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Original Contribution

Heme-induced contractile dysfunction in Human cardiomyocytes caused by oxidant damage to thick filament proteins

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ABSTRACT

Intracellular free heme predisposes to oxidant-mediated tissue damage. We hypothesized that free heme causes alterations in myocardial contractility via disturbed structure and/or regulation of the contractile proteins. Isometric force production and its Ca^{2+}-sensitivity (pCa_{50}) were monitored in permeabilized human ventricular cardiomyocytes. Heme exposure altered cardiomyocyte morphology and evoked robust decreases in Ca^{2+}-activated maximal active force (F_o) while increasing Ca^{2+}-independent passive force ($F_{passive}$). Heme treatments, either alone or in combination with H_2O_2, did not affect pCa_{50}. The increase in $F_{passive}$ started at 3 μM heme exposure and could be partially reversed by the antioxidant dithiothreitol. Protein sulfhydryl (SH) groups of thick myofilament content decreased and sulfenic acid formation increased after treatment with heme. Partial restoration in the SH group content was observed in a protein running at 140 kDa after treatment with dithiothreitol, but not in other proteins, such as filamin C, myosin heavy chain, cardiac myosin binding protein C, and α-actinin. Importantly, binding of heme to hemopexin or alpha-1-microglobulin prevented its effects on cardiomyocyte contractility, suggesting an allosteric effect. In line with this, free heme directly bound to myosin light chain 1 in human cardiomyocytes. Our observations suggest that free heme modifies cardiac contractile proteins via posttranslational protein modifications and via binding to myosin light chain 1, leading to severe contractile dysfunction. This may contribute to systolic and diastolic cardiac dysfunctions in hemolytic diseases, heart failure, and myocardial ischemia–reperfusion injury.

1. Introduction

Oxidative stress, characterized by excess production of reactive oxygen species (ROS), has been implicated in several cardiovascular pathologies including chronic heart failure [1–3], myocardial ischemia–reperfusion injury [4], and cardiovascular complications of hemolytic diseases [5,6]. High levels of ROS cause lipid peroxidation, DNA damage, mitochondrial dysfunction, and oxidation of myocardial proteins [7]. ROS production can modify protein function by oxidative posttranslational modifications such as protein sulfhydryl (SH) group oxidation to disulfide bonds and/or to sulfenic acid [8]. Nevertheless, the molecular mechanisms leading to impaired myocardial function during oxidative stress are obscure, and effective antioxidant therapies are lacking.

Abbreviations: A1M, Alpha-1-microglobulin; BSA, Bovine serum albumin; cMyBPC, Cardiac myosin binding protein C; CPK, Creatinine phosphokinase; DMF, Dimethylformamide; DTDP, 2,2'-Dithiodipyridine; DTT, Dithiothreitol; ECL, Enhanced chemiluminescence; EGTA, Ethylene glycol tetra-acetic acid; *F*, Isometric force; *Fo*, Force at saturating [Ca^{2+}]; $F_{passive}$, Passive force; F_{total}, Total peak isometric force; HO-1, Heme oxygenase 1; IC_{50}, Half maximal inhibitory concentration; LDH, Lactate dehydrogenase; MHC, Myosin heavy chain; MLC-1, Myosin light chain 1; MLC-2, Myosin light chain 2; pCa_{50}, Ca^{2+} sensitivity of isometric force production; PMSF, Phenylmethylsulfonyl fluoride; ROS, Reactive oxygen species; SCD, Sickle cell disease; SH, Sulfhydryl groups

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Heme, a complex of iron with protoporphyrin IX, is an essential component of hemoproteins involved in cellular oxidation–reduction reactions in all aerobic organisms [9]. In the heart, heme plays prominent roles as a catalytic subunit of mitochondrial electron transport chain complexes, myoglobin, antioxidant enzymes, and cytochrome p450 [10]. On the other hand, free heme is potentially toxic [11–13] and due to its hydrophobic nature it can diffuse through cell membranes [14–16]. Hence, it is not surprising that cellular levels of heme are tightly controlled by a fine balance between its mitochondrial biosynthesis and incorporation into hemoproteins or catabolism by heme oxygenase 1 (HO-1) [17]. Earlier investigations have reported increased myocardial heme levels in failing human hearts [18]. In neonatal rat primary myocyte cultures, heme-modified cell morphology reduced the beating rate and led to the release of markers of myocardial damage, lactate dehydrogenase (LDH) and creatinine phosphokinase (CPK) [19]. A specific association between heme and cardiac myosin was also demonstrated in the hearts of guinea pigs and in rabbit skeletal muscles [20]. In another study on porcine skeletal muscles, the formation of both reducible and nonreducible cross-links between myosin molecules and an activated hemoprotein was demonstrated [21]. Moreover, heme also interfered with actin polymerization through binding to F-actin in rabbit skeletal muscles [22].

Myocardial performance depends on the mechanical properties of cardiomyocyte sarcomeres [23] and ultimately on two closely interacting myofibrillar protein systems: the contractile machinery (generating cardiac contractions and relaxations) and the cytoskeleton (providing the necessary structural background) [24,25]. Accordingly, systolic and diastolic ventricular functions are coordinated through Ca^{2+}-regulated active [26,27] and Ca^{2+}-independent passive characteristics of myofilament proteins [28,29]. Thick myofilaments play an important role in systolic function (myosin heavy chain, myosin light chain 1, and cardiac myosin binding protein C) and during the diastole (cardiac myosin binding protein C and titin) [30]. Importantly, hemolytic diseases (e.g., sickle cell disease and thalassemia) are associated with systolic and diastolic dysfunction [5,6,31,32], and in mouse models, administration of the heme scavenger hemopexin decreased ROS production and restored cardiovascular function in a murine model of hemolytic diseases [33]. These data suggest that myocardial proteins can be affected by free heme through the formation of ROS. Nevertheless, it is currently unknown how heme-mediated ROS production affects myofilament proteins in human cardiomyocytes and, consequently, systolic and diastolic function.

Here, we investigated the effects of free heme on myocardial contractility in permeabilized left ventricular cardiomyocytes of the human heart in vitro. To this end, the mechanical properties of cardiomyocytes were investigated before and after test incubations in the presence of heme and/or hydrogen peroxide (H_2O_2), catalase, superoxide dismutase (SOD), hemopexin, and alpha-1-microglobulin (A1M). These results were interpreted in view of additional tests in the presence of agents with known oxidant and/or antioxidant properties: 2,2'-dithiodipyridine (DTDP) and/or dithiothreitol (DTT), respectively. Our results suggest that heme binds to myosin light chain 1 (MLC-1) and then evokes oxidation of SH groups to disulfide bonds and to sulfenic acid in various myofilament proteins, resulting in cardiomyocyte contractile dysfunction.

2. Methods

2.1. Ethical approval

The experiments on human tissues performed in this study complied with the Helsinki Declaration of the World Medical Association and were approved by the Institutional Ethical Committee at the University of Debrecen, Hungary (No. DEOEC RKEB/IKEB 2553-2006) and by the Hungarian Ministry of Health (No. 323- 8/2005-1018EKU).

2.2. Myocardial tissue samples

Frozen left ventricular myocardial tissue samples were obtained from four unused human donor hearts (53- and 57-year-old males and 41- and 46-year old females). The donors did not have any signs of cardiac abnormalities and had not received any medication (except short-term mannitol, noradrenaline, and dopamine). The cause of death was cerebral contusion and cerebral hemorrhage due to accident or subarachnoid hemorrhage after a stroke.

Frozen tissue blocks were first defrosted and mechanically disrupted by a homogenizer in cell isolation solution (1 mM $MgCl_2$, 100 mM KCl, 2 mM EGTA, 4 mM ATP, and 10 mM imidazole, pH 7.0). The suspension was incubated in this solution, supplemented with 0.5% Triton X-100 (Sigma, St. Louis, MO, USA) for 5 min to permeabilize all the membranous structures. Then the preparations were washed theee times (centrifugation at 1300 rpm for 1 min) and subsequently kept at 4 °C for a maximum of 24 h.

2.3. Force measurements in single-myocyte-sized preparations

A permeabilized single cardiomyocyte was mounted between two thin needles with silicone adhesive (DAP Aquarium, Baltimore, USA) while viewed under an inverted microscope (Axiovert 135, Zeiss, Germany).[34,35] One needle was attached to a force transducer element (SensoNor, Horten, Norway) and the other to an electromagnetic motor (Aurora Scientific Inc., Aurora, Canada). The measurements were performed at 15°C and the average sarcomere length was adjusted to 2.3 μm as described previously [36]. The compositions of the relaxing and activating solutions used during force measurements were calculated as described previously [37,38]. The pCa (-log[Ca^{2+}]) values of the relaxing and activating solutions (pH 7.2) were 9 and 4.75, respectively. Solutions with intermediate free [Ca^{2+}] levels were obtained by mixing activating and relaxing solutions. All the solutions for force measurements contained 6.41 mM $MgCl_2$, 37.11 mM MgATP, 7 mM EGTA, and 10 mM NN-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), pH 7.2; protease inhibitors: 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 40 μM Leupeptin, and 10 μM E-64. The ionic equivalent was adjusted to 150 mM with KCl, resulting in an ionic strength of 186 mM.

The isometric force production was measured at varying Ca^{2+}concentrations. When a steady force level was reached, cardiomyocyte length was reduced by 20% within 2 ms and then quickly restretched. As a result, the force first dropped from the peak isometric level to zero (difference=total peak isometric force, F_{total}) and then started to redevelop. About 8 s after the onset of force redevelopment, the cardiomyocyte was returned to the relaxing solution, where a shortening to 80% of the original length with a long slack duration (8 s) was performed to determine $F_{passive}$. F_o was calculated by subtracting $F_{passive}$ from F_{total} for each Ca^{2+} concentration.

After the first activation at pCa 4.75, the resting sarcomere length was readjusted to 2.3 μm, if necessary. The second activation at pCa 4.75 was used to calculate the maximal isometric force (F_o). The cells were subsequently exposed to a series of test solutions with various concentrations of heme for 20 min in the presence or in the absence of H_2O_2 at pCa 9.0, and subsequently pCa 4.75 without heme, to assess the concentration dependence of heme on F_o. To test reversibility, some preparations were also

exposed to the reducing agent ditiothreitol (DTT) following incubations in the presence of 20 μM heme. F_o at submaximal levels of activation was normalized to that at maximal activation to characterize the pCa_{50}. When reexposure to pCa 4.75 at the end of the test protocols yielded a value below 80% of the initial value, the measurements were discarded. In some cases, cardiomyocytes were treated only with DTT.

2.4. Heme treatment

A stock solution (4 mM, pH 12.65, 15°C) of hemin chloride (Sigma-Aldrich, St. Louis, MO, USA) was prepared in a dark room on the day of use for each experiment. Relaxing solution (pH 7.3, 15°C) was used to achieve final concentrations of heme (as hematin) (1, 3, 10, 30, and 100 μM) and/or 30 μM H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA). Catalase and SOD (from bovine liver and bovine erythrocytes, respectively, Sigma-Aldrich, St. Louis, MO, USA) were used at a final concentration of 100 μg/mL. Hemopexin and A1M (human recombinant A1M was prepared as described previously) [39,40] were reconstituted in 10 mM Tris-HCl, 0.125 M NaCl, pH 8 to a final concentration of 10 μM. Heme together with hemopexin or A1M was used in a 1:1 ratio. Samples of 100 μL of the test solutions were subsequently placed on a cover slip. During cardiomyocyte force measurements, basal mechanical parameters were first recorded and then single cardiomyocyte preparations were incubated in test solutions for 20 min at room temperature. The mechanical consequences of the resultant myofilament protein alterations of test incubations were then assessed.

2.5. Protein sulfhydryl group determination

To determine the SH content, permeabilized cardiomyocytes (prepared similarly to the mechanical measurements) were treated with heme (20 μM) in the presence or absence of H_2O_2 (30 μM) or DTT (10 mM). In a separate study, cardiomyocytes were exposed to DTDP (2.5 mM) and/or DTT in relaxing solution. SH content was determined by incubating with the SH-sensitive Ellman's reagent [5,5-dithio-bis-(2-nitrobenzoic acid); Sigma-Aldrich, St. Louis, MO, USA] for 15 min at room temperature [41]. The absorbance of the solutions was measured at 412 nm using a NOVOstar Microplate Reader and calculated by reference to calibration curves (standard: N-acetyl-L-cysteine from Sigma-Aldrich, St. Louis, MO, USA) fitted to a single exponential function. Experiments were performed in triplicate.

2.6. Identification of protein SH groups in myofilament proteins

Human permeabilized cardiomyocytes (25 mg) were incubated in the presence of test solutions under the conditions employed in the myocyte force measurements. Then the reagents were removed by three washing steps and the protein concentrations were adjusted to 1 mg/mL. Subsequently, preparations were exposed to EZ-Link iodoacetyl-LC-biotin (Termo Scientific, Rockford, IL, USA) at room temperature for 60 min under dark conditions to biotinylate the SH groups of the proteins in a reaction buffer (EDTA 5 mM, Tris-HCl 50 mM, pH 8.3). Biotin was dissolved in dimethylformamide (DMF, Sigma-Aldrich, St. Louis, MO, USA). After biotinylation, cardiomyocytes were solubilized in sample buffer (urea 8 M, thiourea 2 M, SDS 3%, DTT 75 mM, Tris-HCl, glycerol 10%, bromo-phenol blue, E64 10 μM, and leupeptin 40 μM, pH 6.8). The protein concentrations were tested by a dot-blot-based method; thereafter 10 μg protein homogenates were applied on 1.5%, 4% or 10% polyacrylamide gels (Biorad, Hercules, CA, USA). After the separation of proteins by SDS-PAGE, proteins were transferred to nitrocellulose membranes. Protein amounts were determined by fluorescent Sypro Ruby Protein Blot Stain (Invitrogen, Eugene, OR, USA). The membranes were blocked in 10% milk powder (1 h) and incubated with a streptavidin-peroxidase conjugate (Jackson Immuno Research, West Grove, PA, USA) for 30 min. Signal intensities of biotin-labeled SH groups were visualized by enhanced chemiluminescence (ECL) and normalized for those assessed by Sypro Ruby Protein Blot Stain.

2.7. Heme magnetic beads

Heme magnetic beads (HMB) were used to assess the binding of cardiac proteins by heme. HMB were custom manufactured by MagnaMedics Diagnostic B.V., Geleen, Netherlands. Briefly, human heart samples were homogenized in a lysis buffer with the composition 50 m*M* Tris-HCl, 150 m*M* NaCl, 1% Igepal, 0.5% sodium deoxycholate, 6% sodium dodecyl sulfate (SDS) and 1% protease inhibitor cocktail, pH 8 (Sigma-Aldrich, St. Louis, MO, USA). Two hundred microliters of the sample with a final concentration of 1 mg/mL were incubated with 50 μL of HMB for 120 min at a room temperature in a rocking machine. The resulting complexes were collected by a magnet rack and washed four times with 200 μL PBS buffer. The complexes were resuspended in PBS buffer, the proteins were eluted from the HMB by boiling for 15 min, and the beads were separated by the magnet rack. Proteins were separated in 12% SDS-PAGE and visualized by silver staining [42].

2.8. LC-MS/MS based protein identification

Following silver staining, gel pieces were digested with trypsin. Then pieces were soaked in 1% $K_3Fe(CN)_6$, 1% $Na_2S_2O_3$ solution followed by extensive washing with ultrapure water. Before digestion, a reduction step was performed using 20 mM DTT. This was followed by alkylation with a solution containing 55 mM iodoacetamide. Overnight trypsinization was performed using TPCK-treated stabilized MS grade bovine trypsin (ABSciex) at room temperature and the digested peptides were extracted and lyophilized. The peptides were redissolved in 10 μL 1% formic acid and used for LC-MS/MS analysis. Prior to mass spectrometry analysis, the peptides were separated using a 90 min water/acetonitrile gradient and a 300 nL/min flow rate on an EasynLC (Bruker) nano HPLC. The separation was done on a 10 cm reverse-phase Easy-Column (ID 75 μm, 3 μm, 120 Å, ReproSil-Pur C18-AQ) analytical column (Thermo Scientific).

Mass spectrometry analysis was performed on a 4000 QTRAP (ABSciex) mass spectrometer, operating in positive ion mode (spray voltage 2800 V, ion source gas 50 psi, curtain gas 20 psi, source temperature 70°C). Information Dependent Acquisition was applied; first a survey scan (+EMS: 440–1400 amu) was recorded, followed by an enhanced resolution scan (ER) in order to determine the charge state of the three most intensive ions. Using this information, the proper collision energies were calculated by the Analyst 1.4.2 software (ABSciex), the selected parent ions were fragmented by collision induced dissociation (CID), and the product ions were scanned (EPI MS/MS).

The acquired LC-MS/MS data were used for protein identification with the help of ProteinPilot 4.5 software (ABSciex) using the Paragon algorithm and the UniProtKB/Swiss-Prot database (2014.06.11 version, 545536 entry). Peptide sequences having at least 95% confidence were accepted and for protein identification at least two peptides were used.

2.9. Sulfenic acid labeling

Permeabilized cardiomyocytes were treated as described above for the identification of protein SH groups. Then the preparations were exposed to biotin-1-3-cyclopentanedione (BP1, a sulfenic acid-specific reagent) dissolved in 5 mM tris-citric acid at pH 5

[43]. After labeling with BP1, cardiomyocytes were solubilized in sample buffer, and 10 μg protein homogenates were loaded on 4% polyacrylamide gels. Separated proteins were transferred onto nitrocellulose membranes after SDS-PAGE. Western blot was used to detect sulfenic acid-containing proteins (biotinylated proteins were visualized by peroxidase-labeled streptavidin and ECL).

2.10. Western immunoblot

Samples separated by SDS–polyacrylamide gradient gel electrophoresis (4 and 12% gradient gels with 10 μg of protein homogenates in each lane) were transferred to nitrocellulose membranes and blocked for 30 min with 2% bovine serum albumin in Tris-buffered saline containing 0.1% Tween. Primary antibodies recognizing filamin C (Sigma, 1:100), α-actinin (Sigma 1:1,000), MYBPC3 (G-7, Santa Cruz Biotechnology, 1:20,000). and myosin light chain 1 and 2 (Developmental Studies Hybridoma Bank, maintained at the University of Iowa, Department of Biology, Iowa City, IA 52242, 1:200) were used. Membranes were washed and subsequently incubated for 30 min at room temperature in the presence of a peroxidase-labeled anti-mouse specific secondary antibody (1:40,000). Signal intensities were visualized by the ECL method.

2.11. Data analysis

Peak active force levels measured during repeated activation/relaxation cycles at various Ca^{2+} concentrations were fitted to a modified Hill equation. Values presented in the current study are given as mean ± S.E.M. Statistical significance was determined using paired or unpaired Student's *t*-test or one-way ANOVA followed by Dunnett's multiple comparison tests when appropriate. Half maximal inhibitory concentration (IC_{50}) values were calculated in nonlinear regression analyses for concentration–response relationships using GraphPad Prism 5.02 software (GraphPad Software, Inc., La Jolla, CA, USA). P values < 0.05 were considered statistically significant.

3. Results

Exposure to heme-containing solutions induced morphological and mechanical alterations in permeabilized cardiomyocytes of human hearts. Fig. 1A illustrates a typical experiment where, following a 20 min incubation in the presence of 10 μM heme, F_o decreased by 45.7%, while $F_{passive}$ increased by about 474.75%. Moreover, these mechanical changes were associated with the deterioration of the cross striation pattern of the cardiomyocyte preparation. Cumulative concentration dependencies of heme-induced mechanical effects were obtained during repeated

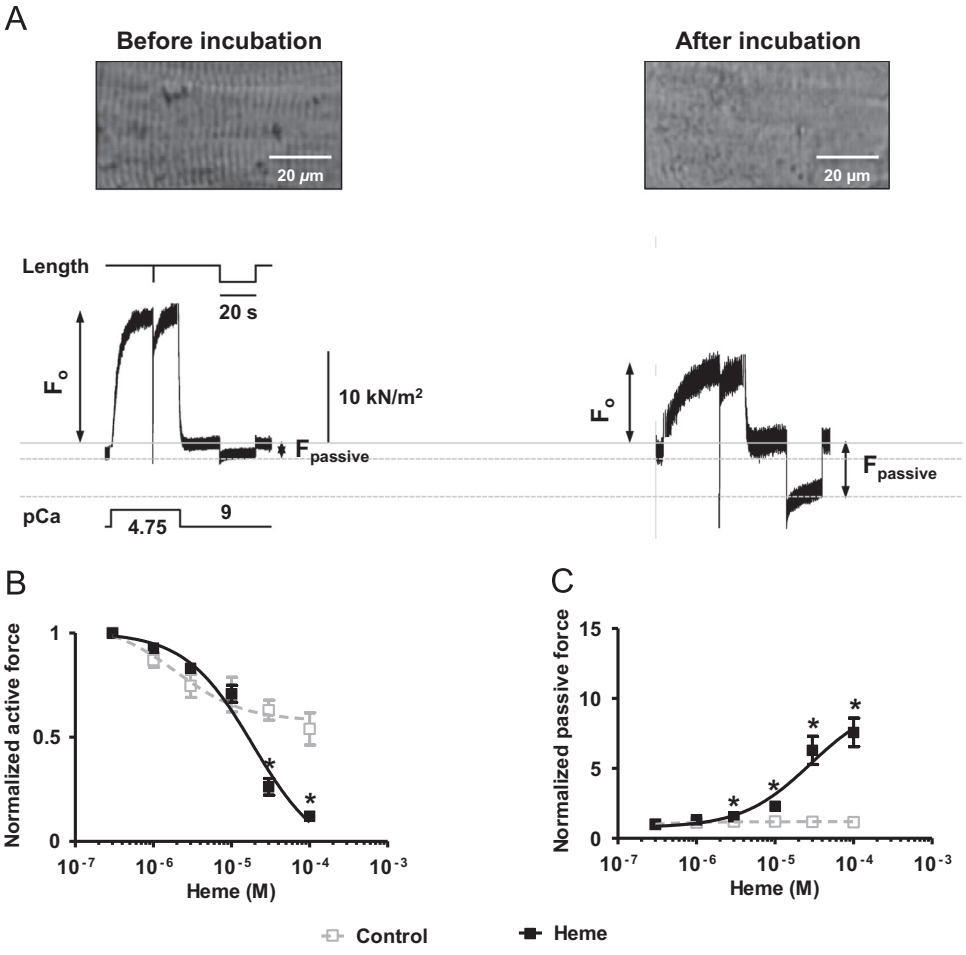

Fig. 1. Morphological and functional changes in a human cardiomyocyte preparation following heme exposure. (A) A permeabilized cardiomyocyte was incubated in the presence of 10 μM heme for 20 min. In the upper panels photomicrographs of a single isolated human cardiomyocyte attached to needles by silicon adhesive illustrate the normal cross striation pattern before and its deterioration after heme incubation. Below, original force recordings illustrate corresponding changes in F_o and $F_{passive}$. (B) Normalized concentration response curves of heme for F_o and (C) $F_{passive}$ (closed symbols) together with their corresponding time controls (open symbols). Data are means ± SEM and statistical significance is expressed as ∗ when $P < 0.05$ throughout all figures (control: $n=4$, heme: $n=8$).

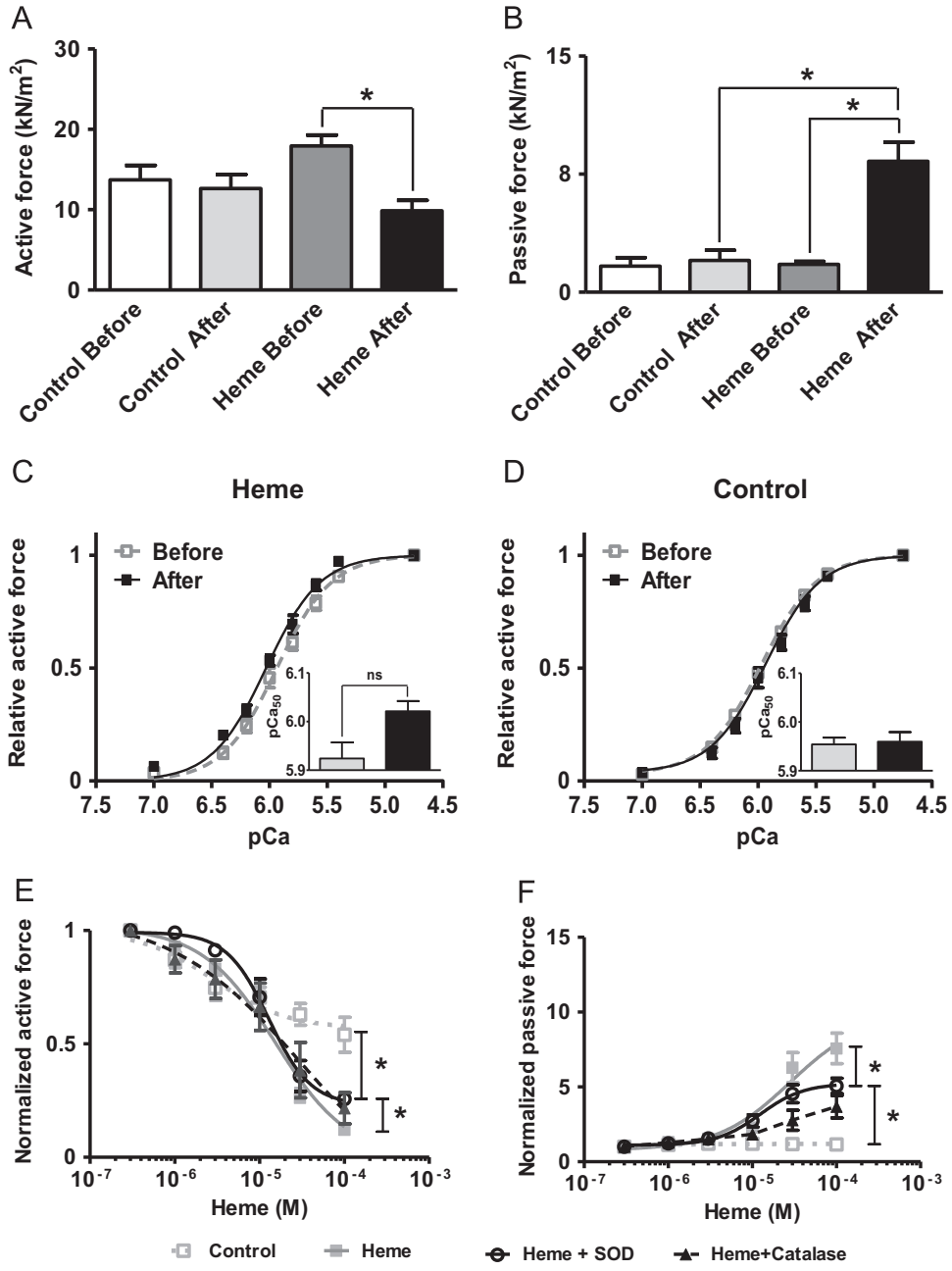

Fig. 2. Effects of 10 μM heme on F_o, $F_{passive}$, and Ca^{2+}-force relationships. (A) Upon exposure to 10 μM heme F_o decreased, while (B) $F_{passive}$ increased significantly. The pCa–relative force relationship constructed from the force recording indicated that 10 μM heme decreased maximal Ca^{2+}–activated force close to ~50% at various Ca^{2+} concentrations. When peak active forces were normalized to their respective maxima following incubation in the presence of 10 μM heme (C) or the vehicle (control) (D) for 20 min, no significant differences could be observed between the means of pCa_{50} values of isometric force productions. The concentration responses of heme-mediated effects on F_o (E) and $F_{passive}$ (F) are also shown. Data are means ± SEM and statistical significance is expressed as ∗ when $P < 0.05$ throughout all figures (number of observations: control, 7; heme, 8; heme+SOD, 5; heme+catalase, 5).

activation–relaxation cycles in cardiomyocytes following exposures to progressively increasing heme concentrations (1, 3, 10, 30, and 100 μM), and the recorded force values were contrasted with those obtained during time controls (i.e., repeated activation–relaxation cycles in the absence of heme). The results showed that F_o decreased in a concentration-dependent manner from 100% to 11.27 ± 2.67% with an IC_{50} of 19.7 ± 2.83 μM (Fig. 1B). Heme-induced decreases in F_o were anti-paralleled by the increases in $F_{passive}$ with heme concentrations, where the first significant effect was seen at 3 μM (increase to 154 ± 8.2%, $P < 0.05$) and the highest effect was seen at 100 μM heme (increase to 644.9 ± 66.48%, $P < 0.05$) (Fig. 1C).

To obtain a more detailed picture of heme-induced mechanical effects, force recordings were repeated at various submaximal [Ca^{2+}] levels before and after the application of a single concentration of heme (10 μM) (Fig. 2). Heme decreased the maximal Ca^{2+}-activated force generation (F_o) from 18.83 ± 1.48 to 10.47 ± 1.3 kN/m^2 ($P < 0.05$) (Fig. 2A), while incubation with the solvent was without effect (from 14.54 ± 1.74 to 13.83 ± 1.67 kN/m^2) (Fig. 2A). Incubation with heme increased the Ca^{2+}-independent passive force ($F_{passive}$) from 1.78 ± 0.18 to 8.32 ± 1.20 kN/m^2 ($P < 0.05$) (Fig. 2B), while the solvent was without effect (from 1.11 ± 0.15 to 1.45 ± 1.19 kN/m^2) (Fig. 2B). Heme decreased Ca^{2+}-activated force production at submaximal Ca^{2+} levels in a concentration-dependent manner. To visualize potential differences in the Ca^{2+}–force relations before and after

heme treatments, peak active forces at submaximal Ca^{2+} levels were normalized to their respective maximal value before and after heme exposure. This analysis did not reveal significant changes in the Ca^{2+} sensitivity of force production either in the heme-treated group of cardiomyocytes (Fig. 2C) or in the control group (incubation with the solvent of heme, Fig. 2D). The effects of SOD and catalase were also tested on the heme-induced changes in cardiomyocyte force generation. Limited effects for SOD and catalase were found on F_o at maximal (100 μM) heme concentration (normalized forces: control, 0.50 ± 0.09; heme, 0.12 ± 0.02, $P < 0.05$ versus control; heme+SOD, 0.26 ± 0.02, $P < 0.05$ versus heme alone; heme+catalase, 0.22 ± 0.07, $P < 0.05$ versus heme alone) (Fig. 2E) and on $F_{passive}$ (normalized forces: control, 1.43 ± 0.27; heme, 7.57 ± 1.02, $P < 0.05$ versus control; heme+SOD, 5.07 ± 0.51, $P < 0.05$ versus heme alone; heme+catalase, 3.67 ± 0.75, $P < 0.05$ versus heme alone) (Fig. 2F).

Next, we set out to investigate the effect of a potential interaction between heme and H_2O_2 on cardiomyocyte function. The selected concentrations of H_2O_2 and heme (30 and 3 μM, respectively) had only negligible effects on cardiomyocyte mechanical

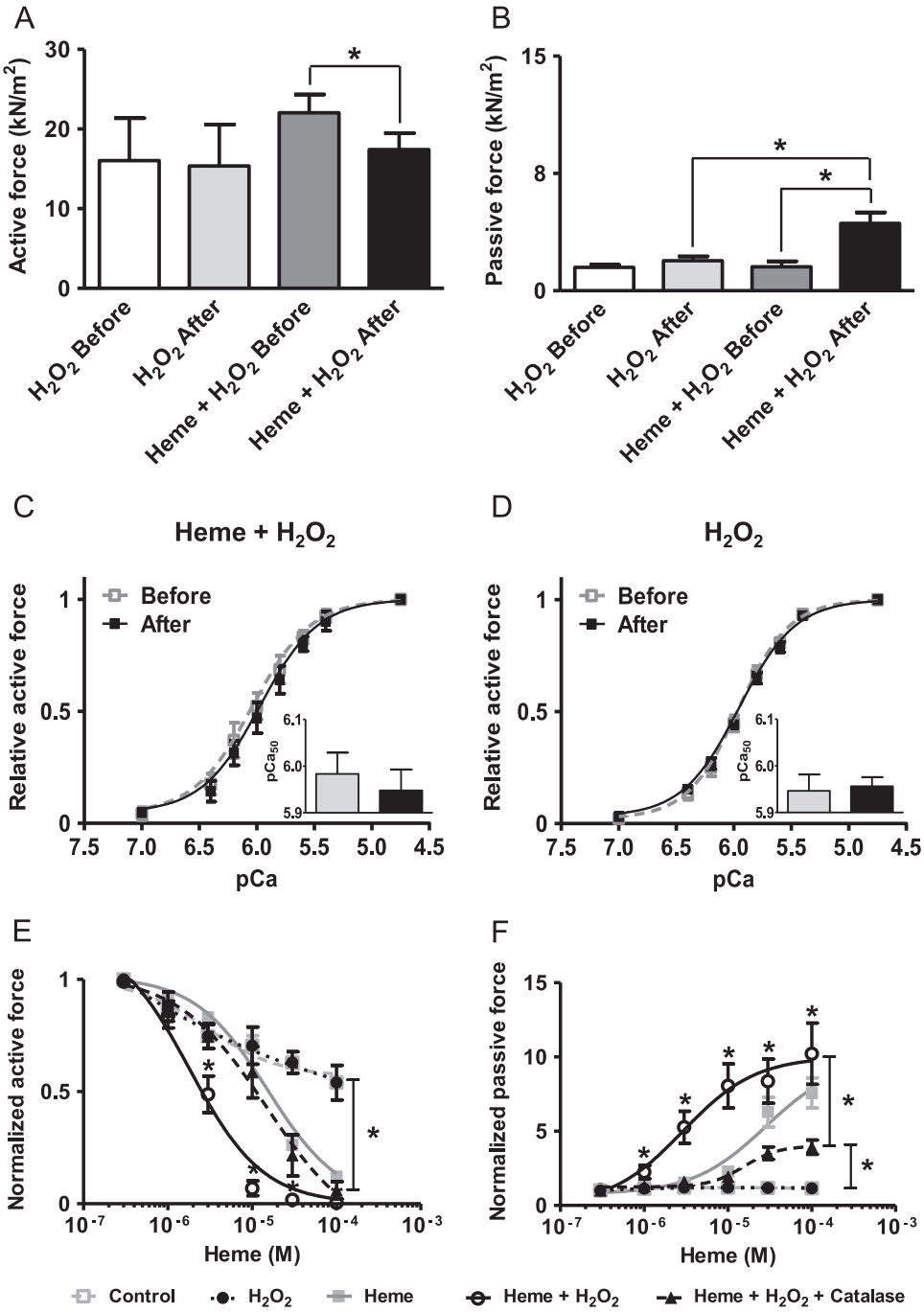

Fig. 3. H_2O_2 augments the heme induced mechanical effects. Mean values are shown for the effects following 20 min incubation in the presence of 3 μM heme in cardiomyocyte preparations for F_o (A) and $F_{passive}$ (B). pCa-normalized force relationships with mean pCa_{50} values corresponding to conditions before and after incubations in the presence of H_2O_2 in the presence (C) or in the absence (D) of 3 μM heme. Normalized heme concentration response curves for F_o and (E) $F_{passive}$ (F) in the absence (open squares) and presence (closed circles) of 30 μM H_2O_2, heme alone (closed squares), heme and H_2O_2 (open circles), and heme and H_2O_2 and catalase (closed triangles). Data are means ± SEM and statistical significance is expressed as * when $P < 0.05$ throughout all figures (control $n=4$, H_2O_2 $n=5$, heme $n=8$, heme+H_2O_2 $n=8$, heme+H_2O_2+catalase $n=5$).

function when employed alone. This combination of heme+H_2O_2 evoked a decrease in F_o from 21.4 ± 2.6 to 16.5 ± 2.3 kN/m^2 ($P < 0.05$), while no effect of H_2O_2 alone was observed (F_o from 15.97 ± 7.54 to 15.31 ± 7.39 kN/m^2) (Fig. 3A). Similarly, $F_{passive}$ increased from 1.9 ± 0.3 to 4.9 ± 0.6 kN/m^2 ($P < 0.05$), while H_2O_2 alone was without effect (from 1.35 ± 0.06 to 1.65 ± 0.14 kN/m^2)

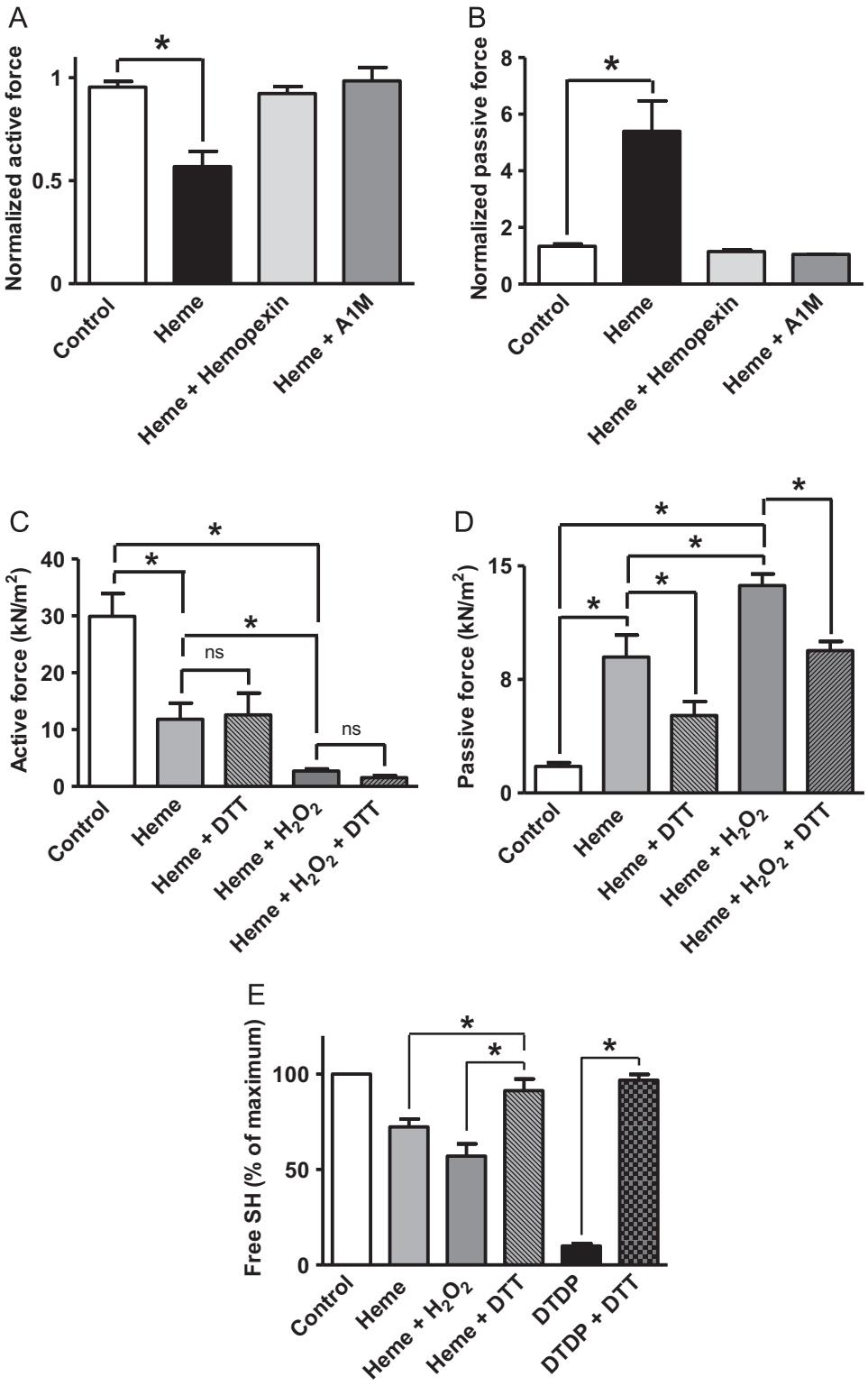

Fig. 4. Mechanical and biochemical parameters reflecting heme-evoked oxidative effects. Normalized force values for solvent (control) heme, heme+hemopexin and for heme+alpha-1-microglobulin (A1M) are shown for F_o (A) and $F_{passive}$ (B). Controls before treatments are taken as 1.0. F_o values are 0.95 ± 0.02% ($n=5$), 0.51 ± 0.06% ($n=5$), 0.92 ± 0.03% ($n=3$), 0.98 ± 0.06% ($n=3$), respectively. $F_{passive}$ values are 1.33 ± 0.07% ($n=5$), 5.39 ± 1.07% ($n=5$), 1.14 ± 0.06% ($n=3$), 1.05 ± 0.01% ($n=3$), respectively. (C) F_o dropped following exposure to 20 μM heme alone or in combination with 30 μM H_2O_2 and did not recover after a subsequent exposure to 10 mM DTT (30 min). In contrast, the increase in $F_{passive}$ generated by 20 μM heme alone or in combination with 30 μM H_2O_2 (D) was partially reversed by 10 mM DTT ($n=5$). (E) Relative protein SH group content as reported by Ellman's assays showed that heme promoted SH group oxidation (in the absence and presence of H_2O_2) and that DTT could reverse this effect similarly to that induced by DTDP ($n=3$). Data are means ± SEM and statistical significance is expressed as * when $P < 0.05$ throughout all figures.

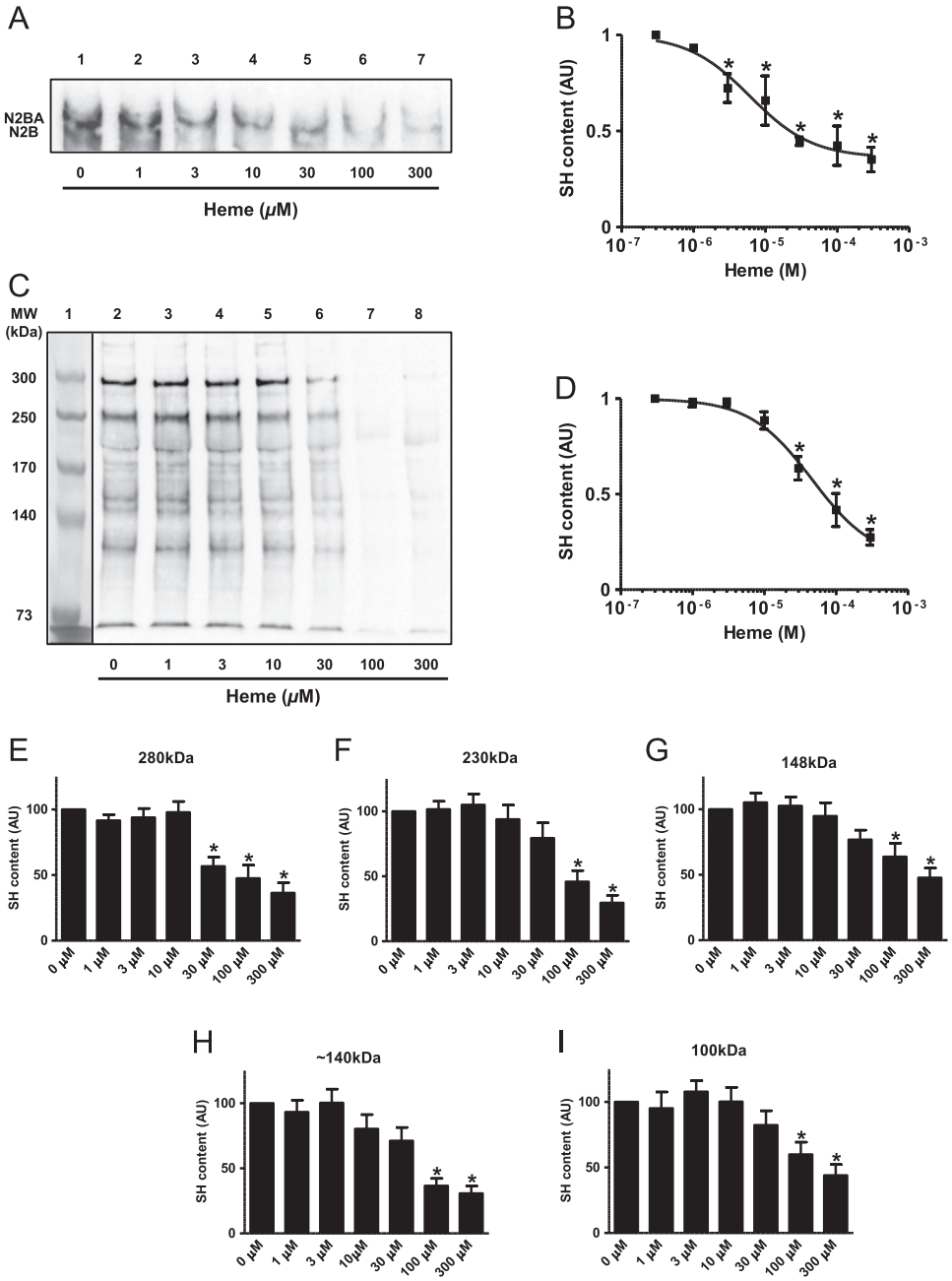

Fig. 5. Determination of heme-dependent SH group oxidation in myocardial proteins by biotinylation assays. (A) 1.5% acrylamide strengthened by 3% agarose gel followed by western immunoblotting show significantly SH group oxidation on titin from 3 μM heme. Typical result of a SDS-PAGE using a 4% gel followed by western immunoblotting (C) illustrating increased SH group oxidation with added heme (lanes 6, 7, and 8). Cumulative signal intensities of titin (B) and all proteins (D) allowed an approximation SH group oxidation from the quantitative viewpoint (AU: arbitrary units, $n=3$). Proteins at molecular weights of 280, 230, 148, 140, and 100 kDa (comigrating with filamin C (280 kDa, E), MHC (230 kDa, F), cMyBPC (148 kDa, G), and α-actinin (100 kDa, I) and a protein close to 140 kDa (H) were all susceptible for heme-induced SH group oxidation. Data are means ± SEM and statistical significance is expressed as ∗ when $P<0.05$ throughout all figures.

(Fig. 3B). As a next step, force recordings were repeated at various submaximal [Ca^{2+}] levels before and after the application of a single heme concentration (3 μM) in the presence of H_2O_2 (30 μM) to test a potential effect of the combination of H_2O_2 and heme on Ca^{2+} sensitivity of force production. The normalized Ca^{2+}–force relationship did not reveal statistical differences in the means of pCa_{50} values in this double-treated group (5.96 ± 0.04 vs. 5.96 ± 0.02, before and after incubation, respectively) (Fig. 3C) or in the H_2O_2-treated group (6.03 ± 0.07 vs. 5.97 ± 0.07, before and after incubation, respectively) (Fig. 3D). As a next step, a concentration–response relationship was obtained with increasing heme concentrations in the presence of 30 μM H_2O_2 and the results were contrasted with those in the absence of H_2O_2 and with time controls (recorded in the presence of the solvent). The results of force recordings in these protocols revealed that the applied 30 μM H_2O_2 alone was without effect (Figs. 3E and 3F); nevertheless it enhanced the heme-induced negative effects: i.e., the concentration–response curve of heme was shifted to the left, resulting in a reduced heme concentration value for its IC_{50} (3.48 μM) on F_o (Fig. 3E). Similarly to the above findings, the increase in $F_{passive}$ was also augmented by H_2O_2, and its heme-concentration dependency was also shifted to the left relative to that in the absence of H_2O_2 (Fig. 3F). This H_2O_2-mediated enhancement of the heme effect was blocked by catalase on F_o (Fig. 3E) and on $F_{passive}$ (Fig. 3F).

Both hemopexin and alpha-1-microglobulin (A1M) blocked heme

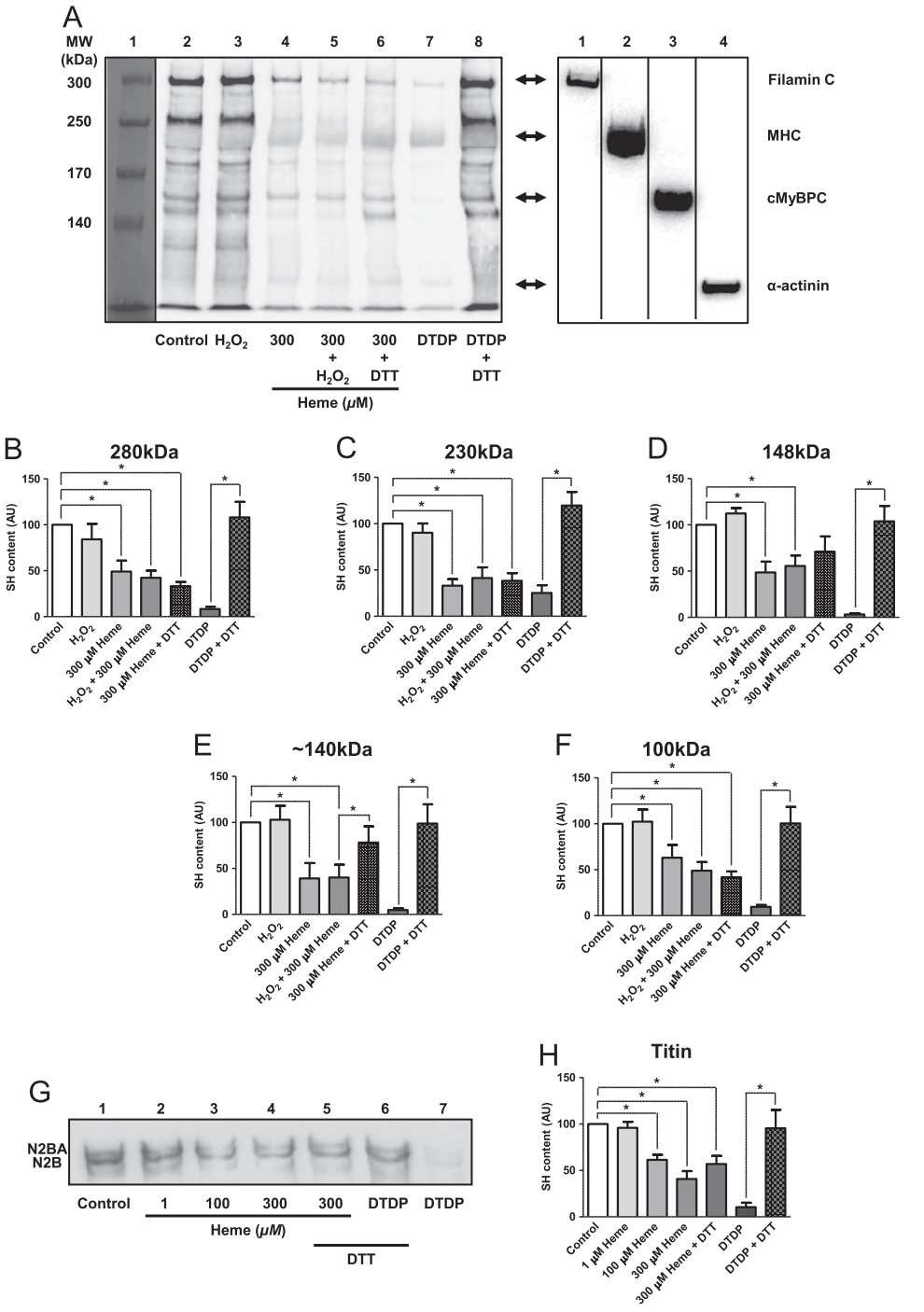

Fig. 6. Partial reversibility of heme-induced SH group oxidation in protein biotinylation assays. (A) Typical result of SDS-PAGE with 4% gel followed by western immunoblotting illustrating SH group oxidation in proteins with various molecular weights following incubation with 30 μM H_2O_2 with or without 300 μM heme and 50 mM DTT. Representative western blots are also shown on filamin C, MHC, cMyBPC, and α-actinin in the same samples as reference. Complete oxidation by DTDP was reversed by the addition of DTT. Changes in signal intensity at the molecular weights of 280 kDa (comigrating with filamin C, B), 230 kDa (comigrating with MHC, C), 148 kDa (comigrating with cMyBPC, D), 140 kDa (E), and 100 kDa (comigrating with α-actinin, F) were quantified. 1.5% acrylamide strengthened by 3% agarose gel followed by western immunoblotting showed partial reversibility of titin after 300 μM heme (G-H). Complete oxidation by DTDP was reversed by the addition of DTT (AU: arbitrary units, $n=3$). Data are means ± SEM and statistical significance is expressed as * when $P<0.05$ throughout all figures.

effects on F_o (normalized forces for control, heme, heme+hemopexin, and heme+A1M were 0.95 ± 0.02%, 0.51 ± 0.06%, 0.92 ± 0.03%, and 0.98 ± 0.06%, respectively) (Fig. 4A) and $F_{passive}$ (normalized forces for control, heme, heme+hemopexin, and heme+A1M were 1.33 ± 0.07%, 5.39 ± 1.07%, 1.41 ± 0.06%, and1.05 ± 0.01%, respectively) (Fig. 4B). To test whether the heme-induced mechanical effects were reversible, cardiomyocytes were exposed (for 30 min) to the reducing agent DTT (10 mM) after exposure to 20 μM heme. Upon heme administration, F_o first decreased from 29.92 ± 4 to 11.82 ± 2.82 kN/m^2 ($P<0.05$), and this reduction could not be reversed by DTT (F_o: 12.58 ± 3.84 kN/m^2, $P>0.05$) (Fig. 4C). DTT was also ineffective in reversing heme+H_2O_2-mediated effects on F_o (F_o for heme+H_2O_2 was 2.72 ± 0.37 kN/m^2 and 1.56 ± 0.35 kN/m^2 for heme+H_2O_2+DTT) (Fig. 4C). In contrast, after heme exposure, $F_{passive}$ increased from 1.76 ± 0.004 to 8.94 ± 1.48 kN/m^2 ($P<0.05$) and DTT partially restored this increase in $F_{passive}$ (i.e., it was decreased by 42.5% to

Table 1
Protein identification by mass spectrometry.

Protein	Peptide sequence
Filamin C	YGGPQHIVGSPFK
Myosin 7 MHC	AGLLGLLEEMRDER
	KGSSFQTVSALHR
	KVQHELDEAEER
	MFNWMVTR
	SVNDLTSQR
	AVVEQTER
	MVSLLQEK
	LDEAEQIALKGGKK
	LFDNHLGK
	QLDEKEALISQLTR
cMyBPC	LLCETEGR
	LTVELADHDAEVK
	NGLDLGEDAR
	VGQHLQLHDSYDR
	VIDVPDAPAAPK
	YGLATEGTR
	ATNLQGEAR
	PEPGKKPVSAFSK
	RVHSGTYQVTVR
	QGVLTLEIR
	YIFESIGAK
α-actinin	ATLPEADGER
	DYESASLTEVR
	LASELLEWIR
	MVSDIAGAWQR
	TINEVETQILTR
	VIQSYNIR
	VLAVNQENER

5.11 ± 0.93 kN/m^2, $P < 0.05$) (Fig. 4D). Similarly, DTT was also effective to reverse heme + H_2O_2-mediated effects on $F_{passive}$ ($F_{passive}$ for heme + H_2O_2 was 13.70 ± 0.08 kN/m^2 and 9.40 ± 0.06 kN/m^2 for heme + H_2O_2 + DTT) (Fig. 4D). The potential involvement of SH group oxidation was confirmed using Ellman's assays, as demonstrated in Fig. 4E. The relative SH group content of cardiomyocyte proteins (untreated control: 100%) decreased following 20 μM heme treatments (to 72.33 ± 4.16%, $P < 0.05$) and following the combination of this heme concentration with 30 μM H_2O_2 to 57.04 ± 6.42% ($P < 0.05$). Exposure to the SH oxidant DTDP (2.5 mM) served as a positive control in these assays and decreased the reduced SH content to 9.94 ± 1.35% ($P < 0.05$). Following DTT treatments, the reduced SH group content of cardiomyocytes was fully restored (i.e., it reached 91.34 ± 6.13%, $P < 0.05$, following heme treatment and 96.83 ± 2.98%, $P < 0.05$, following DTDP exposure).

To obtain further insight into the heme-induced oxidative processes, a protein biotinylation assay was employed, allowing the identification of changes in the levels of protein SH group oxidation for individual proteins (Fig. 5). To this end, suspensions of permeabilized cardiomyocytes were exposed to increasing heme concentrations (from 0 to 300 μM) and subsequently the protein SH groups were biotinylated. Then myofilament proteins were separated by SDS-PAGE on 1.5%, 4%, or 10% gels, followed by western immunoblotting. The observed signal intensities were considered to be proportional to the relative levels of SH group content of myocardial proteins. In 1.5% gels, two titin isoforms and clear reductions in their intensities could be observed following treatments (Fig. 5A), and decreasing SH group content was detected at 3 μM heme concentrations and higher (Fig. 5B). Both in 4% and in 10% SDS-PAGE gels, decreasing SH group content was observed at heme concentrations of 30 μM and higher, showing almost complete oxidation of all myofilament proteins in the presence of 300 μM heme (Figs. 5C and 5D). Calculated IC_{50} values for the apparent SH oxidative effects of heme were 43.2 ± 9.8 and 57.1 ± 13.9 μM for the 4% and 10% gels, respectively ($P > 0.05$). Moreover, the heme-concentration dependencies of SH-group oxidation in individual proteins appeared to be very similar, as indicated by the protein bands at molecular weights of 280 kDa (comigrating with filamin C) (Fig. 5E), 230 kDa (comigrating with myosin heavy chain) (Fig. 5F), 148 kDa (comigrating with cardiac myosin binding protein C) (Fig. 5G), 140 kDa (Fig. 5H), and 100 kDa (comigrating with α-actinin) (Fig. 5I).

Next we set out to evaluate the potential reversibility of the observed decreases in SH group oxidation using a single high concentration of heme in the presence of H_2O_2, followed by the application of the reducing agent DTT (50 mM) (Fig. 6A). In addition, western immunoblots were used to confirm the mobility of selected myofilament proteins (Fig. 6A). During these assays, the oxidative effects of heme appeared to be comparable to those found in the absence of H_2O_2. Interestingly, reversal of SH group oxidation by DTT could be observed only in some of the proteins investigated. For example, partial restoration of SH group content was observed in the 140 kDa band (SH content decreased to 39.23 ± 16.55% then recovered to 78.21 ± 17.52% following DTT, $P < 0.05$) (Fig. 6E). In contrast, no similar recoveries in SH group oxidation by DTT were observed either for the 280 kDa band (comigrating with filamin C, from 49.12 ± 11.78 to 33.24 ± 4.5%) (Fig. 6B), for the 230 kDa band (comigrating with myosin heavy chain, from 33.08 ± 6.99 to 38.33 ± 8.17%) (Fig. 6C), for the 148 kDa band (comigrating with cardiac myosin binding protein C, from 48.56 ± 11.63 to 71.06 ± 16.39%) (Fig. 6D), or for the 100 kDa band (comigrating with α-actinin, from 63.06 ± 13.82 to 41.76 ± 6.35%) (Fig. 6F). H_2O_2 alone did not evoke changes in the SH content of proteins, whereas oxidation and reduction of protein SH groups could be clearly followed by DTDP exposures followed by DTT treatment. SH content of titin decreased from 61.47 ± 5.35% (100 μM) to 40.78 ± 8.47% by 300 μM heme and was not reversed by DTT (SH content being 56.92 ± 8.7% after DTT) (Figs. 6G and 6H). Identities of the proteins were also confirmed by mass spectrometry. Some protein fragments were identified from the excised bands (Table 1). These peptides corresponded with the comigrating proteins detected by western blot (Fig. 6A).

Sulfenic acid formation was also tested upon the same treatments (Fig. 7). Sypro staining was used to visualize the protein content in the samples (Fig. 7A). This was followed by the detection of sulfenic acid residues on the same blots (Fig. 7B). Three protein bands were identified with sulfenic acid modification at apparent molecular masses of 230, 148, and 100 kDa. Sulfenic acid-specific signal was evaluated by densitometry (Figs. 7C–7E). Among these groups only those with heme showed elevated levels of sulfenic acid content, but these elevations were noted for each protein band mentioned above. In particular, 300 μM heme evoked an increase from 100% (control) to 218.04 ± 39.72% ($P < 0.05$), which was not affected by the presence of H_2O_2 (278.20 ± 41.42%) nor reversed by DTT (339.31 ± 57.82%) in the case of the 230 kDa protein (Fig. 7C). DTDP alone or in combination with DTT had no effect on protein sulfenic acid formation, as expected, and served as an internal control.

As a next step we used heme magnetic beads (HMB) to identify possible interactions between heme and contractile proteins. Silver-stained SDS gels displayed a single band between 26 and 15 kDa (Fig. 8A). Western immunoblotting (WB) demonstrated the presence of MLC-1 (MW 21 kDa) and MLC-2 (MW 17 kDa) in a control sample and the ability of MLC-1 to bind to HMB (Fig. 8A, right panel). Mass spectrometry was performed and the myosin light chain 1 (MLC-1, UniProtKB/Swiss-Prot number P08590) protein was identified with 59% sequence coverage (Fig. 8B) encompassing five different peptide sequences (ALGQNPTQAEVLR, HVLATLGER, ITYGQCGDVLR, NKDTGTYEDFVEGLR, and VFDKEGNGTVMGAELR) (Fig.8C).

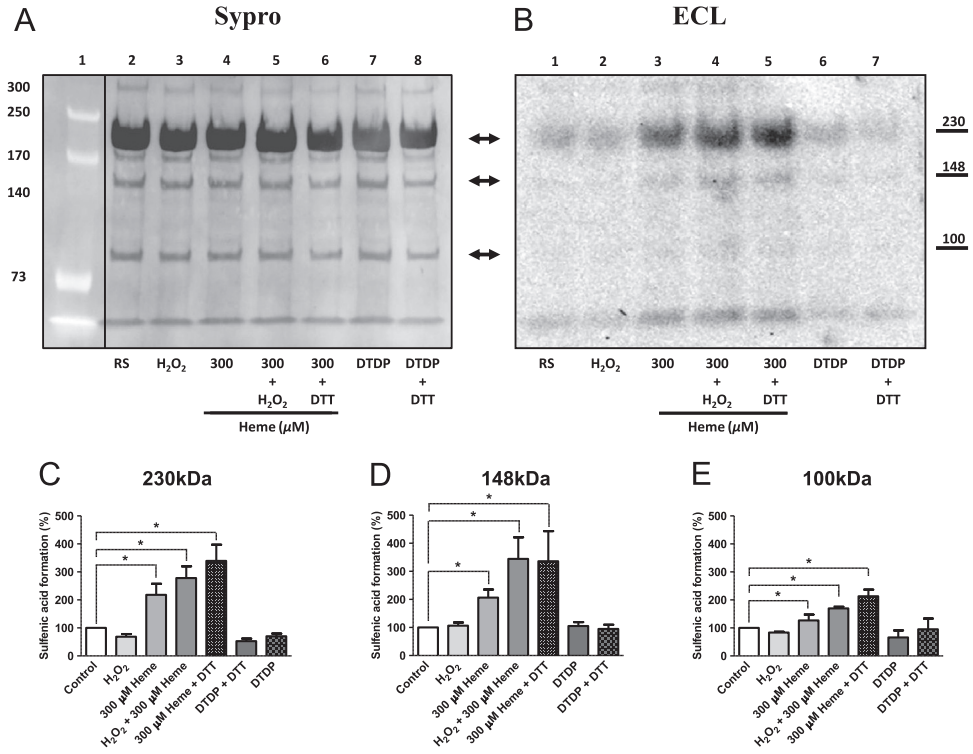

Fig. 7. Sulfenic acid formation upon heme treatments. (A) Typical result of SDS-PAGE with 4% gel followed by western immunoblotting. (B) Sulfenic acid formation in proteins with various molecular weights following incubation with 30 μM H_2O_2, 300 μM heme, 30 μM H_2O_2+300 μM heme, 30 μM H_2O_2+300 μM heme+50 mM DTT, 2.5 mM DTDP, and 2.5 mM DTDP+50 mM DTT. Proteins with the apparent molecular masses of 230 kDa (comigrating with MHC, C), 148 kDa (comigrating with cMyBPC, D) and 100 kDa (comigrating with α-actinin, E). The number of independent observations is 3–4. Bars show means ± SEM and statistical significance is expressed as ∗ when $P < 0.05$ throughout all figures.

4. Discussion

Results of this study revealed free heme as a potential limiting factor of the contractile function of the human heart. Heme-catalyzed pro-oxidative insults compromised the function of myofilaments, leading to reductions in Ca^{2+}-regulated force production and to robust increases in Ca^{2+}-independent passive force. These changes can lead to the development of systolic and diastolic dysfunction during clinical conditions, complicated by increased free heme levels.

The physiological plasma level of free heme is considered to be less than 1 μM, but in hemolytic diseases it can be as high as 20 μM in SCD [17] or even higher (between 50 and 280 μM) in thalassemia [44]. In this in vitro study the heme concentrations employed ranged from the physiological to the pathological levels (0–300 μM). There is considerable evidence for the involvement of oxidative stress in several cardiovascular disorders [1,45,46]. The inclusion of H_2O_2 in our system enhanced the toxic effects of heme at lower heme concentrations. In this respect it is surprising that the addition of exogenous peroxide exacerbates some impacts of heme contractile dysfunction, yet did not appear to further augment SH oxidation. There are some potential explanations for additional posttranslational modifications alternative to thiol oxidation: (1) H_2O_2 produces hydroxyl radical (OH) and protein carbonyls by Fenton reactions [47]; (2) H_2O_2 causes cross-linking of the heme to proteins at tyrosine residues [48]; and (3) H_2O_2-mediated alteration of heme produces iron hydroxychlorine formation [49]. Moreover, sulfenic acid formation was also observed in a heme-dependent fashion, which was not affected by the presence of H_2O_2 nor reversed by DTT. Sulfenic acid (SOH) formation is regarded as a transient redox state for the sulfur (oxidation number is 0), which is readily converted to the initial thiol (SH, oxidation number is -2) or disulfide (S–S, oxidation number is -1) or the overoxidized sulfinic acid (SO_2H, oxidation number is +2). Indeed, sulfenic acid-containing organic compounds are reported to be reversed to thiol by reducing agents, such as DTT [50]. As a matter of fact, it was a challenge to synthesize stable sulfenic acid-containing molecules, as reviewed by Lo Conte and Carroll [51]. However, protein cysteinyl may be modified to yield relatively stable sulfenic acids (Cys-SOH) depending on the chemical environment of the cysteinyl residue. In particular, the Cys-SOH-4-(ethylthio)cyclopentane-1,3-dione adduct was found to be resistant to DTT in the C46 of mutated (C165S) bacterial protein alkyl hydroperoxide reductase (AhpC), where C165S mutation was necessary to stabilize the Cys-SH in C46 to sulfenic acid (Cys-SOH in C46), allowing the quantitative analysis of sulfenic acid formation [43]. This suggests that sulfenic acids can be stabilized by intramolecular interactions within proteins, and their 4-(ethylthio)cyclopentane-1,3-dione adduct can be resistant to reducing agents, such as DTT. As a matter of fact, we have identified three sulfenic acid-containing proteins in human cardiomyocytes, which were resistant to 50 m*M* DTT after reacting with biotin tagged 4-(ethylthio)cyclopentane-1,3-dione), which was claimed to be specific for sulfenic acid [43]. These data suggest that heme evokes protein sulfenic acid formation, which leads to stable Cys-SOH formation in some human myofibrillar proteins. This process may represent a new regulatory mechanism in myocardial tissues. Accordingly, some of the oxidative effects of heme, which cannot be reversed by DTT, may be mediated by sulfenic acid formation in myofilament proteins with molecular masses of 230, 148, and 100 kDa (comigrating with myosin heavy chain, cardiac myosin binding protein C, and α-actinin). It is important to note that some proteins are implicated in the study according to their apparent comigration with major myofilament proteins (as assessed by western blot on different membranes of the same samples) or by direct mass spectroscopy on the excised bands.

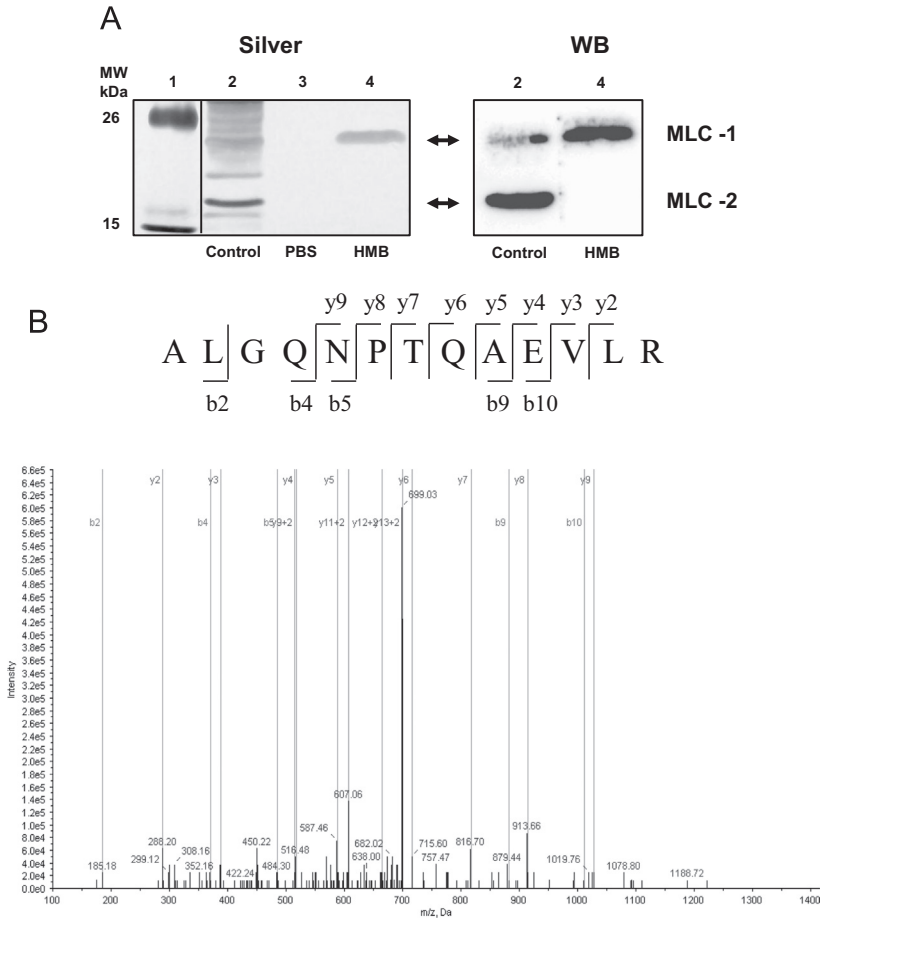

Fig. 8. Myosin light chain 1 identification. (A) Silver-stained gels after SDS-PAGE show the molecular weight standard (lane 1), a control cardiomyocyte sample (lane 2), the loading control (lane 3), and the eluent of the heme magnetic beads (HMB, lane 4). Western immunoblot was also performed on samples loaded on lane 2 (control) and 4 (HMB eluent). The blot was developed by specific antibody for MLC (but not selective to either isoform). Mass spectrometry analysis of overlapping of the amino acid sequences of the HMB eluted protein and MLC-1 (B). The LC-MS/MS spectra of the most common peptide sequences are indicated. The identified peptides are highlighted with bold letters (C).

There are different cellular mechanisms to control the levels of free heme, one of the most important being HO-1 acting as a cardioprotective mechanism by metabolizing heme and generating less toxic by-products (e.g., carbon monoxide, bilirubin) [52]. Another potentially important player in this system is hemopexin, a scavenger protein, which binds heme with high affinity [53,54]. In this study we used skinned cardiomyocytes lacking protective mechanisms; therefore these preparations allowed testing the direct effects of heme on the contractile protein machinery of human cardiomyocytes. The first alteration that we observed following heme exposure was disruption of the cross-striation pattern, meaning that heme has the potential to disturb the cytoskeletal fine architecture in cardiomyocytes [17,55]. This alteration per se may modify the mechanical integrity and functionality of sarcomeric proteins. The observed ultrastructural alterations are consistent with those seen earlier in primary cell cultures by others [19]. Importantly, both hemopexin and another heme-binding protein, A1M, completely blocked the heme-mediated effects on cardiomyocyte contractility. This implies that the first steps of heme-induced effects may involve binding of free heme to myofilament proteins, in particular to MLC-1, as shown here. Further, it appears that proteins with heme-binding abilities may protect cardiomyocytes from heme-evoked oxidation and/or allosteric interactions. On the other hand, heme binding to MLC-1 may direct oxidative effects of heme toward other myofilament proteins. Furthermore, we demonstrate that heme oxidizes filamin C, which is an actin crosslinking protein localized in the Z disk. Its main function is to maintain the mechanical integrity of the cardiomyocytes [56].

An association between morphological changes and decreases in F_o was observed in a previous study by our research group where permeabilized human cardiomyocytes were exposed to peroxynitrite. In that study, apparent links were observed between the deterioration of the cross-striation pattern, the decrease in F_o (to 45% by 50 μM peroxynitrite), and the slight modification (a 19% increase) in $F_{passive}$. Moreover, nitration of the Z-line protein α-actinin was considered as a potential mediator of the observed mechanical changes [57]. In the present study, exposure to 10 μM heme decreased F_o about 50%, accompanied by disturbances in the cytoarchitecture of cardiomyocytes, while $F_{passive}$ was increased by a factor of ~5. Moreover, heme also induced apparently irreversible oxidation in a myofilament protein, α-actinin. Hence,

myofilament disarray following heme exposure may limit the transmission of tension along the myofibrils [58]. In this context, a previous study using rabbit skeletal muscle preparations pointed to the significance of actin polymerization, indicating that heme binding to F-actin can lead to actin depolymerization, thereby limiting sarcomeric function [22]. Although in the present study we did not aim to verify direct interactions between heme and F-actin, molecular interactions of this kind cannot be excluded.

Systolic function of the heart largely depends on the interaction between MHC and actin [59], in which cMyBPC acts as a modulator [60]. Oxidation of these myofilament proteins changes their structure and ultimately leads to functional alterations [61]. For example, MHC has been recognized not only as a powerful force generator during systolic function, but also as a protein whose SH oxidation or carbonylation decreases force production [62]. In this study, we found that following heme treatment F_o was decreased in an irreversible manner. One of the possible explanations behind this finding might be SH group oxidation of MHC, preventing normal cross bridge formation. Theoretically, an alternative mechanism could relate to disturbances in the Ca^{2+} regulation of cardiac contraction, as ROS, in general, can affect not only absolute levels of active and passive forces, but also pCa_{50} through post-translational protein modifications [61], including phosphorylation and/or oxidation of the regulatory proteins (e.g., cTnI, cTnT) [63]. Interestingly, heme did not evoke major changes in SH oxidation of regulatory proteins (data not shown), and accordingly we did not observe significant changes in the pCa_{50} values following heme exposure. Accordingly, we found direct binding of heme to MLC-1 by employing heme magnetic beads. MLC-1 promotes the binding of the myosin head to actin by stronger interaction of myosin cross-bridges, leading to force production. Muthu et al. and Wang et al. found that mutation of this protein leads to decreased active force production by decreasing the strongly attached cross-bridges, interfering with their kinetics and the amount of force generation [64,65]. Previous results in our laboratory showed this protein as being resistant to oxidation [45], and heme did not alter its SH content.

One of the most important findings of this study is the enormous increase in $F_{passive}$ upon heme exposure. $F_{passive}$ is considered a major regulator of ventricular diastolic passive stiffness depending to a large degree on the giant sarcomeric protein titin [27,66,67]. Here we found that titin is sensitive to heme oxidation and therefore we postulate that the observed SH group oxidation in titin can be involved in the increase in $F_{passive}$. Nevertheless, sarcomeric proteins other than titin may also contribute to $F_{passive}$ [68]. In this context, cMyBPC was recently implicated as a critical element of normal diastolic function by phosphorylation [69], and our present results on the oxidation of cMyBPC are in line with this proposal. Of note, the partial restoration of SH group oxidation by DTT at the level of a protein in 140 kDa mirrored the partial recovery in $F_{passive}$ in heme-treated cardiomyocytes following DTT treatment. In accordance, SOD and catalase significantly inhibited heme effects on $F_{passive}$. Nevertheless, additional mechanisms (e.g., changes in titin–actin interaction) cannot be excluded by the above findings [70].

Our results may help explain some of the pathophysiological mechanisms in chronic diseases associated with hemolysis or other disorders leading to increased interstitial heme. For example, in failing human hearts, increased levels of heme and oxidative stress were noted [18]. Moreover, in patients with ischemia–reperfusion injury, a phenomenon seen in myocardial infarction and heart transplantation, hemolysis and ROS production has been found [71]. Expression of HO-1 has a protective role in this alteration, suggesting a potential involvement of free heme in ischemia–reperfusion injury leading to cardiovascular damage. [4]. Based on our present results, this could contribute to the contractile dysfunction that is observed in patients with myocardial infarction. Furthermore, cMyBPC was recently proposed as a marker for diastolic dysfunction in patients with acute myocardial infarction [72].

Another potential medical field where our findings may have functional implications relates to hemolytic diseases. In this regard SCD is particularly interesting, because in this chronic disease left ventricular diastolic dysfunction occurs in 9.7–18% of the cases without iron overload [32]. The link between heme and cardiac dysfunction is also supported by the study from Vinchi et al., in a rat model of SCD and thalassemia where the effects of hemopexin in heme overload were also tested. Importantly, both mouse models of hemolytic diseases had systolic and diastolic dysfunction as a consequence of the release of free heme, but after treatment with hemopexin, these alterations returned completely to control levels, together with a reduction in ROS production [33]. In this context, it should be pointed out that the cardiovascular dysfunction in thalassemia has traditionally been attributed to iron overload [67]. However, diastolic dysfunction in thalassemia is also present in patients without iron overload [31], suggesting the involvement of potential alternative mechanisms. Our present results are consistent with possible pro-oxidative mechanisms where heme is involved in the development of diastolic dysfunction and to some degree also in systolic dysfunction during hemolytic disorders.

In summary, our results suggest that free heme specifically alters the thick myofilament proteins by two mechanisms, oxidation of the cysteine thiol groups and direct binding with MLC-1, leading to robust mechanical limitations in their physiological functions. The heme-induced antiparallel changes in F_o and $F_{passive}$ may potentially explain part of the systolic and diastolic cardiac dysfunction not only in some hemolytic diseases but also in heart failure and myocardial ischemia–reperfusion injury.

Acknowledgments

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