcomeric and cytoskeletal proteins and is activated in myocardial I/R injury, we address here whether MMP-2 targets titin to contribute to the pathogenesis of myocardial I/R injury.

Methods

Titin Isolation and Purification
Titin was prepared as described previously.27,28 See the online-only Data Supplement.

Skinned Cardiomyocyte Isolation
Skinned cardiomyocytes were isolated as described in the online-only Data Supplement.

Cleavage of Native Titin in Skinned Cardiomyocytes
Skinned cardiomyocytes were incubated with human recombinant MMP-2 catalytic domain (4 to 120 nmol/L; Enzo Life Sciences, Plymouth Meeting, Pa) with or without MMP inhibitors (10 μmol/L o-phenanthroline or ONO-4817) at 37°C for 60 minutes. This concentration of o-phenanthroline inhibits MMP-2 activity under similar in vitro conditions.29 The samples were denatured with 2 mol/L urea sample buffer (8 mol/L urea, 2 mol/L thiourea, 3% SDS, 75 mmol/L DTT, 0.03% bromophenol blue, and 0.05 mol/L Tris-HCl, pH 6.8) at 60°C for 10 minutes, and the proteins were electrophoresed by 1% SDS-agarose and stained with Coomassie brilliant blue.

Isolated Working Rat Heart: Ex Vivo Model of I/R
Male Sprague-Dawley rats (300 to 350 g) were anesthetized with sodium pentobarbital (60 mg/kg IP). Hearts were isolated and paced at 300 bpm during perfusion at 37°C as working hearts30 with 100 mL recirculating Krebs–Henseleit solution containing 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L KH₂PO₄, 1.2 mmol/L MgSO₄, 25 mmol/L NaHCO₃, 11 mmol/L glucose, 100 μU/mL insulin, 2.5 mmol/L Ca²⁺, 0.5 mmol/L EDTA, and 0.1% BSA continuously gassed with 95% O₂/5% CO₂ (pH 7.4). The perfusate enters the left atrium at a hydrostatic preload pressure of 9.5 mm Hg, and the left ventricle ejects it against a hydrostatic afterload of 70 mm Hg. Cardiac work (cardiac output times peak systolic pressure) was used as an index of mechanical function. After 25 minutes of equilibration, hearts were either aerobically perfused for 85 minutes (control; n=6) or subjected to 25 minutes of global, no-flow ischemia followed by 60 minutes aerobic reperfusion without (I/R; n=7) or with 50 μmol/L ONO-4817 (I/R + ONO-4817; n=8). ONO-4817, a

Figure 1. Colocalization of MMP-2 and titin at the Z-disk region of the left ventricular cardiac sarcomere in rat hearts aerobically perfused for 10 minutes (longitudinal sections). MMP-2 shows better colocalization with T12 than the M8 epitope of titin in the sarcomere of the left ventricular myocardium. MMP-2-immunoreactivity reveals at Z lines with high density and M lines with low density. T12 epitope reveals at only Z lines, and M8 epitope reveals at only M-lines. A through C, High density of MMP-2 (green) colocalizes (yellow) with T12 epitope (red) at the Z lines. D through F, Low density of MMP-2 (red) colocalizes (yellow) with M8 epitope (green) at M lines. Scale bar=5 μm for all images except the enlarged portion of C illustrating the Z and M lines.
selective MMP inhibitor (K_i in the nanomolar range for MMP-2 and almost no inhibitory activity up to 100 μmol/L against several other proteases of different classes), was added to the perfusion buffer 10 minutes before the induction of ischemia. All hearts were perfused for a total of 110 minutes. At the end of perfusion, the ventricles were rapidly frozen in liquid nitrogen and processed for titin analysis in ventricular extracts as described below.

Additional series of hearts (control, n = 5; I/R, n = 5; and I/R + ONO-4817, n = 4) were perfused and processed for immunohistochemistry and confocal microscopy analysis for assessment of titin immunostaining. Another 6 hearts were briefly perfused for 10 minutes at 37°C with Krebs-Henseleit buffer at a constant hydrostatic pressure of 70 mm Hg to clear them of blood, followed by processing for immunohistochemistry as described below to investigate the colocalization of titin and MMP-2 in the left ventricle.

This investigation conforms to the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care.

In Vivo Model of I/R
I/R was induced in vivo by modifying a previously described protocol. Briefly, MMP-2 knockout and age-matched wild-type male C57BL6 mice were anesthetized with isoflurane, intubated, and kept on a heating pad to maintain body temperature at 37°C. The heart was exposed, and the left anterior descending coronary artery was temporarily ligated with a 7-0 silk suture, with a piece of 4-0 silk placed between the left anterior descending coronary artery and the 7-0 silk. After 30 minutes of left anterior descending coronary artery occlusion, reperfusion was initiated by releasing the ligature and removing the 4-0 silk. The loosened suture was left in place to help identify the ischemic area of the left ventricle. After 30 minutes of reperfusion, the hearts were excised, and the ischemic and nonischemic regions of the left ventricle were dissected out under a magnifying glass and flash frozen in liquid nitrogen for titin analysis.

Analysis of Titin by Gel Electrophoresis
Titin was analyzed in ventricular extracts using 1% vertical SDS–agarose gel electrophoresis as previously described. See the online-only Data Supplement for details.

Immunohistochemistry and Confocal Microscopy
Colocalization of Titin and MMP-2
Rat hearts perfused aerobically for 10 minutes to flush them of blood, followed by static pressure of 70 mm Hg to clear them of blood, followed by processing for immunohistochemistry as described above to investigate the colocalization of titin and MMP-2 in the left ventricle.

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I-band and A-band titin regions, including near the Z-line terminus of titin. These sites show >60% homology to the 3 MMP-2 cleavage motifs (Figure II in the online-only Data Supplement). Moreover, human recombinant MMP-2 was able to bind to titin prepared from skinned muscle fibers as shown by the overlay assay method (Figure III in the online-only Data Supplement). Next we tested the susceptibility of purified titin to proteolytic degradation by MMP-2 in vitro. Incubation of titin with MMP-2 (60 minutes at 37°C) at increasing molar ratios of MMP-2 to titin (1:500, 1:50, and 1:5) caused titin degradation in a concentration-dependent manner (Figure IVA in the online-only Data Supplement). Inhibition of MMP-2 activity with GM-6001 or ONO-4817 prevented titin cleavage by MMP-2 (Figure IVB in the online-only Data Supplement). To determine whether MMP-2 directly cleaves cardiac titin in situ, we incubated skinned mouse cardiomyocytes with increasing concentrations of MMP-2 (60 minutes at 37°C). This resulted in concentration-dependent cleavage of cardiac titin (T1) as shown by the increased level of the degradation product of titin (T2) with increasing MMP-2 concentration (Figure 2A). Inhibition of MMP-2 activity with o-phenanthroline or ONO-4817 prevented titin cleavage by MMP-2 (Figure 2B).

**Effect of ONO-4817 on Functional Recovery of I/R Hearts**

Isolated working rat hearts were perfused for 110 minutes under 1 of 3 conditions: aerobic perfusion (control); 25 minutes of global, no-flow ischemia and 60 minutes of aerobic reperfusion (I/R); or I/R in the presence of a selective MMP inhibitor, ONO-4817 (I/R + ONO-4817; Figure 3A). Control hearts showed no significant loss of mechanical function over 110 minutes of aerobic perfusion (Figure 3). I/R hearts showed markedly reduced recovery of mechanical function during reperfusion compared with control hearts (Figure 3B). The recovery of cardiac work during reperfusion was significantly improved after MMP inhibition with ONO-4817, compared with the I/R group (Figure 3B).

**Myocardial I/R Causes Titin Cleavage, an Effect Diminished by an MMP Inhibitor**

To investigate whether MMP-2 can cleave titin in the intact heart under pathophysiological conditions, titin content was assessed with 1% vertical SDS–agarose gels in ventricular extracts prepared from the control, I/R, or I/R + ONO-4817 hearts. Ventricular extracts from control hearts revealed a titin band at ~3000 kDa (Figure 4A). I/R caused a significant increase in T2 band density, an effect abolished in the I/R + ONO-4817 hearts (Figure 4A). Quantification of the ratio of total titin to myosin heavy chain (MHC) showed that I/R did not significantly change this ratio compared with control hearts (Figure 4B), whereas the ratio of T2 to MHC was significantly increased in I/R hearts. ONO-4817 abolished the I/R-induced increase in the T2/MHC ratio (Figure 4C). These observations were further confirmed by immunohistochemistry experiments using the anti–titin 9D10 antibody, raised against the proline-glutamate-valine-lysine (PEVK) domain in the spring region of titin. Titin immuno-
staining was significantly reduced by I/R, whereas ONO-4817 treatment preserved titin immunostaining to a level comparable to control (Figure 4D).

Titin Degradation Is Reduced in Hearts From MMP-2 Knockout Mice Subjected to I/R Injury In Vivo

We next determined whether genetic ablation of MMP-2 could influence titin degradation in cardiac muscle. Mouse hearts subjected in vivo to left anterior descending coronary artery ligation for 30 minutes followed by 30 minutes of reperfusion exhibited titin degradation, which was significantly less in MMP-2 knockout hearts than in wild-type control hearts (Figure 5).

MMP-2 Localizes Near the Z-Disk Region of Titin in the Human Heart

Immunostaining of sections prepared from the left ventricle of an explanted heart from a patient undergoing heart transplantation showed colocalization of MMP-2 and titin mainly near the Z disk, with a weaker colocalization at the M line. Compared with the rat heart, MMP-2 immunostaining in the human heart was more diffuse yet still showed a sarcomeric staining pattern (Figure 6).

Discussion

In this study, we demonstrated that titin, the giant sarcomeric protein, is a target of the proteolytic activity of MMP-2 in the setting of acute myocardial I/R injury. Immunohistochemical analysis shows that MMP-2 clearly colocalizes with titin near the Z-disk region of the sarcomere in both rat and human hearts. We established that under in vitro conditions MMP-2 is able to bind to and cleave titin in a concentration-dependent manner. The proteolytic action of MMP-2 is blocked by the selective MMP inhibitors GM-6001 and ONO-4817, verifying that the cleavage is indeed due to MMP activity. ONO-4817 not only improves the functional recovery after I/R in isolated rat hearts but also prevents the significant increase in the titin degradation product T2 caused by I/R injury, indicating that titin degradation is reduced when MMP activity is
intrinsic localization and functions of MMP-2 to various cardiac pathologies. However, the discovery of the degradation and remodeling of extracellular matrix proteins were not identified.

Cardiomyocytes caused a time- and concentration-dependent contractile dysfunction that was abrogated with MMP inhibitors. In vitro, peroxynitrite was shown to directly inhibit MMP-2 knockout mice subjected to in vivo I/R injury show less titin degradation compared with wild-type controls. Titin proteolysis has been observed in various human heart diseases associated with increased myocardial oxidative stress, including dilated cardiomyopathy, the terminally failing heart, and Chagas cardiomyopathy; however, the proteases responsible for this were not identified.

MMPs are best known as proteases responsible for the degradation and remodeling of extracellular matrix proteins in both physiological and pathological conditions, including various cardiac pathologies. However, the discovery of the intracellular localization and functions of MMP-2 to proteolyze tropinin I, myosin light chain-1, and α-actinin during myocardial oxidative stress injury challenged the canonical notion of extracellular-only actions of this enzyme. In previous studies, we showed that peroxynitrite biosynthesis in I/R rat hearts peaks within the first minute of reperfusion and that the peak in MMP-2 activity follows at 2 to 5 minutes of reperfusion. Infusion of peroxynitrite into isolated perfused rat hearts or isolated cardiomyocytes caused a time- and concentration-dependent contractile dysfunction that was abrogated with MMP inhibitors. In vitro, peroxynitrite was shown to directly activate MMP-2 via a nonproteolytic mechanism involving S-glutathiolation of the propeptide cysteine sulfydryl group. Indeed, this intracellular activity of MMP-2 on I/R injury caused proteolytic degradation of specific sarcomeric (tropinin I and myosin light chain-1) and cytoskeletal (α-actinin) proteins that are susceptible to its proteolytic activity.

MMP-2 is localized within the cardiac sarcomere, including near the Z disks. These previous observations are supplemented by the present data showing clear colocalization of MMP-2 near the Z-disk region of titin using the T12 clone in rat (Figure 1) and human (Figure 6) hearts. Several studies show that titin interacts with α-actinin at the Z disk of the sarcomere and that this interaction plays a crucial role in Z-disk assembly and sarcomeric integrity. Interestingly, MMP-2 was found not only to colocalize with α-actinin in the Z disk of cardiac sarcomeres but also to degrade it after peroxynitrite infusion into isolated rat hearts. The M8 titin antibody (raised against the M-line region of titin) shows a weaker localization of MMP-2 to this region of titin. Although our data do not rule out the possible localization of MMP-2 to other region(s) of titin, they do suggest that a main MMP-2 anchoring site is near the Z disk of the sarcomere.

Titin is the third myofilament (in addition to thick and thin myofilaments) of the sarcomere that plays an important role in sarcomere integrity and cardiac muscle contraction. Any alterations in its structure could severely affect the contractile performance of the heart. The increase in T2 titin and the decrease in titin immunostaining after I/R injury observed here (Figure 4) were associated with poor cardiac mechanical recovery during reperfusion (Figure 3). These effects are likely due at least in part to titin degradation by MMP-2, given the colocalization of MMP-2 with titin near the Z disk of cardiac sarcomeres, the susceptibility of titin to degradation by MMP-2, and the reduction in I/R-induced titin degradation in hearts from MMP-2 knockout mice or in rat hearts in which MMP activity was selectively blocked with ONO-4817. A significant increase in MMP-2 activity was seen in the heart after experimental Trypanosoma cruzi infection (the parasite responsible for Chagas disease), and mortality was markedly reduced upon treatment with an MMP inhibitor, suggesting a role of MMP-2 in mediating acute Chagas cardiomyopathy. Putative titin degradation products were detected in the plasma of patients with Chagas disease, further supporting a role of MMP-2 in titin degradation. Moreover, myocardial infarction is associated with a significant right shift in the left ventricle pressure-volume relation (an observation consistent with titin degradation in the heart), and the broad-spectrum MMP inhibitor PD-166793 was shown to protect against this shift. Although cardiac mechanical function at the end of perfusion is inversely related to ratios of T2 to MHC in hearts (Figures 3B and 4C), caution is needed in relating this effect exclusively to titin degradation. As mentioned, other sarcomeric/cytoskeletal proteins, including tropinin I, myosin light chain-1, and α-actinin, are also susceptible to degradation by MMP-2 under conditions of myocardial oxidative stress injury. However, our work clearly suggests that titin proteolysis is an important factor that negatively affects myocardial contractility on I/R injury.

Titin content in rat ventricles was investigated here by SDS–agarose gel electrophoresis or immunofluorescence staining against titin epitopes at the PEVK domain. Our electrophoresis results showed the ~60% elevation in the ratio of T2 to MHC in the I/R group compared with aerobic control hearts. Immunofluorescence data also showed a reduction of titin immunostaining in the I/R group using the
9D10 antibody. In addition to degradation, posttranslational modifications of titin may have occurred upon I/R that led to diminished binding of titin antibodies to the specific epitopes. Posttranslational modifications of many cardiac myofilament/cytoskeletal proteins during I/R, including actin and myosin light chain-1, have been reported in previous studies.

Our study does not rule out the possible action of other proteases in titin degradation. Calpains are most likely involved in sarcomeric protein degradation after ischemic episodes more severe than that observed in our model. Indeed, calpain was shown to be able to cleave titin only after 24 hours of doxorubicin treatment of rat cardiomyocytes. The ubiquitin-proteasome system is another proteolytic pathway that may be involved in titin degradation. Increased proteasome activities have been reported in various models of I/R injury. Moreover, the E3 ubiquitin-ligase MURF1 is known to be associated with the M-line region of titin and ubiquitates titin in yeast 2-hybrid screens. In a rat heart failure model, both a loss of titin and an increase in MMP-2 gene expression were observed in diaphragm muscle. However, in our short-term experiments, we did not observe a significant loss of intact titin on I/R injury. We speculate that MMP-2 activation not only results in titin cleavage but also may trigger a cascade of proteolytic events leading to titin loss several hours after reperfusion.

Conclusions
The present results indicate that MMP-2 cleaves titin during either ex vivo or in vivo I/R injury. Furthermore, MMP-2 inactivation by pharmacological or genetic approaches protects against titin degradation. Our previous findings of troponin I, myosin light chain-1, and α-actinin cleavage by MMP-2, in addition to our present results with titin, suggest that MMP-2 plays a crucial role in the pathogenesis of acute I/R injury at the level of the sarcomere and cytoskeleton. Whether MMP-2 causes contractile protein alterations in other cardiac pathologies needs further investigation. Pharmacological inhibition of MMP activity could represent a useful strategy for the prevention and/or treatment of myocardial I/R injury.

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Disclosures
None.

References

**CLINICAL PERSPECTIVE**
In addition to the well-known extracellular effects of matrix metalloproteinases (MMPs), we provide evidence that MMP-2 is localized inside the cardiac myocyte, near the Z-disk region of the sarcomere. We also show that upon acute ischemia/reperfusion injury, MMP-2 is activated and proteolyses titin, the largest known protein that plays a crucial role in both the diastolic and systolic function of the heart. Titin contains several cleavage motifs for MMP-2, and its proteolysis is reduced in hearts protected by pharmacological inhibition of MMP activity and in MMP-2–deficient hearts. This study provides new insights into the pathophysiological mechanism of ischemia/reperfusion injury and suggests that MMP inhibitors might be a useful strategy for reducing reperfusion injury.
SUPPLEMENTAL MATERIAL

Supplemental Methods

Titin isolation and purification

Titin was prepared as described\textsuperscript{1,2}. Briefly, myofibrils were prepared from chilled fresh rabbit longissimus dorsi muscle by homogenisation in 3 volumes of 50 mmol/L KCl, 5 mmol/L EGTA, 1 mmol/L NaHCO\textsubscript{3} and 5 \textmu mol/L E64 (Sigma); (pH 7.0, 4°C). This was followed by three cycles of centrifugation (2000 g) and resuspension in buffer without E64 inhibitor. After the fourth spin the myofibrils were resuspended and extracted on ice for 5 min with stirring in 2 volumes of 0.9 mol/L KCl, 2 mmol/L MgCl\textsubscript{2}, 10 mmol/L imidazole, 2 mmol/L EGTA, 1 mmol/L PMSF, 10 \mu g/ml trypsin inhibitor, 0.5 mmol/L dithiothreitol, 5 \textmu mol/L E64; pH 7.0. The extract was clarified at 20,000 g for 30 min, diluted three times with water (final ionic strength ~0.2 mol/L) and after 1 hr for precipitation, myosin was removed by spinning for 30 min at 20,000 g. The supernatant was diluted five times more (final ionic strength 0.05 mol/L), left for 40 min, and then spun at 11,000 g for 30 min. The crude titin pellet was resuspended in 0.6 mol/L KCl, 30 mmol/L potassium phosphate (pH 7.0), clarified for 30 min at 25,000 g, and then chromatographed in this buffer in a 90 cm × 1 cm Sepharose CL2B column maintained at 1°C. On occasion the titin at this stage was also passed through a Q-Sepharose column as described by Nave et al.\textsuperscript{3} The purification was monitored by SDS-polyacrylamide electrophoresis in gradient slab gels (4% to 15%). Samples for these were dissociated at 56°C for 20 min in SDS/urea, as described by Fritz et al.\textsuperscript{4}

Skinned cardiomyocyte preparation

Skinned cardiomyocytes were isolated as described previously\textsuperscript{5}. Briefly, mice were anesthesitized via isoflurane inhalation, sacrificed via cervical dislocation, and the heart was rapidly (< 90 seconds) cannulated via the aorta. The heart was then perfused for 4 min with perfusion buffer (113 NaCl, 4.7 KCl, 0.6 Na2HPO\textsubscript{4}, 1.2 MgSO\textsubscript{4}, 12 NaHCO\textsubscript{3}, 10 KHCO\textsubscript{3}, 10 HEPES, and 30 taurine, all in mmol/L) and then switched to digestion buffer (perfusion buffer plus 0.148 mg/ml Liberase blendzyme 2, 0.13 mg/ml of trypsin, 12.5 \mu mol/L CaCl\textsubscript{2}) for 8-10 min. When the heart was flaccid, digestion was halted and the heart was placed in myocyte stopping buffer 1 (perfusion buffer plus 0.04 ml bovine calf serum (BCS)/ml buffer and 5 \textmu mol/L CaCl\textsubscript{2}). The left ventricle was cut into small pieces, triturated several times with a
transfer pipet, filtered through a 300 µm nylon mesh filter and the filtered cells were gravity pelleted. We added 10 ml of myocyte stopping buffer 2 (perfusion buffer plus 0.05 ml BCS/ml buffer and 12.5 µmol/L CaCl₂) and then skinned the cells in 1X relaxing solution (N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonate (BES) 40 mmol/L, EGTA 10 mmol/L, MgCl₂ 6.56 mmol/L, ATP 5.88 mmol/L, dithiothreitol 1 mmol/L, K-propionate 46.35 mmol/L, creatine phosphate 15 mmol/L, pH 7.0) (chemicals from Sigma-Aldrich, MO, USA) with 1% Triton-X-100 (Pierce, IL, USA) for 6 min and then quickly washed in 1X relaxing solution without Triton-X-100. Finally, skinned cells were stored in 50% glycerol/50% relaxing solution at -20°C.

**Analysis of titin by gel electrophoresis**

Titin was analyzed in rat ventricular extracts at the end of the 110 min working heart perfusion protocol using 1% vertical SDS-agarose gel electrophoresis as previously described⁶. Briefly, the frozen ventricles were pulverized under liquid nitrogen and homogenized in urea sample buffer (8 mol/L urea, 2 mol/L thiourea, 3% SDS, 75 mmol/L DTT, 0.05 mol/L Tris–HCl, 0.03% bromophenol blue, 25% glycerol and 10 µmol/L leupeptin, 10 µmol/L E64 and 0.5 mmol/L phenylmethylsulfonylfluoride, pH adjusted to 6.8) (20:1 v/w buffer to tissue ratio). Samples were thoroughly vortexed and then heated at 60°C for 10 min. The samples were again vortexed and subsequently centrifuged (12,000g for 5 min) at 4°C. The supernatant was removed and stored at -80°C until use. The integrated optical density of T1 titin (full-length titin), T2 titin (degradation product) and myosin heavy chain (MHC) were determined as a function of the volume of the solubilized protein sample that was loaded (a range of volumes was loaded onto each gel). The slope of the linear range of the relation between integrated optical density and loaded volume was obtained for each protein. The total titin (T1+T2):MHC and titin’s degradation product T2:MHC ratios were calculated as the slope of titin (either T1 + T2 or T2 alone) divided by the slope of MHC.

**Immunohistochemistry and confocal microscopy**

**α- Co-localization of titin and MMP-2**

Rat hearts perfused aerobically for 10 min to flush them of blood or left ventricular tissue from the explanted heart of a heart transplant patient was fixed with 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4) and
cryoprotected with 30% sucrose in 0.1 mol/L sodium phosphate buffer. The cryoprotected hearts were cryosectioned into 6 µm thick sections which were attached to glass slides coated with poly-L-lysine (Cat. No. 63410, Electron Microscopy Sciences, Hatfield, PA, USA) and dried at room temperature. The dried cryosections were rinsed twice (5 min each) in 0.5% Triton-X 100 in phosphate buffered saline (PBS, pH 7.4) and rinsed once in PBS for 5 min. Double immunolabeling was accomplished by sequential staining of each primary antibody (mouse anti-MMP-2 IgG, 1:200 final dilution, Cat. No. 3308, Chemicon International; mouse anti-titin T12 IgG, 1:10 final dilution; rabbit anti-titin M8 IgG, 1:200 final dilution, both titin antibodies provided by Dr. Elisabeth Ehler, King’s College London, UK) for 16 hr. The T12 antibody labels titin near the Z disc region of titin and the M8 antibody recognizes an epitope at the M line region of titin. Therefore, T12 and M8 antibodies were used to address the proximity of MMP-2 localization to either the N-terminus or C-terminus regions of titin, respectively. Secondary antibodies conjugated with fluorescent dyes (Cy3-donkey anti-mouse IgG, 1:25 final dilution, Cat. No. 715-165-151, Jackson ImmunoResearch Laboratories; Alexa488-donkey anti-rabbit IgG, 1:50 final dilution, Cat. No. A-21206, Invitrogen) were applied for 2 hr. During incubation with any of the antibodies, 2% normal donkey serum was added. A solution of 10 µmol/L DRAQ5 (Biostatus Ltd., Leicestershire, UK) was applied to stain nuclei. To determine specificity of immunolabeling, primary or secondary antibodies were omitted.

The immunolabeled cryosections were observed by confocal microscopy (LSM 510, Carl Zeiss Co., Jena, Germany). Cy3 (red) was scanned with a helium/neon green laser (543 nm) with a band pass 565 - 615 nm filter (565 - 615 nm excitation). Alexa488 (green) was captured using an argon laser (488 nm) with band pass 500 - 530 filter (500 - 530 nm excitation). DRAQ5 (blue) for nuclei was obtained with a helium/neon red laser (633 nm) with a long pass 650 nm filter. All confocal images were exported as TIFF files without any modifications by LSM 510 Image.

b- Titin 9D10 immunostaining

At the end of the 110 min working heart perfusion protocol some Control, I/R or I/R + ONO-4817 hearts were fixed and cryoprotected as described above. The cryoprotected hearts were cryosectioned and dried at room temperature. The dried cryosections were rinsed and titin immunolabeling was accomplished using 9D10 antibody (mouse anti-titin 9D10
IgM, 1:100, developed by Dr. Marion Greaser and available at the Development Studies Hybridoma Bank at the University of Iowa). 9D10 antibody is raised against the proline-glutamate-valine-lysine (PEVK) domain in the spring region of titin and is used to measure titin immunostaining in Control, I/R and I/R + ONO-4817 groups. Secondary antibody conjugated with fluorescent dyes (Alexa488-goat anti-mouse IgM, 1:40, Cat. No. 20142, Invitrogen) were applied. DRAQ5 was applied to stain nuclei. To determine specificity of immunolabeling, primary or secondary antibodies were omitted. The immunolabeled cryosections were observed by confocal microscopy for Alexa488 and DRAQ5 as above.

Three dimensional surface rendered image construction

Z-stack images obtained from the LSM 510 were reconstructed to three dimensional images and surface rendered using the Inside 4D module of AxioVison software (Version 4.6, Carl Zeiss Co., Jena, Germany). The same configuration of the Inside 4D module was applied to all images.

In silico analysis

According to Turk et al.\textsuperscript{7} and Chen et al.\textsuperscript{8} three MMP-2 cleavage motifs were chosen. These are PVS↓LRS, PVG↓LLA and L/ISR↓LTA with MMP-2 cleavage site indicated by the arrow. These consensus sequences were shown to be optimal MMP-2 cleavage motifs and, importantly, they showed high selectivity towards MMP-2 in comparison to other MMPs including MMP-1, MMP-3, MMP-7, MMP-9 and MMP-14\textsuperscript{7}. These consensus sequences were aligned vs. N2B mouse and human titin and the result was restricted to the top 20 with more than 60% homology using the SIM Alignment tool for protein sequence.
Overlay assay to determine MMP-2 binding to titin

Skinned muscle fibers, stored in relaxing solution (40 mmol/L BES pH 7.0, 10 mmol/L EGTA, 6.56 mmol/L MgCl₂, 5.88 mmol/L ATP, 1 mmol/L DTT, 46.35 mmol/L potassium propionate and 15 mmol/L creatine phosphate) for 24 hours post-dissection, were incubated with 0.75 µg/ml of trypsin from bovine pancreas (Sigma, T9201) in relaxing solution for 10 min at room temperature. The fibers were solubilized with a glass pestle, in solubilization buffer (750 ul of 8 mol/L urea, 2 mol/L thiourea, 3% SDS, 50 mmol/L Tris-HCL pH 6.8, 0.03% bromphenol blue, 250 ul of 50% glycerol with leupeptin, E-64, and PMSF, and 23 mg of DTT), heated for 5 min at 65°C and centrifuged 11,000 g for 10 min to remove the particulate fraction. The proteins of the samples were separated by electrophoresis on 1% agarose gel SDS-PAGE and run with Fairbank’s buffer (50 mmol/L Trizma base pH 7.5, 384 mmol/L glycine, 0.1% SDS, 10 mmol/L 2-mercaptoethanol) for 3 h and 20 min at 15 mA. Then the proteins were transferred to a PDVF (Millipore) membrane, 2 h 30 min at 1.33 mA/cm², and stained with Ponceau S to visualize the protein bands.

The overlay assay was performed using the transferred membrane as described previously followed by western blot. Briefly, membranes were blocked in blocking buffer (Odyssey, 927-40000) for 1 h followed by a 4 h incubation with recombinant MMP-2 (active human; EMD, PF023), 10 nmol/L in 10 ml blocking buffer (1.3 µl/20 ml). Both steps were performed at room temperature on a gentle rocker. Following incubation, the blot was washed 3 X 5 min with 10 ml of blocking buffer and then incubated with primary antibody (MMP-2 (1:250), Millipore; MAB3308, mouse monoclonal, 2 mg/ml). Following overnight incubation with the primary antibody in blocking buffer (0.05% Tween-20) membranes were washed with PBST (0.1% Tween-20), subjected to secondary antibodies labeled with IR dyes (Goat anti mouse 1:20,000; 800 green) for 1 h, and washed again with PBST. Membranes were scanned using a Li-Cor Odyssey infrared imager at 700 nm and 800 nm.

In vitro degradation of titin

Two micrograms of rabbit longissimus dorsi titin were incubated with human recombinant 64 kDa MMP-2 (Calbiochem, 500:1, 50:1 and 5:1, titin:MMP-2 molar ratios) in 50 mmol/L Tris-HCl buffer (5 mmol/L CaCl₂ and 150 mmol/L NaCl) at 37°C for 60 min. In additional experiments, MMP-2 was preincubated with either of the MMP
inhibitors GM-6001 (100 nmol/L) or ONO-4817 (10 µmol/L) for 15 min at 37°C before adding to titin. This concentration of GM-6001 was previously shown to inhibit the exogenously added MMP-2 activity under similar in vitro conditions\textsuperscript{7,10}. The reaction mixtures were denatured with 2X urea sample buffer (8 mol/L urea, 2 mol/L thiourea, 3% SDS, 75 mmol/L dithiothreitol, 0.03% bromophenol blue, and 0.05 mol/L Tris-HCl; pH 6.8) at 100°C for 3 min, and the proteins were separated by 2% SDS-PAGE strengthened with 0.5% agarose\textsuperscript{11}. Protein bands were visualized with silver stain (Invitrogen kit).
Fig. S1: A, Schematic representation of titin (N2B isoform) showing T12 and M8 epitopes near the Z-disc and at the M-line regions, respectively. B, Three dimensional rendered images of T12 and M8 epitopes of titin in sarcomeres of the left ventricular myocardium in aerobic normal control. a-c show frontal views of distributions of T12 (red) and M8 (green). d-f show clipping views of distributions of T12 (red) and M8 (green). Nuclei are blue in all images.
**Fig. S2:** Possible MMP-2 cleavage sites within mouse and human N2B titin. A, Titin schematic indicating putative MMP-2 cleavage sites (*). Possible cleavage sites are located in both the I-band including near the Z-disc and the A-band of titin. Panel B lists the putative MMP-2 cleavage sites in mouse and human titin shown in A; data is based from three consensus cleavage motifs: PVS↓LRS, PVG↓LLA, and L/ISR↓LTA. Number correlates with the initial amino acid that is in parenthesis listed in the left column.
**Fig. S3:** A, Coomassie blue stain following electrophoretic separation of proteins from skinned muscle fibers treated for 10 min with trypsin, in order to increase T1 degradation to T2, and used for overlay/WB analysis seen in B. B, Overlay assay showing MMP-2 binding to both T1 and T2. C, Quantification of the overlay/WB analysis T1. p < 0.001 (One-way ANOVA, n= 3), MHC (myosin heavy chain).
**Supplementary Fig. 4**

**Fig. S4:** In vitro degradation (60 min, 37°C) of rabbit longissimus dorsi titin by MMP-2. A, MMP-2 cleaved titin in a concentration-dependent manner (1:500, 1:50 and 1:5 MMP-2:titin molar ratios). B, The cleavage of titin by MMP-2 was prevented by inhibiting the activity of MMP-2 with GM-6001 (100 nmol/L) or ONO-4817 (10 μmol/L).
Supplemental References


Molecular Giant Vulnerable to Oxidative Damage: Titin Joins the Club of Proteins Degraded by Matrix Metalloproteinase-2
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Molecular Giant Vulnerable to Oxidative Damage
Titin Joins the Club of Proteins Degraded by Matrix Metalloproteinase-2

Wolfgang A. Linke, PhD

Matrix metalloproteinases (MMPs) are a family of >20 enzymes that have long puzzled scientists for their diversity in tissue location and function. True to their name, MMPs are best known for their role as degradation enzymes cleaving multiple components of the extracellular matrix, including collagen, laminin, elastin, and fibronectin. However, the traditional view that MMPs are proteases whose only function is to target matrix proteins does not hold up anymore. A large body of evidence suggests that the proteolysis of extracellular matrix scaffold proteins causes release of growth factors and other bioactive molecules, indicating that MMPs are involved in the regulation of cell and tissue function. Moreover, recent proteomic screens for MMP substrates have shown that MMPs regulate a host of signaling molecules, such as chemokines, both directly and indirectly. The novel functions of MMPs are also crucial to the cardiovascular system. MMP enzymes can regulate, for instance, the bioavailability of vascular endothelial growth factor and the potent vasodilator adrenomedullin. The diverse roles of MMPs are relevant not only for physiological processes such as heart development but also for pathological processes such as remodeling events resulting from myocardial infarction, hypertensive heart disease, or cardiomyopathy.

Titin Functions and Alterations in Human Heart Disease
Titin, the largest protein of the human body, has multiple important functions in the heart, providing both a scaffold for myofibril assembly and a hub for various molecules participating in myocyte signaling pathways. Perhaps best known is the role of titin as an elastic spring contributing to total myocardial passive (diastolic) stiffness. The titin springs located in the I-band segment of the sarcomere (Figure) are the predominant source of myocardial passive stiffness in the normal human heart, even exceeding the contribution to stiffness coming from the collagen fibers of the extracellular matrix. In end-stage failing hearts of patients with dilated cardiomyopathy, the contribution of titin to total myocardial passive stiffness decreases, whereas the contribution made by collagen increases. Moreover, cardiac titin is expressed in 2 main isoforms: the shorter and stiffer N2B isoform and the longer, more compliant, N2BA isoform (which, in fact, has multiple splice pathways). Importantly, the N2BA:N2B isoform ratio is increased in end-stage failing human hearts compared with donor hearts in both chronically ischemic hearts of patients with coronary artery disease and nonischemic dilated cardiomyopathy hearts. The titin-isof orm switch decreases passive myocyte stiffness, probably as a compensatory mechanism counteracting an increased total passive stiffness related to fibrosis. Dysregulation of titin stiffness in cardiac failure could severely impair various mechanical functions of the heart, including diastolic filling and early diastolic recoil, for which titin is held partially responsible, length-dependent activation as the molecular basis of the Frank-Starling mechanism, and contractile performance in systole, which is supported by the titin springs.

Other alterations to cardiac titin occur in heart disease, such as reduced levels of protein kinase A– or protein kinase G–mediated titin phosphorylation, observed in human dilated cardiomyopathy hearts. This titin phosphorylation deficit in end-stage failing hearts increases myocyte passive stiffness and contributes to impaired diastolic function. Furthermore, cardiac-specific regions in titin are vulnerable to intramolecular disulfide bonding, which could stiffen the titin springs under oxidative stress conditions. These recent findings complement earlier studies of ischemic and failing human hearts, which showed that titin is degraded and appears highly disorganized in the cardiomyocytes when analyzed by immunofluorescence microscopy. Taken together, evidence for titin alterations in human heart disease is manifold; derangement of titin in cardiac myocytes and reduced titin expression are obvious.

Proteases Targeting Cardiac Titin
Little has been known about specific proteases targeting cardiac titin in acute or chronic myocardial dysfunction. On the other hand, recent studies suggest that MMPs could be involved in the degradation of cardiac titin. For example, MMP-2 and MMP-9 have been found to degrade cardiac titin in vitro and in vivo, supporting the hypothesis that MMPs play a role in the proteolytic remodeling of the extracellular matrix in heart disease. Further research is needed to elucidate the mechanisms by which MMPs target cardiac titin and how these proteases contribute to the pathogenesis of heart failure.
hand, titin is notorious for rapid degradation ex vivo: Protease inhibitors are required to keep titin intact. A curious correlation has been found between the extent of titin-isoform transitions in chronic ischemic human heart disease and the degree of cardiac troponin-I degradation or complex formation. A candidate enzyme potentially degrading titin is the ubiquitous Ca\(^{2+}\)-dependent protease calpain-1, which has been implicated in the pathogenesis of myocardial dysfunction after I/R. Indeed, calpain-1 binds titin near the Z-disk and in vitro degrades various cytoskeletal and myofibrillar proteins isolated from human cardiac tissue, including titin. Titin is also proteolyzed in cultured rat cardiomyocytes after doxorubicin treatment to induce calpain-1.

Similar to calpain-1, MMP-2 targets a subset of structural and regulatory sarcomeric proteins, including titin (Figure). The important news is that MMP-2 is activated by oxidative stress after I/R and degrades titin both in an ex vivo working rat heart model of I/R and in an in vivo mouse model of I/R. However, it remains to be answered unambiguously whether the degradation of titin and other intracellular target proteins under oxidative stress in vivo is due mainly to the activity of MMP-2 or calpain-1 or both. The authors address this issue by showing reduced titin degradation in hearts from MMP-2 knock-out mice subjected to I/R in vivo, suggesting that MMP-2 really does the job, at least in part. Along the same line, titin cleavage induced by I/R damage was diminished by an MMP inhibitor. In summary, in light of the importance of titin for diastolic and systolic function, titin degradation by MMP-2 (and perhaps calpain-1) likely contributes to the acute contractile depression developing in stunned myocardium.
Activating MMP-2 During I/R Injury
The intracellular activation of MMP-2 as an effector of acute myocardial I/R injury in cardiomyocytes occurs via generation of peroxynitrite, a highly pro-oxidant species that increases to cardiotoxic levels within the first minutes of I/R. Peroxynitrite activates cytosolic MMP-2 from an inactive precursor state, and MMP-2 now executes its actions on the sarcomic proteins, troponin-I, myosin light chain-1, α-actinin, and titin (Figure). Ali et al propose that MMP-2 binding to titin occurs preferentially near the Z-disk, as suggested by colocalization of immunofluorescence signals for MMP-2 and Z-disk–adjacent titin antibodies. However, the MMP-2 binding site(s) on titin still need to be mapped precisely. Whether the binding sites on titin are also the sites of titin cleavage is another open question. From sequence analysis, the authors predict potential cleavage sites in various regions along the titin filament, including the Z-disk–flanking, I-band, and A-band segments (Figure). Providing experimental evidence in support of these predictions could be a challenging task, given the huge size (3000 to 4000 kDa) of the titin molecule.

Despite convincing evidence showing that MMP-2 degrades titin, it is not clear whether this cleavage, or even that of other sarcomeric proteins, is the principal cause of reduced cardiac work output during I/R injury. More likely, the contractile depression after oxidative stress has additional causes. MMP-2 by itself probably has biological actions in other subcellular compartments, such as caveolae, mitochondria, and the nucleus, as well as in the extracellular space (Figure). The nature of these actions is still largely unknown, but both acute and long-term effects on contractile performance triggered by I/R damage could originate here. Finally, MMP-2 has links to signaling molecules associated with the development of apoptosis or necrosis, and it has been discussed that higher levels of oxidative stress could preferentially induce these pathways, causing irreversible cell injury.

Conclusion
The article by Ali et al is important because it demonstrates for the first time an acute effect of a protease, MMP-2, on the stability of the giant elastic protein titin as a consequence of oxidative stress during I/R. The finding provides another piece of evidence for an intracellular role of MMP-2. The study also uncovers a previously unrecognized link between the 2 components principally responsible for myocardial passive stiffness: extracellular matrix proteins (“classically” degraded by MMPs) and titin filaments. Both components are remodeled in heart disease, with detrimental effects on cardiac performance. Inhibiting MMP-2 activity by pharmacological intervention could be a desirable cardioprotective strategy in I/R injury and possibly also in chronic heart failure.

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Disclosures
None.

References

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