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Citrullination of myofilament proteins in heart failure

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Aims Citrullination, the post-translational conversion of arginine to citrulline by the enzyme family of peptidylarginine deiminases (PADs), is associated with several diseases, and specific citrullinated proteins have been shown to alter function while others act as auto-antigens. In this study, we identified citrullinated proteins in human myocardial samples, from healthy and heart failure patients, and determined several potential functional consequences. Further we investigated PAD isoform cell-specific expression in the heart.

Methods and results A citrullination-targeted proteomic strategy using data-independent (SWATH) acquisition method was used to identify the modified cardiac proteins. Citrullinated-induced sarcomeric proteins were validated using two-dimensional gel electrophoresis and investigated using biochemical and functional assays. Myocardial PAD isoforms were confirmed by RT-PCR with PAD2 being the major isoform in myocytes. In total, 304 citrullinated sites were identified that map to 145 proteins among the three study groups: normal, ischaemia, and dilated cardiomyopathy. Citrullination of myosin (using HMM fragment) decreased its intrinsic ATPase activity and inhibited the acto-HMM-ATPase activity. Citrullinated TM resulted in stronger F-actin binding and inhibited the acto-HMM-ATPase activity. Citrullinated TnI did not alter the binding to F-actin or acto-HMM-ATPase activity. Overall, citrullination of sarcomeric proteins caused a decrease in Ca^{2+} sensitivity in skinned cardiomyocytes, with no change in maximal calcium-activated force or hill coefficient.

Conclusion Citrullination unique to the cardiac proteome was identified. Our data indicate important structural and functional alterations to the cardiac sarcomere and the contribution of protein citrullination to this process.

Keywords Myofilament • Citrullination • Peptidylarginine deiminases • Heart failure • Sarcomere

1. Introduction

Citrullination, the irreversible post-translational modification (PTM) involving the conversion of arginine to citrulline by the family of enzymes peptidylarginine deiminase (PAD), is associated with several diseases.[1–5] Citrullination appears to be a generalized process; autoantibodies targeting citrullinated proteins are relatively specific for rheumatoid arthritis (RA) and, although occasionally observed in other autoimmune conditions, are uncommonly observed in healthy individuals.[6] In our previous study, elevated levels of citrullination were found in the myocardium of RA patients.[7] PADs were detected in cardiomyocytes, resident inflammatory cells, endothelial cells and vascular smooth muscle cells.[7] However, the actual proteins that are citrullinated in myocardium are unknown as is whether (i) myocardial citrullinated proteins are immune targets for circulating autoantibodies, (ii) myocardial citrullinated proteins can themselves induce an autoimmune response, and (iii) citrullinated proteins directly mediate phenotypic modifications to cardiac structure or function. There is precedent for PTMs of myocardial proteins leading to changes in cardiac contractility and structure in heart failure (HF). Phosphorylation, oxidation, and acetylation of sarcomeric proteins cause morphologic changes to proteins that lead to decreased contractile performance

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and adverse cardiac remodelling with HF.[8] However, it is unknown whether citrullination of myocardial proteins plays a similar role.

Citrullination results in a small increase in molecular mass (+0.984 Da) but converts the positively charged guanidine group on an arginine residue into the neutrally charged ureido group on the citrulline amino acid. The loss of charge from an arginine to a citrulline can have dramatic consequences on protein structure,[9] proteolytic susceptibility,[10] protein–protein interactions,[11,12] and intracellular signalling.[12] Since citrullination can lead to profound changes in protein structure and function, it is not surprising that increased citrullination and the PAD enzymes are found in numerous chronic diseases.[13] Furthermore, the conversion of arginine to citrulline is catalyzed in a Ca^{2+}-dependent manner with relatively high intracellular concentration of calcium. Because the cytosolic and nucleoplasmic calcium concentrations are relatively low, PADs should be inactive under normal conditions. However, PADs become activated in injured and dying cells, when calcium concentrations increase because of the influx of calcium ions from the extracellular environment and release from intracellular calcium stores.[14] HF is a chronic maladaptive state in which complex transcriptional, proteomic, and morphological changes result in profound perturbations in intracellular Ca^{2+} cycling that drive the progressive deterioration in cardiac function.[15] Hallmark features of HF pathogenesis are mechanical dysfunction and Ca^{2+} handling dysfunction,[16–19] suggesting a potential for PAD activation.

There have been challenges in identification of citrullinated proteins and the modified amino acid residues.[20] Here for the first time, we use a mass spectrometry (MS) approach, SWATH, to define the citrullinated proteome. In this application, SWATH is a two-step process involving the development of a citrullination-specific peptide ion library that is used to subsequently compare the individual sample MS ion data allowing quantification of site-specific differences between control and ischaemic and dilated cardiomyopathy HF subjects. Citrullination of the high abundant sarcomeric proteins was further analysed using 1 and 2 DE analysis. In addition, we investigate the biochemical and physiological effects of citrullinated sarcomeric proteins, providing the initial evidence supporting the hypothesis that citrullination may play a role in the reduced contractility observed in HF.

2. Methods

An expanded Methods section can be found in the Supplementary material online.

2.1 Reagents and materials

The following reagents were obtained: rabbit skeletal muscle PAD2 (Sigma); PAD cocktail (SignalChem); PAD2, heavy meromyosin (HMM), tropomyosin (TM) (Sigma); F-actin (Cytoskeleton Inc.), cardiac troponin (TnI) (Abcam), anti-modified citrulline antibody (Millipore); sequencing grade Lys-C protease and protease inhibitor cocktail (Roche).

2.2 Human heart tissue

Left ventricular tissue samples were obtained from Cris Dos Remedios, University of Sydney, Australia after informed consent and with approval of the local Ethical Committee. The samples were acquired during heart transplantation surgery, from patients with HF [ischemic heart disease (ISHD) and idiopathic cardiomyopathy (IDCM), $n = 10$ each] and non-failing donor hearts ($n = 10$) as previously described.[21]

2.3 Mouse heart tissue and neonatal myocytes

Male C57BL/6 mice ($n = 3$) (5-day-old neonatal mice, Jackson Laboratories) were obtained. Animal study was approved by The Johns Hopkins University Animal Care and Use Committee and followed established NIH guidelines. Briefly, primary cultured ventricular myocytes isolated from neonatal mice were a kind gift from Dr Koitabashi.[22] Collagenase-digested isolated myocytes were incubated in buffer with increasing concentrations of Ca^{2+}, achieving a final concentration of 1.2 mM Ca^{2+} as in the MEM culture media. Cells were seeded at 25 000 rod-shaped myocytes/mL on 6-well plates or 60 mm dishes coated with laminin. After 1 h incubation in 37°C, 5% CO_2, the culture media were replaced to remove unattached cells.

2.4 Protein extraction and SWATH-MS

Hearts were fractionated into myofilament- and cytosolic-enriched fractions using the IN Sequence protocol.[23] Protein extraction and generation of LysC peptides from subfractions was performed using a filter-aided sample preparation (FASP) protocol.[24] When needed, recombinant proteins or the In Sequence fractions were incubated with PAD's cocktail at a ratio of 1:20 for 2 h at 37°C in 100 mM Tris, pH 7.6, 5 mM DTT, 10 mM $CaCl_2$. The reaction was stopped by addition of 5 mM EDTA prior to digestion. A TripleTOF 6600 mass spectrometer (Sciex) was used for both data-dependent acquisition to build peptide spectral ion library and SWATH-MS (data-independent acquisition) for each individual sample analysis. The raw data were searched with ProteinPilot™ Software 5.0 to create a spectral ion library. Individual SWATH-MS runs were matched against the spectral library created in the presence or absence of PAD (plus and minus PAD) for both the myofilament- and cytosolic-enriched protein fractions (see Supplementary material online).

2.5 Preparation of citrullinated samples

Recombinant proteins and the fractions obtained from IN Sequence were incubated with PAD2 at a ratio of 1:20 for 2 h at 37°C in working buffer (100 mM Tris, pH 7.6, 5 mM DTT, 10 mM $CaCl_2$). The reaction was stopped by addition of 5 mM EDTA.

2.6 Statistical validation peptides, proteins and citrullination residues: acceptance criteria

Bioinformatics analysis was performed with the workflow described in data supplement. The peptide normalization used in this study was based on the iRT peptide retention time,[25] and normalized values were used for downstream analysis. Ensemble protein ID accession numbers were mapped back to their associated encoding Ensemble gene entries. Data analysis and mining were performed using iProXpress (http://proteininformationresource.org/iproxpress2)[26] and Cytoscape.[27] The Kruskal–Wallis test (non-parametric one-way ANOVA) for each peptide was used to calculate P-values. The significance of the biochemical changes was determined by performing a t-test ($P \leq 0.05$) on the differences for all paired data. Unless otherwise stated all biochemical assays were replicated three times.

2.7 SDS–PAGE immunoblot for citrullination

A 1:2000 diluted of the anti-citrulline (Modified) antibody was used for the 1 DE western blot (see Supplementary material online).

2.8 Two-dimensional gel electrophoresis

The independent verification of proteomics data was performed with fluorescence two-dimensional (2DE) gel electrophoresis (2D-DIGE, pI range and SDS–PAGE range) as reported previously.[28] The treatment of sample with PAD2 enzyme, which removes a guanidino group from specific arginine residues within the modified protein, can be used to identify citrullinated proteins based on the change in charge of the protein after treatment (see Supplementary material online).

2.9 Membrane-permeabilized myocytes

Left ventricular tissue from C57BL/6 mice was flash-frozen in liquid nitrogen and stored at −80°C. For analysis, tissue was homogenized in the presence of 0.3% Triton X-100, and protease and phosphatase inhibitors, as described.[29] Myocytes were washed without Triton X-100 to remove the detergent and resuspended in isolation buffer. PAD2 was activated in 10 mM Ca^{2+} and 50 mM DTT for 60 min at 37°C. PAD2-treated myocytes were then exposed to a 1:10 dilution of activated PAD2 in isolation buffer for 30 min at room temperature. Myocytes were then glued with silicone to the tips of 150 μm diameter minutia pins attached to a force transducer and motor arm (Aurora Scientific Inc., Aurora, ON, Canada). Sarcomere length was monitored by video camera (Imperx, Boca Raton, FL, USA) and calculated by the High-speed Video Sarcomere Length Program (Aurora Scientific Inc.). Myocyte sarcomere length was set at 2.1 μm. A complete activation of the myocyte occurred at the beginning and end of the experiment, and the myocyte discarded if there was >10% rundown, as described.[29]

2.10 ATPase activity

The HMM-ATPase activity was analysed at three separate experiments described previously.[30,31] First experiment was carried out at constant HMM and F-actin concentration with citrullinated or non-citrullinated HMM and/or F-actin. Second, HMM-ATPase activity was determined at increasing TM concentrations (citrullinated or non-citrullinated). Third experiment HMM-ATPase activity was determined at constant HMM, F-actin, and TM concentration with increasing citrullinated or non-citrullinated TnI. Each experiment was done in triplicate and three separate times. For more details, see Supplementary material online.

2.11 Actin-binding experiments

Various concentrations of citrullinated and non-citrullinated HMM, TM, and/or TnI were added to F-actin and centrifuged to determine extent of binding. Pellet and supernatant were analysed using 10% SDS–PAGE, and amount of each protein was quantified by densitometry as previously described.[32,33] Each assay was carried out in triplicate. For more details, see Supplementary material online.

2.12 Reverse transcription-PCR

Total mRNA from mouse neonatal cardiac myocytes was extracted using TRI-reagent (Sigma) according to the manufacturer's protocols. cDNA was generated using the SuperScript III First-Standard Synthesis System (Invitrogen) according to the instructions of the manufacturer. Reverse transcription (RT)-PCR was performed using primers specific for the PAD1, PAD2, PAD3, PAD4, and β-actin. The PCR products were separated by electrophoresis on a 1.8% agarose gel and visualized under UV light. Each assay was done in duplicate.

3. Results

3.1 Identification of myocardial citrullinated proteins

To identify citrullinated targets in the heart, we assessed the citrullinome in three groups, ISHD, IDCM, and non-failing donor hearts ($n = 10$ per group) using SWATH-MS.[34] SWATH-MS allowed for the quantification. Fifty-three citrullinated sites were altered with HF compared with the non-failing controls ($P < 0.05$) and are listed in *Table 1* (see Supplementary material online, *Table S1* for details of all citrullinated peptides in this study). These were proteins with diverse cellular functions, including the regulators of transcription and chromatin structure, cytoskeletal and contraction, cellular signalling processes, and metabolism (*Figure 1*). Western blotting of myofilament- and cytosolic-enriched fractions obtained from ISHD, IDCM, and non-failing donor hearts ($n = 10$ per group) using an anti-modified citrulline antibody confirmed that citrullination occurs in intracellular proteins. Although there was no difference (see Supplementary material online, *Figure S1*) in the overall immunoreactivity between groups with regard to the number of bands or band density per blot, in-gel digestion and subsequent MS of the immunoreactive bands identified the major sarcomeric proteins at their expected molecular weight (e.g. myosin heavy and light chains and actin, data not shown). It must be noted that quantitative assessment by immuno-1DE was confounded by the presence of other proteins in the gel bands and challenges of direct site-specific assessment using MS.

To further validate citrullination of the high abundant sarcomeric proteins, DIGE 2D gel electrophoresis (pH 4–7, 10% SDS–PAGE) was carried out. Myofilament- and cytosolic-enriched fractions of ISHD, IDCM, and non-failing donor hearts ($n = 4$ per group) were pretreated with PAD2 to induce maximum citrullination, combined at a 1:1 ratio with the matching untreated samples and simultaneously resolved by 2D gel electrophoresis. The sarcomeric proteins, including actin, TM, and myosin light chains were shown to be citrullinated with ectopic treatment of PAD2 (*Figure 2*).

3.2 Biochemical assessment of modified sarcomeric proteins

To test whether citrullination can affect sarcomeric protein function, actin, HMM, TM, and troponin were citrullinated by ectopic PAD2 and then compared with respective unmodified recombinant proteins to determine whether citrullination affects their biochemical, structural, or enzymatic properties. The binding of citrullinated or untreated HMM, TM, or TM–TnI to F-actin was determined using classical co-sedimentation assays (*Figure 3A–C*). Citrullination of actin did not alter F-actin formation and over 95% of the actin was pelleted upon centrifugation (data not shown). Binding of citrullinated TM to F-actin was enhanced compared with unmodified TM (*Figure 3A*). Since TM and HMM can affect binding of each other to F-actin in a co-operative manner,[35] we tested for co-operativity under conditions in which binding of TM alone to F-actin is poor, but increased upon the binding of myosin heads to F-actin.[36] As illustrated in *Figure 3B*, at low salt concentration, citrullinated TM in the presence of HMM was able to bind to F-actin.

The binding of cardiac TnI to F-actin in the presence of TM was performed by co-sedimenting citrullinated TnI in the presence of F-actin and TM. It was found that both citrullinated and non-citrullinated forms of cardiac TnI bound to F-actin equally well (*Figure 3C*).

3.3 Inhibition of HMM-ATPase activity

To verify a possible modulation of the actomyosin HMM-ATPase activity by citrullination of sarcomeric proteins, experiments were performed in

Table I List of citrullinated protein ($P = 0.05$) with citrullinated peptide sequence

	Protein	Peptide	UP identifier	p_kw
Cytoplasm	Adenylate kinase isoenzyme 1	RGETSGR[Dea]VDDNEETIK	Q5T9B7	0.03
Cytoplasm	Alcohol dehydrogenase 1B	AAGAAR[Dea]IIAVDINK	ADH1B	0.04
Cytoplasm	Beta-enolase	SPDDPAR[Dea]HITGEK	ENOB	0.00
Cytoplasm	Carbonic anhydrase 3	DIR[Dea]HDPSLQPWSVSYDGGSAK	CAH3	0.01
Cytoplasm	Fatty acid-binding protein, heart	LILTLTHGTAVC[CAM]TR[Dea]TYEK	FABPH	0.04
Cytoplasm	Peptidyl-prolyl *cis-trans* isomerase A	TAENFR[Dea]ALSTGEK	PPIA	0.01
Cytoplasm	Serum deprivation-response protein	FQHPGSDMR[Dea]QEK	SDPR	0.01
Cytoplasm	Serum deprivation-response protein	VSPLTFGR[Dea]K	SDPR	0.01
Cytoplasm	Heat shock protein beta-7	RHPHTEHVQQTFR[Dea]TEIK	HSPB7	0.05
Cytoplasm	Alpha-crystallin B chain	GLSEMR[Dea]LEK	CRYAB	0.01
Cytoskeleton	Actin[a]	C[CAM]DIDIR[Dea]K	ACTA1	>0.05
Cytoskeleton	Actin[a]	QEYDEAGPSIVHR[Dea]K	ACTA2	>0.05
Cytoskeleton	Filamin-C	SSSSR[Dea]GSSYSSIPK	FLNC	0.03
Cytoskeleton	LIM domain-binding protein 3	TSPEGAR[Dea]DLLGPK	LDB3	0.00
Cytoskeleton	Myosin-binding protein C, cardiac-type	EPVFIPR[Dea]PGITYEPPNYK	A8MXZ9	0.03
Cytoskeleton	Myozenin-2	R[Dea]VATPFGGFEK	MYOZ2	0.01
Cytoskeleton	Myozenin-2	AELPDYR[Dea]SFNR[Dea]VATPFGGFEK	MYOZ2	0.02
Cytoskeleton	Troponin I, cardiac muscle	NIDALSGMEGR[Dea]K	TNNI3	0.02
Cytoskeleton	Troponin I, cardiac muscle	ESLDLR[Dea]AHLK	TNNI3	0.02
Cytoskeleton	Troponin T, cardiac muscle	PR[Dea]SFMPNLVPPK	E7EPW4	0.01
Cytoskeleton	Tropomyosin[b]	ETR[Dea]AEFAERSVTKLEK	TPM1	0.002
Cytoskeleton	Vimentin	FADLSEAAN[Dea]RNNDALR[Dea]QAK	VIME	0.03
Cytoskeleton	Glyceraldehyde-3-phosphate dehydrogenase	LWR[Dea]D[Dhy]GRGALQN[Oxi]IIPASTGAAK	G3P	0.01
Cytoskeleton	Myosin-7	AEETQR[Dea]SVNDLTSQR[Dea]AK	MYH7	0.05
Mitochondrion	Aconitate hydratase	SYLR[Dea]LRPDRVAMQDATAQ[Dea]M[Oxi]AMLQFISSGLSK	ACON	0.01
Mitochondrion	Aconitate hydratase	ANSVR[Dea]NAVTQEFGPVPDTAR[Dea]YYK	ACON	0.03
Mitochondrion	Aconitate hydratase	IVYGHLDDPASQEIER[Dea]GK	ACON	0.03
Mitochondrion	Adenylate kinase 4	LLR[Dea]AVILGPPGSGK	KAD4	0.00
Mitochondrion	Alcohol dehydrogenase class-3	VAGASR[Dea]IIGVDINK	ADHX	0.05
Mitochondrion	ATP synthase subunit alpha	R[Dea]TGAIVDVPVGEELLGR[Dea]VVDALGNAIDGK	ATPA	0.00
Mitochondrion	ATP synthase subunit alpha	AIEEQVAVIYAGVR[Dea]GYLDK	ATPA	0.01
Mitochondrion	ATP synthase subunit alpha	GIRPAINVGLSVSR[Dea]VGSAAQ[Dea]TRAMK	ATPA	0.01
Mitochondrion	ATP synthase subunit alpha	QGQYSPMAIEEQVAVIYAGVR[Dea]GYLDK	ATPA	0.03
Mitochondrion	ATP synthase-coupling factor 6	SGGPVDASSEYQQELER[Dea]ELFK	ATP5J	0.00
Mitochondrion	Chloride intracellular channel protein 4	YR[Dea]NFDIPK	CLIC4	0.04
Mitochondrion	Cytochrome c (Fragment)	EER[Dea]ADLIAYLK	C9JFR7	0.01
Mitochondrion	Delta-1-pyrroline-5-carboxylate dehydrogenase	VLR[Dea]NAAGNFYINDK	AL4A1	0.00
Mitochondrion	Delta-1-pyrroline-5-carboxylate dehydrogenase	AADMLSGPR[Dea]R[Dea]AEILAK	AL4A1	0.01
Mitochondrion	Delta-1-pyrroline-5-carboxylate dehydrogenase	VANEPVLAFTQGSPER[Dea]DALQK	AL4A1	0.03
Mitochondrion	Enoyl-CoA hydratase	EMVLTGDR[Dea]ISAQDAK	ECHM	0.01
Mitochondrion	ES1 protein homologue	VLR[Dea]GVEVTVGHEQEEGGK	ES1	0.00
Mitochondrion	Heat shock protein HSP 90-alpha	HLEINPDHSIIETLR[Dea]QK	HS90A	0.04
Mitochondrion	NAD(P) transhydrogenase	AATITPFR[Dea]K	NNTM	0.05
Mitochondrion	Phosphate carrier protein	VYFR[Dea]LPRPPPPEMPESLK	MPCP	0.00
Mitochondrion	Pyruvate dehydrogenase E1 component subunit alpha, somatic form	C[CAM]DLHR[Dea]LEEGPPVTTVLTR[Dea]EDGLK	ODPA	0.01
Mitochondrion	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit	FAIYR[Dea]WDPDK	SDHB	0.05
Mitochondrion	Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha	TR[Dea]LIGPNC[CAM]PGVINPGEC[CAM]K	SUCA	0.02
Mitochondrion	Succinyl-CoA:3-ketoacid coenzyme A transferase 1	ADR[Dea]AGNVIFR[Dea]K	SCOT1	0.04
Mitochondrion	Voltage-dependent anion-selective channel protein 1	SR[Dea]VTQSNFAVGYK	VDAC1	0.02
Mitochondrion	60 kDa heat shock protein	FDR[Dea]GYISPYFINTSK	CH60	0.04

Continued

Table 1 *Continued*

	Protein	Peptide	UP identifier	p_kw
Mitochondrion	Trifunctional enzyme subunit beta	PNIR[Dea]NVVVVDGVR[Dea]TPFLLSGTSYK	ECHB	0.02
Mitochondrion	Aconitate hydratase	TGR[Dea]EDIANLADEFK	ACON	0.00
Mitochondrion	Aconitate hydratase	R[Dea]LQLLEPFDK	ACON	0.01
Nucleus	Dual specificity protein phosphatase 3	LGITHVLNAAEGR[Dea]SFMHVNTNANFYK	DUS3	0.01
Nucleus	Neuroblast differentiation-associated protein AHNAK	FGVSTGR[Dea]EGQTPK	AHNK	0.01
	Collagen alpha-3(VI) chain	DVVFLLDGSEGVR[Dea]SGFPLLK	E7ENL6	0.03

Citrullinated proteins were grouped by cellular component.
[a]P_{kw} (P) < 0.05.
[b]Based on 2DE gel analysis.

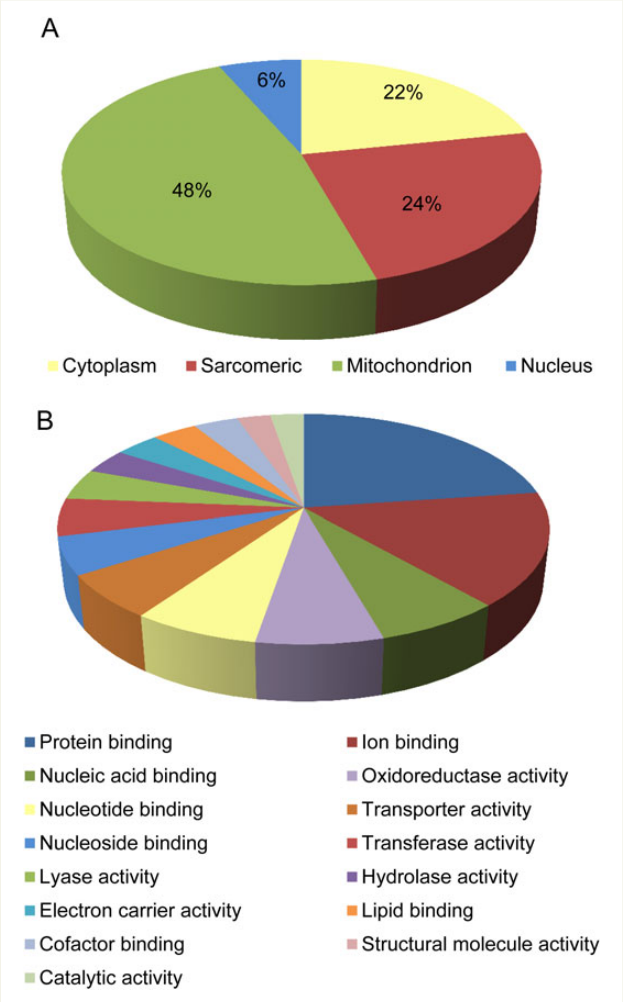


Figure 1 The cardiac citrullinated proteome. Diagrams show citrullinated proteins with significant *P*-value group by (*A*) cellular component and (*B*) molecular function. Details can be found in *Table 1*.

the presence of citrullinated HMM, F-actin, and TM. The data summarized in *Figure 4A* show that, in the presence of citrullinated HMM (0.5 μmol/L HMM and 7 μmol/L F-actin), the actomyosin HMM-ATPase activity decreased from 0.32 ± 0.01 to 0.22 ± 0.002 nmol Pi, corresponding to 30% decrease in the enzyme activity. In contrast, citrullination of F-actin enhanced actomyosin HMM-ATPase activity up to 55% on the enzyme activity (*Figure 4A*). When both F-actin and HMM were citrullinated, the rate of ATP hydrolysis remained greater than control (0.368 ± 0.005 to 0.32 ± 0.01 nmol Pi). *Figure 4B* showed that actomyosin HMM-ATPase activity was affected by TM citrullination. The inhibition of actomyosin HMM-ATPase conferred by non-citrullinated and citrullinated TnI was compared. Addition of non-citrullinated TnI to HMM-F-actin-TM caused a decrease in the ATPase rate at 37 °C. Citrullinated TnI acted similar to non-citrullinated TnI and caused a decrease in the ATPase with no significant deference to non-citrullinated form (data not show).

3.4 PAD2 reduces calcium sensitivity in skinned myocytes

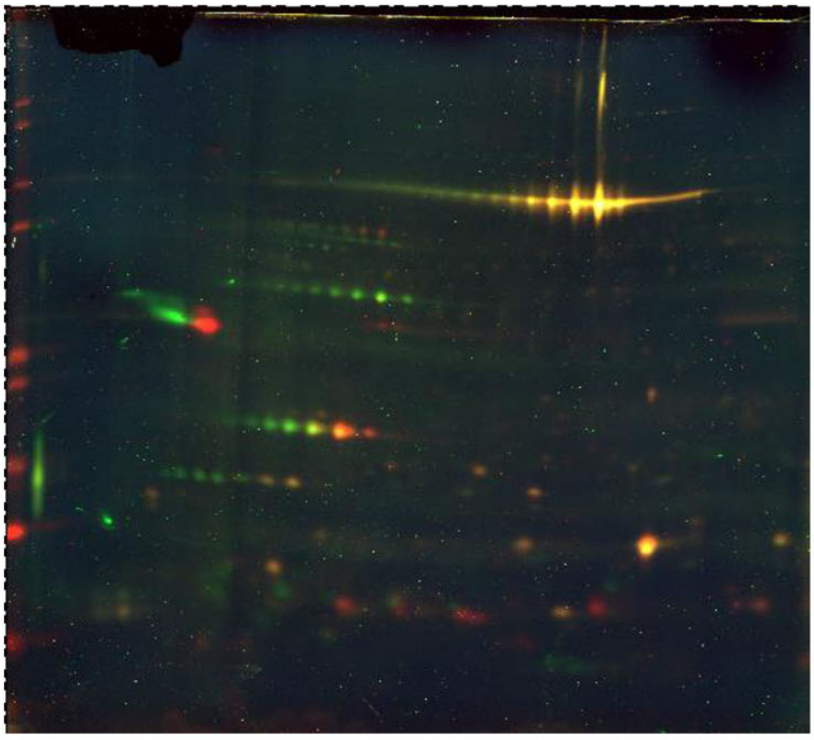
Chemically skinned cardiomyocytes isolated from the left ventricle of wild-type C57Bl6 male mice were exposed to varying concentrations of calcium ($n = 8$ myocytes from three mice per group, *Figure 4C–F*). PAD2 treatment had no effect on either maximal calcium-activated force (F_{max}) or hill coefficient (nH). However, PAD2 caused a rightward shift in the force–calcium relationship that indicates an increase in EC_{50} or a decrease in calcium sensitivity ($P = 0.009$, *Figure 4C*). This suggests that citrullination of myofilament proteins causes a loss-of-function phenotype, reducing its ability to generate force in response to intracellular calcium.

3.5 Analysis of PAD mRNA expression

To determine the cell specificity of the PAD isoform expression in the myocardium, mRNA expression level was determined using isoform-specific primers by Nested-PCR on isolated cardiac myocytes and fibroblasts (*Figure 5*). PAD2 was the primary isoform expressed in both cardiomyocytes and cardiac fibroblasts, although fibroblasts also had significant expression of PAD1 and PAD4 mRNA. In contrast, PAD3 mRNA was not detected in any of the cell types and conditions tested (*Figure 5*, note no band at 200 bp, see Supplementary material online for more details).

4. Discussion

Our experimental findings characterize citrullinated proteins in the normal and HF myocardium. The analysis revealed that protein citrullination has broad cellular distribution (*Figure 1*) but is highly enriched in the mitochondria and sacromeric subproteome. A close connection between these two subproteomes is not unexpected due to the high energetic requirements of the sarcomere. We speculated that



Gel view	MS/MS ID*	Accession number	Coverage %	Observed pI/MW	Theoretical pI/MW	$P < 0.05$
1	Fatty acid binging protein, heart	P05413	63.2	6.0/ 14.500	6.29/ 14.858	0.20*
2	L-lactate dehydrogenase B chain	P07195	35.9	5.8/ 36.000	5.71/ 36.638	0.13*
3	Vinculin	P18206	4.5	6.2/ 170.00	5.83/ 123.799	0.19*
4	Actin, alpha cardiac	P62736	30.1	5.5/ 44.000	5.24/ 42.009	0.020
5	Tropomyosin	P09493	6.2	4.6/ 38.000	4.69/ 32.709	0.0020
6	Myosin light chain	P08590	32	5.0/24.000	5.03/ 21.932	0.021
7	Myosin regulatory light chain 2	P10916	39.8	4.9/18.000	4.92/ 18.789	0.0018

* Not statistically significant

Figure 2 2DE DIGE analysis with samples treated with PAD2. Samples labelled with Cy2 (internal control), Cy3 (untreated), and Cy5 (treated) as described in Methods section. Details can be found in insert table.

citrullination, similar to phosphorylation and acetylation, could potentially regulate muscle-contractile proteins in a co-ordinated manner. Interestingly, the multiple enzymes involved in metabolic pathways/metabolism were up-regulated in ischaemia but down-regulated in IDCM (*Figure 6*). To understand the topology and functional annotation of citrullinated protein–protein interaction in the heart system, the protein network was constructed using the STRING database (see Supplementary material online, *Figure S2*).[37] The entire protein network consisted of high scoring interaction partners (STRING relevant confidence score ≥ 0.5, ischaemia/IDCM vs. control). The visual analysis of this sub-network showed that citrullinated proteins are interacted to each other and are involved in the metabolism and respiratory chain

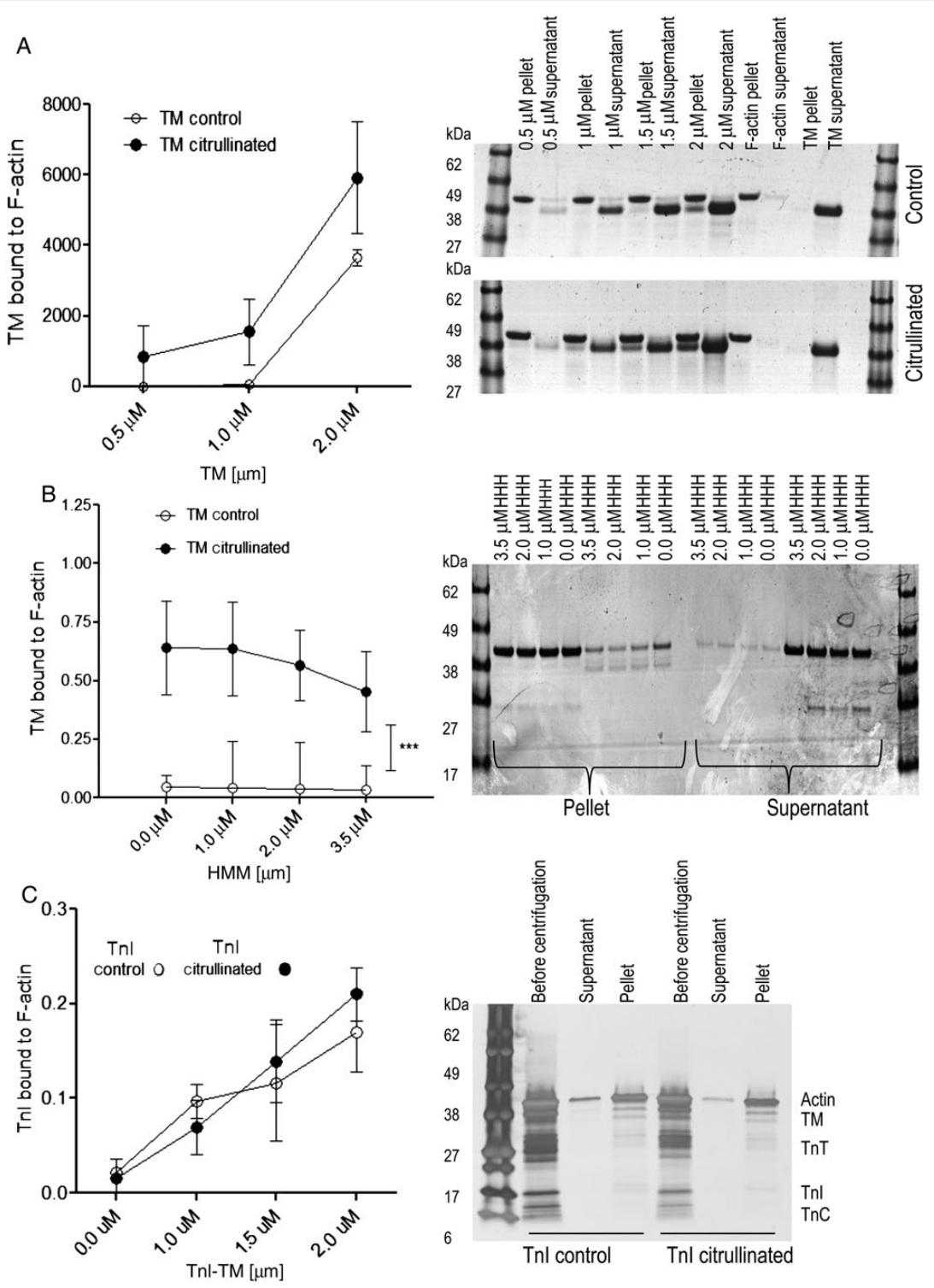


Figure 3 Actin-binding studies. Increasing concentrations of (*A*) TM (0.5–2 μM) were incubated with F-actin or (*B*) actin-HMM and (*C*) TnI (0.5–2 μM) in buffer containing 40 mM Tris–HCl (pH 7.6), 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT. Binding of TM to F-actin was carried out at 25°C for 30 min and ultracentrifuged at 156 565 *g* for 25 min, 20°C, in a Beckman model TL-100.2. Both pellet and supernatant (unbound protein) were analysed. Representative silver-stained gels show proteins composition of the supernatants and pellets. A triplicate set of gels was analysed by densitometry. Each data point is an average (and range) of the values obtained from the three sets of gels.

targets, contraction and signal transduction systems.[38] As an initial assessment to understand potential functional consequences of the citrullination, recombinant sarcomere proteins HMM, F-actin, TM, and TnI were used as model proteins to study the interactions that govern the thick and thin filament function and by measuring the contractile properties of single skinned myocytes treated with PAD2.

Ca^{2+}-dependent alterations to Tn alter an azimuthal movement of TM on the actin surface, which allows myosin binding and cross-bridge

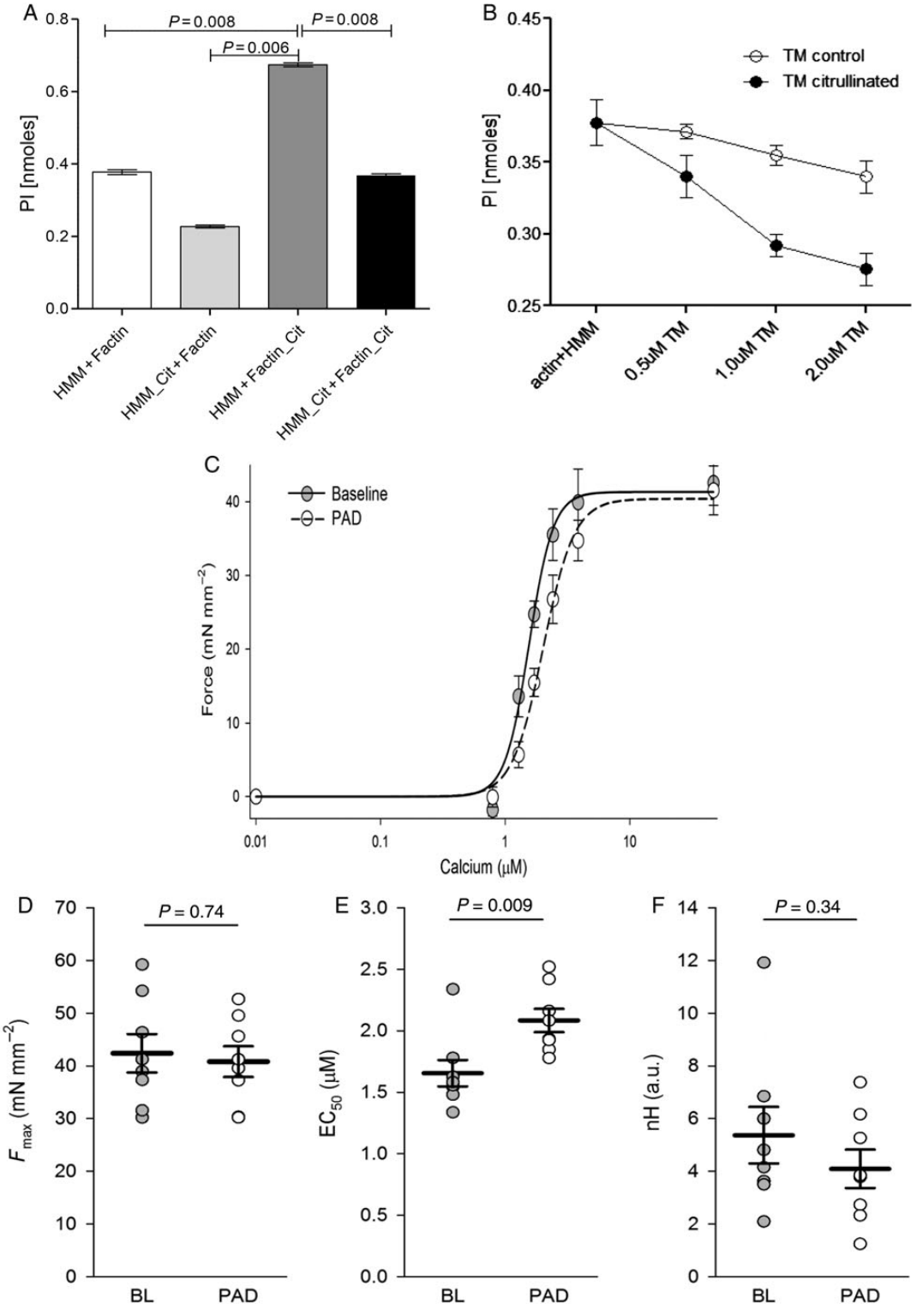


Figure 4 Citrullination of sarcomeric proteins, biochemical and physiological effects. (*A*) Regulation of the actomyosin HMM-ATPase activity by citrullinated F-actin and/or citrullinated HMM. (*B*) Inhibition of actomyosin HMM-ATPase activity by TM. ATPase activity was measured as a function of TM concentration. The results are the average of four independent experiments for each protein at each TM concentration. Assay conditions: 0.2 mg/mL F-actin, 0.02 mg/mL HMM, 0–2.0 μM TM in 10 mM Hepes, pH 7.5, 30 mM NaCl, 5 mM $MgCl_2$, 4 mM ATP. PAD2 treatment reduced myofilament calcium sensitivity. (*C*) Force–calcium relationships for untreated membrane-permeabilized myocytes from untreated control ($n = 8$ myocytes from three mice, grey circles) and PAD2-treated ($n = 8$ myocytes from three mice, open circles) groups. (*D*) There was no difference in maximal calcium-activated force (F_{max}) between the two groups. (*E*) PAD2 treatment caused a significant ($P = 0.009$) increase in EC_{50} (calcium required to generate 50% F_{max}), indicating a decrease in calcium sensitivity. (*F*) While the hill coefficient (nH) trended to be decreased by PAD2 treatment (see steepness of curve in *C*), the difference was not significant ($P = 0.34$).

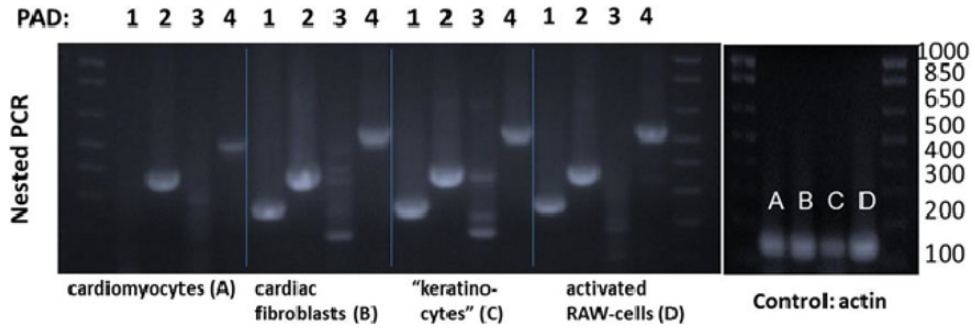


Figure 5 RT-PCR analysis of expression level of PAD isoforms in (*A* and *B*) heart from control mouse, (*C*) mouse keratinocytes, and (*D*) mouse macrophage cell line activated by lipopolysaccharide. The PCR product PAD2 is seen in all types of samples; PAD4 and PAD1 are seen in cardiac fibroblast, keratinocytes, and macrophage cell line. PAD3 has not been detected. (MW: PAD1, 285 bp; PAD2, 390 bp; PAD3, 200 bp; PAD4, 550 bp).

Ischemia		IDCM	
Protein ID	Peptide	Protein ID	Peptide
LIM domain-binding protein 3	TSPEGAR[Dea]DLLGPK	Filamin-C	SSSSR[Dea]GSSYSSIPK
Adenylate kinase 4	LLR[Dea]AVILGPPGSGK	Collagen alpha-3(VI) chain	DVVFLLDGSEGVR[Dea]SGFPLLK
Cytochrome c	EER[Dea]ADLIAYLK	Phosphate carrier protein,	VYFR[Dea]LPRPPPPEMPESLK
Carbonic anhydrase 3	DIR[Dea]HDPSLQPWSVSYDGG SAK	ATP synthase subunit alpha,	AIEEQVAVIYAGVR[Dea]GYLDK
Neuroblast differentiation-associated protein AHNAK	FGVSTGR[Dea]EGQTPK	Myozenin-2	R[Dea]VATPFGGFEK
Peptidyl-prolyl cis-trans isomerase A	TAENFR[Dea]ALSTGEK		
Succinyl-CoA ligase [ADP/GDP-forming]	TR[Dea]LIGPNCPGVINPGECK		
Troponin I, cardiac muscle	ESLDLR[Dea]AHLK		
Myozenin-2	AELPDYR[Dea]SFNR[Dea]VATPFGGFEK		

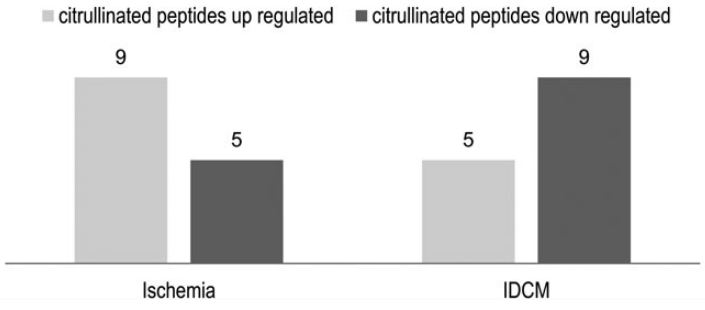


Figure 6 Citrullinated peptides up-/down-regulated between heart failure groups, ischaemia, and IDCM.

isomerization to strong binding, force-producing states, and muscle contraction. At low intracellular Ca^{2+}, Tn-TM sterically blocks myosin-binding sites on actin (blocked state), while in the presence of elevated Ca^{2+} Tn-TM moves and partially exposes the myosin-binding sites on F-actin (closed state). Myosin binding in the presence of Ca^{2+} is required for full activation.[39,40] It is a combination of Ca^{2+}-induced TnI conformational change which is, in part, transmitted via TnT to TM (especially the T1 region that binds along TM) as well as myosin binding that influence the exact positioning of TM on the actin filament. Based on our MS data, citrullination sites on myosin, actin, TM, TnI, and TnT are in regions what can influence these interactions and thus the actomyosin HMM-ATPase activity and contraction. Below outlines the potential impact based on the biochemical and physiological experiments presented in this manuscript.

First, intrinsic actomyosin HMM-ATPase was inhibited by citrullination, but this was overcome in the presence of F-actin regardless of

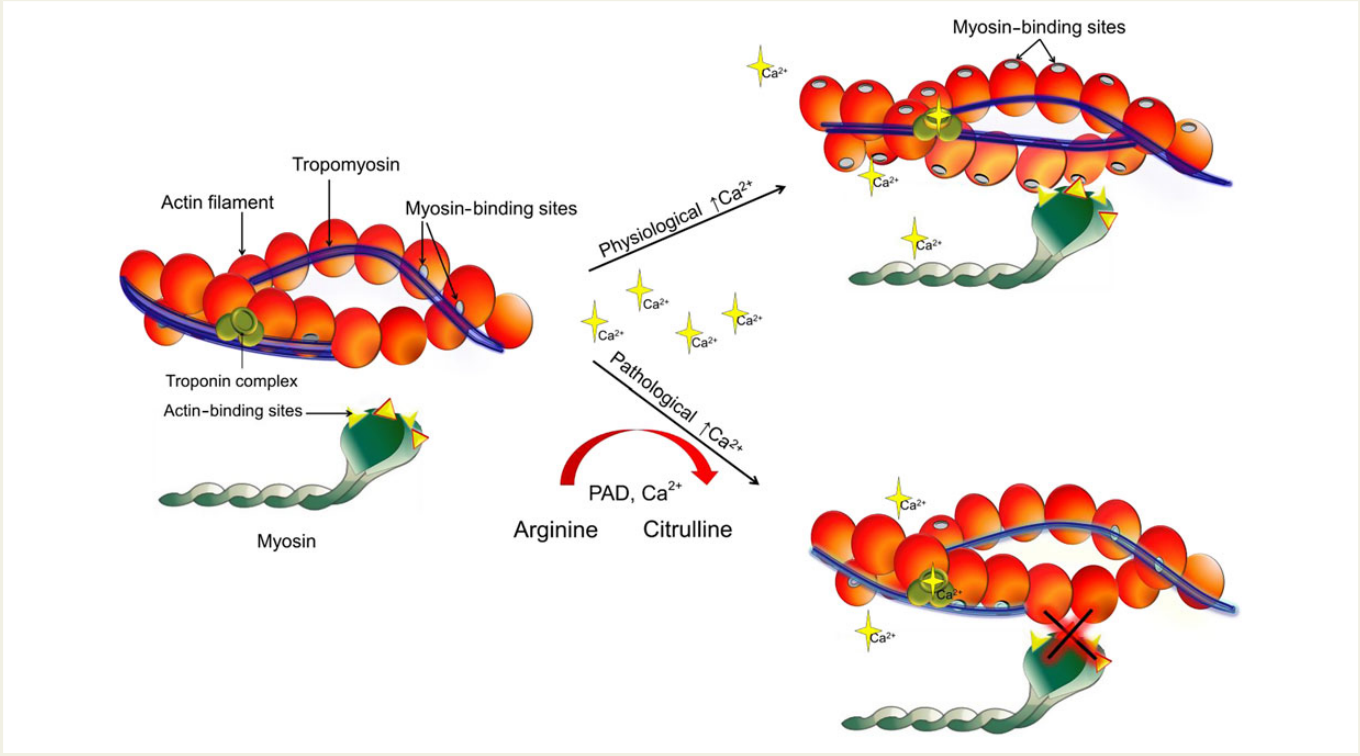


Figure 7 Citrullination of the contractile proteins could affect different aspects of regulatory function. It could either trigger a structural change or stabilize a conformation that is necessary for actin-activated release of Pi and completion of the ATPase cycle.

whether F-actin is citrullinated or not. Citrullinated F-actin was a more potent modulator of HMM enzymatic activity and increased actomyosin HMM-ATPase rate by 55% compared with the unmodified F-actin. This suggests that citrullination of F-actin changes the confirmation of the actin filament to alter the ease of ATP hydrolysis by the myosin once it is bound. The actin-HMM interaction was also affected by the citrullination of TM. Citrullinated TM displayed enhanced binding to F-actin compared with unmodified TM. This was also observed in the presence of HMM (based on the centrifugation assays). This correlated to an inhibition of the citrullinated TM-actin-HMM-ATPase activity compared with the non-citrullinated TM, suggesting that citrullinated TM altered the ability of HMM to bind to the actin filament (*Figure 7*). Intriguingly, citrullinated TnI, like the unmodified protein complex, bound tightly to actin-TM based on centrifugation studies. *Figure 3C* shows that >95% of the unmodified and modified TnI was present in the pellet.

Biochemical results were related to our physiological finding and showed that citrullination of the sarcomeric proteins caused a decrease in Ca^{2+} sensitivity in the skinned cardiac myocyte (*Figure 4C*). On TnI, C-terminus residues 191–210, which contains the citrullinated residue 203, is primarily responsible for maintaining the TM conformation that prevents cross-bridge cycling.[41] Thus, not only does TnI promote the blocked state, but also contributes to the stabilization of TM in the closed state. In mice, cardiac TnI containing cardiomyopathy mutations, R192H or R204H (which are equivalent to the human sequence of 191 and 203 the latter being the same citrullinatable residue as discussed above), increases the binding affinity of Tn for actin-TM.[35] Perhaps this is augmented by the citrullination of TnT at residue 77, which is located in the N-terminal tail (TnT1) that lies along TM and has been shown to be involved in co-operativity of the actin-TM-Tn filament. In addition, binding of Ca^{2+} or myosin to actin-TM-Tn can displace TnI residues 151 and 188, which flank the second citrullinated residue 169, away from the outer domain of F-actin.[42] This is consistent with an azimuthal displacement to TnI–TM by Ca^{2+} that can expose the high-affinity binding site on F-actin for myosin.[40] Studies on familial hypertrophic cardiomyopathic mutations occurring on thick filament proteins (TM, MHC, MyBP-C, ELC, and RLC) show that a change in one amino acid side chain can have an enormous effect on cardiac morphology and function.[43,44] Furthermore, factors that lead to abnormal contraction and relaxation in the failing heart include metabolic pathway abnormalities that result in decreased energy production, energy transfer, and energy utilization.[45]

Finally, to better understand the involvement of citrullinated proteins in the heart, it is important to obtain insight about cell specificity. Previous data acquired by the immunohistochemistry showed that PADs 1–3 and, to some degree, PAD6 were detected in cardiomyocytes with PADs 2 and 4 found in endothelial cells and vascular smooth muscle cells.[7] In the present study, we relied not on antibodies and tissue slices, but rather examined the mRNA expression of PAD family members in isolated mouse cardiomyocytes and cardiac fibroblasts. The data showed that PAD2 was primarily expressed in both cardiomyocytes and cardiac fibroblasts, whereas PAD1 and PAD4 mRNA were the major forms in the cardiac fibroblasts.

In summary, we have presented previously unexplored roles for citrullination in the heart. Ultimately, identification of citrullination of the majority of the sarcomeric proteins and alterations in their biochemical properties suggest that there is potentially a new PTM regulation of cardiac contractility. Citrullination at some of these residues was increased in the myocardium of individuals with HF compared with controls, suggesting that citrullination could play a role in the decrease in contractile dysfunction in HF.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: none declared.

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