Original Contribution

Myeloperoxidase impairs the contractile function in isolated human cardiomyocytes

Judit Kalász, Enikő Pásztorné Tóth, Miklós Fagyas, Ágnes Balogh, Attila Tóth, Viktória Csató, István Édes, Zoltán Papp and Attila Borbély*

Division of Clinical Physiology, Institute of Cardiology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

Abbreviations: ACS, acute coronary syndrome; ADHP, 10-acetyl-3,7-dihydroxyphenoxazine; APF, 2-(6-(4-aminophenoxy)-3-oxo-3H-xanten-9-yl)-benzoic acid; BSA, bovine serum albumin; CAD, coronary artery disease; CI, carbonylation index; CV, cardiovascular; DNPH, 2,4-dinitrophenylhydrazine; DMF, dimethylformamide; DTDP, dithiodipyridine; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); DTT, dithiotreitol; ECL, enhanced chemiluminescence; EGTA, ethyleneglycoltetraacetic acid; F_{active}, cardiomyocyte active force; $F_{passive}$, cardiomyocyte passive force; HDL, high-density lipoprotein; HF, heart failure; HOCl, hypochlorous acid; H_2O_2, hydrogen peroxide; Iso, isolating solution; LDL, low-density lipoprotein; LV, left ventricular; MetSO, methionine sulfoxide; MHC, myosin heavy chain; MI, myocardial infarction; MLC-1, myosin light chain–1; MPO, myeloperoxidase; MPO-I, MPO inhibitor (4-aminobenzhydrazide); MyBP-C, myosin-binding protein C; N2B, stiff titin isoform; N2BA, compliant titin isoform; NAC, N-acetyl-L-cysteine; NO, nitric oxide; NOS, nitric oxide synthase; NTB, 2-nitro-5-thiobenzoic acid; PBS, phosphate-buffered saline; pCa_{50}, measure of calcium sensitivity; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; SDS, sodium dodecyl sulphate; SH, sulfhydryl; Tm, tropomyosin.

*Corresponding author at: Division of Clinical Physiology, Institute of Cardiology, Faculty of Medicine, University of Debrecen, H-4032 Debrecen, Móricz Zsigmond krt. 22., Hungary, Telephone/Fax: +36 52 255928, E-mail: borbelya@med.unideb.hu

Abstract

Purpose: We set out to characterize the mechanical effects of myeloperoxidase (MPO) in isolated left ventricular human cardiomyocytes. Oxidative myofilament protein modifications (sulfhydryl (SH) group oxidation and carbonylation) induced by the peroxidase and chlorinating activities of MPO were additionally identified. The specificity of the MPO-evoked functional alterations was tested with an MPO inhibitor (MPO-I) and the antioxidant amino acid Met.

Results: The combined application of MPO and its substrate, hydrogen peroxide (H_2O_2), largely reduced the active force (F_{active}), increased the passive force ($F_{passive}$) and decreased the Ca^{2+} sensitivity of force production (pCa_{50}) in permeabilized cardiomyocytes. H_2O_2 alone had significantly smaller effects on F_{active} and $F_{passive}$ and did not alter pCa_{50}. The MPO-I blocked both the peroxidase and chlorinating activities, while Met selectively inhibited the chlorinating activity of MPO. All of the MPO-induced functional effects could be prevented by the MPO-I and Met. Both H_2O_2 alone and MPO+H_2O_2 reduced the SH content of actin and increased the carbonylation of actin and myosin-binding protein C to the same extent. Neither the SH-oxidation nor the carbonylation of the giant sarcomeric protein titin was affected by these treatments.

Conclusions: MPO activation induces a cardiomyocyte dysfunction by affecting Ca^{2+}-regulated active and Ca^{2+}-independent passive force production and myofilament Ca^{2+} sensitivity, independently of protein SH oxidation and carbonylation. The MPO-induced deleterious functional alterations can be prevented by the MPO-I and Met. Inhibition of MPO may be a promising therapeutic target to limit myocardial contractile dysfunction during inflammation.

Keywords: cardiomyocyte contractile function, myeloperoxidase, hydrogen peroxide, oxidative post-translational protein modifications, antioxidants

Introduction

Oxidative stress-related myofilament protein alterations have been shown to play key roles in the impaired cardiomyocyte contractility in response to myocardial inflammation, ischemia-reperfusion injury and left ventricular (LV) remodeling following a myocardial infarction (MI) [1, 2]. In particular, reactive oxygen species (ROS) oxidize cellular components [3], leading to cardiomyocyte contractile dysfunction, myocyte apoptosis or cardiac hypertrophy [4, 5].

Myeloperoxidase (MPO; EC 1.11.2.2) is a member of the heme peroxidase superfamily, synthesized by neutrophils, monocytes and macrophages, stored in their azurophilic granules and released in substantial amount upon leukocyte activation [6]. MPO has beneficial effects in the innate host defense mechanisms [7]. Considerable evidence has emerged to suggest, that ROS formation by MPO promotes various deleterious action in the cardiovascular (CV) system and contributes to the development of CV diseases [6]. Individuals with a total or subtotal MPO deficiency (a defect with a frequency of ≈1 in every 2000 to 4000 Caucasians) are protected from CV diseases [6]. An elevated level of circulating MPO is a prognostic marker of mortality and predicts the risks of subsequent major adverse cardiac events in patients with acute coronary syndrome (ACS) [8], particularly in association with a low LV ejection fraction [9]. MPO also contributes to adverse LV remodeling after a MI [10]. MPO exerts adverse effects on the vasculature, oxidizes low-density lipoprotein (LDL) [11], impairs the high-density lipoprotein (HDL) function [12] and reduces the bioavailability of nitric oxide (NO) [13]. MPO can therefore serve as a valuable biomarker of inflammation in coronary artery disease (CAD) and ACS [14]. The serum level of MPO correlates positively with the severity of the LV dysfunction and seems to be an essential factor in the development and exacerbation of heart failure (HF) [15, 16]. Interestingly, the MPO concentration was earlier found not to differ in ischemic and

non-ischemic cardiomyopathy, suggesting that MPO has an independent pathogenic role in the LV dysfunction [17].

MPO is known to generate numerous reactive oxidants and diffusible radical species via its peroxidase and chlorinating activities, which are capable of promoting an array of reversible and irreversible post-translational protein modifications [18, 19]. The relative concentrations of chloride and the reducing substrate determine whether MPO uses its substrate hydrogen peroxide (H_2O_2) for peroxidation or chlorination. MPO amplifies the oxidative potential of H_2O_2 [20-22], which may originate from a number of sources *in vivo*, including leukocyte NADPH oxidases, xanthine oxidase and uncoupled NO synthase (NOS) [23, 24]. The perfusion of isolated rat hearts with H_2O_2 led to disulfide cross-bridge formation in actin and tropomyosin (Tm) [25]. In one of our previous studies, the sulfhydryl (SH) oxidation of actin and myosin light chain-1 (MLC-1) was suggested as the mechanism in the H_2O_2-evoked depressed cardiomyocyte contractility [26].

MPO is unique in its ability to create hypochlorous acid (HOCl, a potent antimicrobial agent) through its chlorinating activity [22]. Interestingly, the cardiac tissue is highly susceptible to oxidation even by physiological concentrations of HOCl [27]. Importantly, HOCl is much more effective than H_2O_2 in oxidizing proteins in the myocardium [27], it causes SH oxidation [28] and carbonylation in myofilament proteins [29], it disturbs Ca^{2+} homeostasis and Ca^{2+} handling [30], it increases the intracellular Ca^{2+} concentration in isolated rat [31] and rabbit [32] ventricular cardiomyocytes, and it induces cardiomyocyte death in rats [33]. It is also very important to consider, how far H_2O_2 or HOCl can diffuse on the cellular scale and whether these substances are capable to penetrate the cell membranes. H_2O_2 is stable [34], membrane permeable [35], although, *in vivo* concentration of H_2O_2 highly depends on its generation and consumption rates [36, 37]. HOCl appears to be more toxic and reactive and can also penetrate through cell membranes, but has a much shorter

lifespan. An *in vitro* study revealed that HOCl production by neutrophils can be as high as 450 mM/h, which was shown to be less in an *in vivo* model [38]. MPO generates HOCl in micro-molar concentration [39], but in inflammatic tissue it is estimated to be as high as 5 mM [40].

The antioxidant amino acid Met acts as a scavenger of HOCl and has been shown to prevent the HOCl-induced morphological changes and contractile dysfunction in murine myocytes [41]. Moreover, the fact that MPO-derived chlorinating compounds can serve as specific biomarkers for disease progression has attracted considerable interest in the development of therapeutically useful MPO inhibitors (MPO-Is) [42].

Although the role of MPO-derived oxidants in the pathogenesis of myocardial ischemia and HF is relatively well established, only limited data are available as concerns the exact cellular and subcellular mechanisms through which MPO could directly affect the contractility of the myocardial cells, especially at the level of the myofibrillar proteins. In this study, therefore, we set out (1) to characterize the functional effects of MPO and its substrate H_2O_2 on single, permeabilized human cardiomyocytes; (2) to identify the biochemical alterations induced by the peroxidase and chlorinating activities of MPO; (3) to investigate the specificity of the MPO-induced contractile changes by using the MPO inhibitor (MPO-I) 4-aminobenzhydrazide and the antioxidant amino acid Met; and (4) to explore the MPO-related reversible and irreversible oxidative myofilament protein modifications in the human LV myocardium.

Materials and methods

I. Human myocardial samples

LV myocardial tissue was obtained from the hearts of four general organ-donor patients (41- and 46-year-old women, and 53- and 57-year-old men). All of these patients were free of any cardiac abnormalities and had not received any medication except for plasma volume expanders, dobutamine and furosemide. The cause of death included cerebral contusion, cerebral hemorrhage and subarachnoidal hemorrhage. All biopsies were transported in cardioplegic solution (pH 7.4; in mM: NaCl 110, KCl 16, $MgCl_2$ 1.6, $CaCl_2$ 1.2, $NaHCO_3$ 5) and were frozen in liquid nitrogen and stored at -80 °C at the laboratory. The experiments on human tissues complied in full with the Helsinki Declaration of the World Medical Association and were approved by the Hungarian Ministry of Health (No. 323-8/2005-1018EKU) and by the Institutional Ethical Committee at the University of Debrecen, Hungary.

II. Force measurements in permeabilized cardiomyocyte preparations

Force measurements were performed as described previously [43]. In brief, frozen tissue samples were first defrosted and mechanically disrupted in cell isolation solution (Iso) (in mM: KCl 100, ethyleneglycoltetraacetic acid (EGTA) 2, $MgCl_2$ 1, Na_2ATP 4, imidazole 10; pH 7.0) containing phenylmethylsulfonyl fluoride (PMSF, 0.5 mM, Sigma-Aldrich, St. Louis, MO, USA), leupeptin (40 μM, Sigma, St. Louis, MO, USA) and E-64 (10 μM, Sigma-Aldrich, St. Louis, MO, USA) protease inhibitors. The mechanically isolated cells were skinned by incubation in Iso supplemented with 0.5% (v/v) Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 5 min. Triton-X-100 was removed by washing at least three times in Iso (1 ml in each washing step) and the skinned myocytes were kept in cell Iso on ice until

the measurements. A skinned single cardiomyocyte was mounted between two thin needles, which were attached to a force transducer element (SensoNor, Horten, Norway) and an electromagnetic motor (Aurora Scientific Inc., Aurora, Canada) through the use of silicone adhesive (DAP, Baltimore, MD, USA) for determination of the mechanical parameters. The measurements were performed at 15°C on the stage of a light microscope. The average sarcomere length was adjusted to 2.3 μm.

The compositions of the relaxing and activating solutions used during force measurements were calculated as described previously [43]. Both solutions were supplemented with protease inhibitors: leupeptin (40 μM) and E-64 (10 μM). The pCa, i.e. the $-\log_{10}[Ca^{2+}]$ values of the relaxing and activating solutions (pH 7.2), were 9.0 and 4.75, respectively. Solutions with intermediate free $[Ca^{2+}]$ levels were obtained by mixing activating and relaxing solutions [44]. Isometric force production was measured after the preparation had been transferred from the relaxing solution to a set of Ca^{2+}-containing solutions. When a steady force level had been reached, the length of the myocyte was reduced by 20% within 2 ms, and the myocyte was then quickly restretched (release-restretch maneuver). 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 6 s after the onset of force redevelopment, the cardiomyocyte was returned to the relaxing solution, where the length of the myocyte was again reduced by 20% for 8 s to determine the Ca^{2+}-independent passive force component ($F_{passive}$). The Ca^{2+}-activated isometric force (F_{active}) was calculated by subtracting $F_{passive}$ from F_{total}. F_{active} at submaximal levels of activation was normalized to that at maximal activation (pCa 4.75). Thereafter, the normalized force values were plotted against the Ca^{2+} concentration of the activating solutions to create a sigmoidal curve, in order to determine the Ca^{2+} sensitivity of force production (pCa_{50}). Maximal active

force was also tested at the end of the experiments at pCa 4.75. Experiments that yielded a value below 80% of the initial value were discarded.

To determine the mechanical consequences of myofilament protein oxidation, cardiomyocytes were exposed to Iso supplemented with H_2O_2 (30 μM, Sigma-Aldrich, St. Louis, MO, USA) for 15 min; MPO+H_2O_2 (8 U/l, Abcam, Cambridge, UK) for 15 min; MPO+H_2O_2+MPO-I 4-aminobenzhydrazide (50 μM, Cayman Chemicals, Ann Arbor, MI, USA) for 15 min; or MPO+H_2O_2+Met (10 mM, Sigma-Aldrich, St. Louis, MO, USA) for 15 min at 15 °C. The reversibility of MPO+H_2O_2 evoked effects were examined by the application of the reducing agent dithiotreitol (DTT, Sigma-Aldrich, St. Louis, MO, USA, 10 mM, 30 min) to MPO+H_2O_2-treated cardiomyocytes. Force-pCa relationships and pCa_{50} values were determined before and after the application of these agents. The effects of the applied agents on F_{active} and $F_{passive}$ were expressed relative to their control (untreated, before application of the agent at pCa 4.75 and pCa 9.0, respectively). Changes in F_{active} and $F_{passive}$ upon application of the agents were compared with the force values measured after incubation of the cardiomyocytes in Iso for 15 min (time control).

III. Measurements of MPO activities

MPO chlorination and peroxidation assay kits (Cayman Chemicals, Ann Arbor, MI, USA) were used. The chlorination activity assay utilizes a nonfluorescent substrate (APF, 2-(6-(4-aminophenoxy)-3-oxo-3H-xanthen-9-yl)benzoic acid), which is cleaved by the MPO-generated hypochlorite (OCl^-) to produce highly fluorescent fluorescein. The peroxidase activity assay uses a nonfluorescent substrate (ADHP, 10-acetyl-3,7-dihydroxyphenoxazine) which is converted by MPO to the fluorescent resorufin. Fluorescence was detected with a NovoStar Microplate Reader (BMG Labtech, Ortenberg, Germany) at λ_{ex} 485 nm, λ_{em} 520 nm in the chlorination assay, and at λ_{ex} 544 nm, λ_{em} 590 nm in the peroxidase assay. The reaction

solution contained the nonfluorescent substrate (APF (18 μM) or ADHP (45 μM)), assay buffer (phosphate-buffered saline (PBS), pH 7.4) and H_2O_2 (30 μM), or MPO+H_2O_2 (38 U/l), or MPO+H_2O_2+MPO-I (50 μM) or MPO+H_2O_2+Met (10 mM). Activities were measured for 5 min at 24-s intervals. Fluorescence intensities were fitted by linear regression analysis (before saturation) and the slope of this relation was used to calculate MPO activities. Values were corrected for the background (the activity determined in the absence of MPO).

IV. Biochemical assays for the identification of oxidative protein modifications

1. Ellman's reaction

Overall myofilament SH group content was determined by Ellman's reaction. Skinned cardiomyocytes were treated with Iso (time control) or with Iso supplemented with H_2O_2 and MPO as described for the mechanical experiments. Washing steps followed the treatments and the cardiomyocytes were then incubated for 15 min in Ellman's reagent (5,5'-dithio-bis(2-nitrobenzoic acid), DTNB; Sigma-Aldrich, St. Louis, MO, USA), which reacts with myofilament SH groups and produces the yellow 2-nitro-5-thiobenzoic acid (NTB). The absorbance of NTB was measured with NovoStar Microplate Reader at 412 nm. N-Acetyl-L-cysteine (NAC, Sigma-Aldrich, St. Louis, MO, USA) was used to calibrate the NTB absorbance in relation to the amount of SH groups. A known concentration of NAC was reacted with Ellman's reagent and the absorbance at 412 nm, fitted with a single exponential, served as calibration curve. The SH contents in 1-mg lyophilized myocardial samples were calculated from the measured absorbance, the tissue weight and the calibration curve. Measurements were performed in triplicates.

2. Protein SH oxidation

Cardiomyocytes were isolated from LV myocardial samples (25 mg wet weight) similarly as for the functional measurements, and were treated in Iso (150 µl) containing H_2O_2 (30 µM) or MPO+H_2O_2 (38 U/l) for 15 min. Cardiomyocytes exposed to dithiodipyridine (DTDP, 2.5 mM, for 2 min) were used as positive control. Protein SH groups were labeled with EZ-Link Iodoacetyl-LC-Biotin (Thermo Scientific, Rockford, IL, USA, for 60 min in the dark, at room temperature) in a reaction buffer (containing EDTA 5 mM, Tris-HCl 50 mM pH 8.3 and 0.1 mg/ml biotin) according to the manufacturer's instructions (biotin was solved in dimethilformamide (DMF, Sigma-Aldrich, St. Louis, MO, USA) and diluted in reaction buffer to 0.1 mg/ml). After the biotinylation process, the myocytes were solubilized in sample buffer (containing 8 M urea, 2 M thiourea, 3% (w/v) sodium dodecyl sulphate (SDS), 75 mM DTT, Tris-HCl pH 6.8, 10% (v/v) glycerol, bromophenol blue, 10 µM E-64 and 40 µM leupeptin (1 h, under continuous agitation). Protein concentration was determined in the supernatant with a dot-blot-based method, using bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) as a standard. Protein concentration was adjusted to 1 mg/ml. 2% (strengthened with 0.5% agarose), 4%, 10% and 15% polyacrylamide gels and 4-15% gradient gels (BioRad, Hercules, CA, USA) were used to separate myofilament proteins before blotting to nitrocellulose membranes. Protein was quantitated with the fluorescent Sypro Ruby Protein Blot Stain (Invitrogen, Eugene, OR, USA). Membranes were blocked with 10% (w/v) milk powder diluted in PBS containing 0.1% (v/v) Tween-20 (PBST). Biotin-labeled SH groups were probed with peroxidise-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA, USA) at a final concentration of 5 ng/ml for 30 min. Signal intensities of biotin-labeled SH groups were visualized by an enhanced chemiluminescence (ECL) method and normalized for those assessed with the Sypro Ruby Protein Blot Stain.

3. Protein disulfide cross-bridge formation

Similarly to the experiments by *Canton et al.* [45] human LV myocardial samples were solubilized in reducing (1x Laemmli sample buffer (Sigma-Aldrich, St. Louis, MO, USA) containing 2% SDS, 10% glycerol, 5% β-mercaptoethanol (β-ME), 0.0625 M Tris-HCl, pH 6.8) and non-reducing (same buffer without β-ME) sample buffer after H_2O_2 or MPO+H_2O_2 treatment. SDS-PAGE was performed using 10% polyacrylamide gels, thereafter proteins were transferred onto nitrocellulose membranes. After blocking the non-specific binding sites membranes were probed with monoclonal anti-tropomyosin (1:10.000, clone CH1) or monoclonal anti-actin (1:1000, clone HHF35, Dako Cytomation, Glostrup, Denmark) antibodies.

4. Detection of protein carbonyl groups

Cardiomyocytes from LV myocardial tissue (15 mg wet weight) were incubated with H_2O_2 and MPO, as described above. Cardiomyocytes treated with Fenton reagent (50 μM $FeSO_4$, 6 mM ascorbic acid and 1.5 mM H_2O_2 for 7 min) were used as positive controls for protein carbonylation. Cardiomyocytes were washed after treatment and solubilized in sample buffer containing 8 M urea, 3% (w/v) SDS, 50 mM Tris-HCl (pH 6.8), 10 μM E-64 and 40 μM leupeptin for 1 h by vortexing. The samples were then centrifuged (16,000 g for 5 min) and the supernatants were used for cabonyl group derivatization based on the formation of 2,4-dinitrophenylhydrazone (DNPhydrazone) from 2,4-dinitrophenylhydrazine (DNPH) (OxyBlot^{TM} Protein Oxidation Detection Kit, Millipore, Billerica, MA, USA). After derivatization (15 min), samples were centrifuged (1000 g for 1 min) and the pellet was dissolved in a buffer containing 8 M urea, 2 M thiourea, 3% (w/v) SDS, 75 mM DTT, 0.05 M Tris-base (pH 14), 10% (v/v) glycerol and bromophenol blue (30 min, shaking). Derivatized samples were centrifuged (16,000 g for 5 min) and the protein concentrations of the

supernatants were determined with a dot-blot-based method, using a BSA standard. The protein concentration of the samples was adjusted to 1 mg/ml. Polyacrylamide gel electrophoresis with 2% (strengthened with 0.5% agarose), 4%, 10% and 15% polyacrylamide gels and 4-15% gradient gels was carried out to separate myofilament proteins. Proteins were transferred onto nitrocellulose membranes and visualized with the Sypro Ruby Protein Blot Stain. The membranes were then blocked with 2% (w/v) BSA in PBST for 30 min and probed with primary and secondary antibodies (rabbit anti-DNP antibody 1:150, 1 h and goat anti-rabbit IgG 1:300, 1 hour) diluted in 1% (w/v) BSA-PBST according to the manufacturer's intructions. Protein bands were visualized by the ECL method. Signal intensities determined by OxyBlot^{TM} assay were normalized for those assessed with the Sypro Ruby Protein Blot Stain. The extent of carbonylation was expressed as carbonylation index (CI=1 in the time control samples).

V. Data analysis and statistics

Cardiomyocyte force generation was measured with a custom-built system (utilizing the DAQ platform produced by National Instruments, Austin, TX, USA) and recorded by a custom-built LabVIEW (National Instruments) module. Results were evaluated in Excel (Microsoft, 2007) and GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, California, USA).

Ca^{2+}-force relations were fitted to a modified Hill equation:

$$F_{total} = F_{max}[Ca^{2+}]^{nHill}/(pCa_{50}{}^{nHill} + [Ca^{2+}]^{nHill}) + F_{passive}$$

where F_{max} is the maximal force, $F_{passive}$ is the passive force, $F_{total} = F_{max} + F_{passive}$, $[Ca^{2+}]$ is the calculated Ca^{2+} concentration, nHill is a constant, and pCa_{50} corresponds to the $[Ca^{2+}]$ at which $F_{total} - F_{passive} = F_{max}/2$.

The results of the measurements for each cardiomyocyte were fitted individually. F_{active} and $F_{passive}$ values were normalized to the cardiomyocyte cross-sectional area and expressed in kN/m^2. The number of experiments in each group varied between 5 and 12 from 3 or 4 different hearts.

Western immunoblot assays were performed in triplicates. Intensities of protein bands were quantified by determining the area under intensity curves by a Gaussian fit using ImageJ (NIH, Bethesda, MD, USA) and Magic Plot (Saint Petersburg, Russia) software. Graphs were created in GraphPad Prism 5.0 software.

Differences between groups were calculated by analysis of variance (ANOVA followed by Bonferroni's post hoc test) or multilevel mixed-effects linear regression analysis, to appropriately address non-independence between multiple observations from the same heart. The null hypothesis for all group means being equal was tested, followed by pairwise between-groups comparisons based on the variance-covariance matrix of the fixed effects. Comparisons of normalized pCa-force relationships determined upon subsequent applications of the agents were performed with paired and unpaired t tests. Group descriptions were based on the mean and SEM values. Statistical significance was accepted at $p < 0.05$.

Results

MPO+H_2O_2 impairs the contractile function in human cardiomyocytes

When permeabilized human LV cardiomyocytes (Fig. 1A) were treated with isolating solution (Iso) containing MPO (8 U/l) and H_2O_2 (30 μM), a significant decrease in the maximal Ca^{2+}-dependent (pCa 4.75) F_{active} and a marked increase in the Ca^{2+}-independent (pCa 9.0) $F_{passive}$ were observed (to 57.7±4.1% and 179.6±14.6% of untreated, respectively, *n=12*) (Fig. 1B). The decrease in the isometric force at various free Ca^{2+} concentrations was significantly larger in response to MPO+H_2O_2 application than that in the presence of H_2O_2 alone (Fig. 1C). Incubation of cardiomyocytes with Iso (time control) resulted in only a minor change in F_{active} (to 89.0±1.6%). The MPO-induced increase in $F_{passive}$ was significanly higher than that evoked by H_2O_2 alone (79.6±14.6% vs. 23.9±7.4%, $p<0.001$) (Fig. 1D). When the peak contractile forces measured at intermediate Ca^{2+} concentrations were normalized to their respective maximum, a significant rightward shift in the pCa-force relationship, i.e. a decrease in the Ca^{2+} sensitivity of force production (pCa_{50}) was observed after MPO+H_2O_2 treatment (from 5.83±0.02 to 5.66±0.02, $p<0.001$) (Fig. 1E). In contrast, the application of H_2O_2 alone did not alter pCa_{50} (5.85±0.05 vs. 5.82±0.03, $p=0.55$) (Fig. 1F). The differences in the baseline cardiomyocyte maximal F_{active}, $F_{passive}$ and pCa_{50} were 5.4%, 5.5% and 0.9%, respectively. The light microscopic morphology did not reveal visible alterations in the cross-striation pattern of the cardiomyocytes upon MPO+H_2O_2 or H_2O_2 treatments (data not shown).

Met inhibits the chlorinating, but not the peroxidase activity of MPO

To identify the biochemical mechanism underlying the functional effects of MPO, we measured its chlorinating and peroxidase activities in the presence of the MPO-I and Met

(Fig. 2A, B). The MPO-I diminished both the chlorinating and the peroxidase activities of MPO (to 0.3±0.2% and 10.4±6.0%, respectively, $p<0.001$, *n=4*). However, Met selectively inhibited the chlorinating activity of MPO (to 2.3±1.3%, $p<0.001$, *n=4*), without significantly affecting on its peroxidase activity (78.4±8.6%, *n=4*).

MPO-I and Met completely prevent, while DTT partially reverses the MPO-induced cardiomyocyte dysfunction

To assess whether the MPO-I or Met is also able to prevent the deleterious mechanical effects of MPO, cardiomyocytes were incubated with MPO+H_2O_2 in the presence of the MPO-I (50 μM) or Met (10 mM). Both the MPO-I and Met prevented the MPO-induced decrease in F_{active} (to 80.0±5.3% and 80.1±3.6% of untreated, respectively, $p<0.001$) (Fig. 3A) and the increase in $F_{passive}$ (to 147.7±6.1% and 139.9±8.7% of untreated, respectively, $p<0.05$, *n=5-6*) (Fig. 3B). F_{active} and $F_{passive}$ measured after the application of the MPO-I or Met to MPO+H_2O_2 were similar to those determined after H_2O_2 treatment. Moreover, the MPO-I (Fig. 3C) or Met (Fig. 3D) completely abolished the rightward shift in the pCa-force relationships observed upon combined MPO+H_2O_2 treatment (5.88±0.07 vs. 5.66±0.02, $p<0.05$ and 5.81±0.04 vs. 5.66±0.02, respectively, $p<0.001$ vs. MPO+H_2O_2, *n=5-6*). The changes in pCa_{50} measured after H_2O_2, MPO+H_2O_2, MPO-I and Met treatments are illustrated in Fig. 3E. The reversibility of the MPO+H_2O_2-evoked functional alterations was tested by application of the reducing agent DTT (10 mM) to the cardiomyocytes (n=6). The increase in $F_{passive}$ after MPO+H_2O_2 ($\Delta F_{passive}$ 89.3±27.3% compared to untreated) was almost completely reversed after DTT treatment ($\Delta F_{passive}$ 9.7±10.4% compared to untreated, $p<0.05$). DTT, however, did not significantly affect F_{active} (to 57.7±4.1% and to 43.8±5.1% of untreated after MPO+H_2O_2 and DTT administration, respectively, $p=0.13$) (figure not shown).

Effects of MPO+H_2O_2 on the SH oxidation and carbonylation of myofilament proteins

Attempts were made to identify the changes in the oxidative status of myofilament proteins contributing to the MPO-induced cardiomyocyte dysfunction in parallel with the functional measurements. Relative SH contents were determined in human LV skinned cardiomyocytes. The baseline SH content of myofilament proteins in the donor heart samples varied between 98.0±4.6% and 104.1±3.9% (p=0.35). Ellman's reaction revealed a small, but significant decrease in the overall amount of SH groups in response to H_2O_2 (to 90.4±1.5%, p<0.05, n=3) or MPO+H_2O_2 treatments (to 86.7±4.0%, p<0.01, n=3) (Fig. 4A). An SH group biotinylation assay was applied to identify individual myofibrillar proteins affected by MPO-mediated SH oxidation. Samples treated with the oxidative agent DTDP were used as positive controls. H_2O_2 and MPO+H_2O_2 lowered the SH content of actin to similar extents (to 75.9±7.1%, p<0.01, n=4, and 84.2±4.4%, p<0.05 vs. time control, respectively, n=9) (Fig. 4B). In contrast, the SH contents of myosin-binding protein C (MyBP-C, Fig. 4C) and the more compliant (N2BA) and stiffer (N2B) isoforms of the giant sarcomeric protein titin were not affected by these treatments (Fig. 4D-F). Using immunoblots a Tm and an actin containing complex was observed at on approximately 90 kDa molecular weight level under non-reducing conditions (in a buffer not containing β-ME), however, no increase in its intensity and that of Tm and actin could be detected after H_2O_2 and MPO+H_2O_2 treatments (Fig. 5).

Protein carbonylation assays revealed a modest, but significant increase in the carbonylation of actin upon H_2O_2 treatment (CI=1.1±0.05, p<0.05 vs. the time control, n=5), which was not further affected by the addition of MPO (CI=1.1±0.05, p=0.1 vs. the time control, n=11) (Fig. 6A). Similarly as for actin, a slight, but significant increase in the carbonyl content of MyBP-C was observed both after H_2O_2 (CI=1.5±0.2, p<0.05 vs. the time control, n=2) and after MPO+H_2O_2 application (CI=1.4±0.2, p<0.05 vs. the time control,

n=4) (Fig. 6B). The extent of carbonyl group formation in the N2BA and N2B titin isoforms remained unaltered after H_2O_2 or MPO+H_2O_2 treatment (CI=0.9±0.2 and CI=1.0±0.2 for N2BA; CI=1.0±0.1 and CI=0.9±0.1 for N2B, respectively) (Fig. 6C-E).

Discussion

This is the first reported investigation of the direct effects of MPO on the contractile function of single, isolated human myocardial cells. The *in vitro* model experiments revealed that (1) MPO impairs Ca^{2+}-dependent isometric force generation, increases the Ca^{2+}-independent $F_{passive}$ and decreases the Ca^{2+} sensitivity of force production; (2) the MPO-induced functional changes can be prevented by an MPO-I and the antioxidant Met; (3) the levels of SH oxidation in actin and of carbonylation in actin and MyBP-C are increased by the application of MPO+H_2O_2 or H_2O_2 alone; (4) the MPO-evoked functional effects are probably mediated by the chlorinating activity of MPO.

Myocardial inflammation and ischemia-reperfusion injury are characterized by enhanced extents of oxidative stress and contractile dysfunction [46]. The application of MPO+H_2O_2 to human cardiomyocytes appreciably reduced the Ca^{2+}-activated F_{active} and markedly decreased pCa_{50}. In contrast, H_2O_2 (30 μM) alone induced a smaller decrease in F_{active}. Consistent with our findings, a lower concentration of H_2O_2 (10 μM) did not result in a decrease in the maximal Ca^{2+}-activated force in skinned rat heart preparations [47, 48]. This suggests that the action of H_2O_2 on contractile force generation is concentration-dependent. Lower concentrations have no measurable effects, whereas higher concentrations affect the cardiomyocyte contractility. The deleterious effect on F_{active} can be explained by the MPO-mediated H_2O_2-derived production of HOCl. In a previous study, HOCl treatment alone (10 μM and 50 μM for 1 min) evoked a significant decrease in the maximum Ca^{2+}-activated force [47], similarly to the result of MPO+H_2O_2 treatment in the present study. Interestingly, neither the H_2O_2- nor the MPO-induced functional changes were related to any deterioration in the cross-striation pattern of the cardiomyocytes under the light microscope. It is important

to note, however, that electron microscopy has revealed a myofilament lattice disruption after HOCl treatment [47].

The subtle increase after H_2O_2 application and the marked elevation in the Ca^{2+}-independent $F_{passive}$ upon MPO+H_2O_2 treatment in the present study are consistent with the observations that H_2O_2 at low (<10 μM) concentration did not alter $F_{passive}$, while HOCl (10 μM and 50 μM) induced a significant rise in $F_{passive}$ of skinned rat trabeculae [48]. It is well established that the giant sarcomeric protein titin plays a key role in the development of $F_{passive}$ in permeabilized cardiomyocytes by acting as a molecular spring in the sarcomere [49]. The cardiomyocyte $F_{passive}$ can be modulated by the titin isoform switch (between the short and stiff N2B and the longer and more compliant N2BA isoforms [50]) and by several post-translational modifications, including phosphorylation [51], SH oxidation [52] and potentially carbonylation. One elegant study demonstrated that the oxidative stress-induced formation of disulfide bridges within the titin molecule (N2B unique sequence, N2B-Us) reduced the contour length of the N2B-Us, leading to stiffening of the whole titin molecule [52]. In the present study, neither SH oxidation nor carbonylation of the N2B and N2BA titin isoforms was found to be affected by MPO or H_2O_2 treatment. This may be explained by the distinct sensitivities of the titin N2B isoform, actin and MyBP-C to oxidative changes based on the differences in their ultrastructures and SH group contents. Our results indicate that modifications other than titin SH oxidation or carbonylation might be responsible for the marked elevation in $F_{passive}$ after MPO treatment in human cardiomyocytes.

The significant decrease observed in pCa_{50} after MPO+H_2O_2 in this study is in marked contrast with the previous finding of an increase in pCa_{50} in skinned rat trabeculae in response to HOCl treatment [48]. This apparently conflicting result might be explained by (1) the different concentration of HOCl produced by the MPO under our experimental conditions; (2) a difference in susceptibility of the myofilaments to HOCl between the two

species; and (3) the difference in the experimental setting, permeabilized, single cardiomyocytes presenting a negligible diffusion obstacle in comparison with trabeculae. Further, the pronounced MPO-induced decrease in pCa_{50} suggests that different myofilament protein modifications occur and contribute to pCa_{50} in the course of MPO and H_2O_2 treatments. Under these experimental conditions H_2O_2 more probably induced a structural, rather than a regulatory alteration in the contractile apparatus because pCa_{50} was not affected. The deleterious effect on the maximal F_{active} and the modest increase in $F_{passive}$ upon H_2O_2 administration implies that the H_2O_2-induced contractile alterations could be explained by a reduction in the number of force-generating cross-bridges due to the diminished longitudinal transmission of force along the sarcomeres. These findings are consistent with the observations of *MacFarlane et al*., who exposed the superoxide anion (from which H_2O_2 formed endogenously through spontaneous or superoxide dismutase-catalyzed dismutation) to chemically skinned rat cardiac muscles. They also found a dose-dependent reduction in the maximal F_{active} without any alteration in the pCa_{50} and concluded that some aspect of the cross-bridge behavior is particularly vulnerable to superoxide [53].

A substantial number of data indicate that the inhibition of MPO may well be useful in CV pathologies characterized by elevated MPO levels (myocardial inflammation, ischemia-reperfusion injury and acute MI). Thus, despite the fact that MPO-Is may have adverse effects on the function of MPO in the innate host-defense mechanisms, potential therapeutic interventions through which to inhibit MPO have aroused considerable interest [42]. In the present study, both the MPO-I 4-aminobenzhydrazide (50 μM) and the antioxidant amino acid Met (10 mM) were equally able to prevent all of the MPO-evoked deleterious contractile effects in skinned human cardiomyocytes, the latter potentially by scavenging the HOCl generated by MPO. MPO activity assays suggested that the Met-inhibited chlorinating activity is responsible for the MPO-evoked functional changes. HOCl

reacts most rapidly with the sulfur-containing residues (Met and Cys) [54]. It is likely, therefore, that the high concentration of Met used in this study diminished the HOCl-evoked oxidative capacity. The oxidation of Met residues results in the generation of Met-sulfoxide (MetSO), a process that may be reversed by MetSO reductase [55]. Met is therefore considered to play a protective role against the deleterious effects of protein oxidation [28]. Interestingly, the incomplete reversion and oxidation of physiologically relevant Met residues has been shown to contribute to the impaired function of proteins [56], including actin [57]. It is important to note, that other HOCl scavenging substances than Met (e.g. glutathione, taurine and L-ascorbic acid) were also tested recently in HOCl scavenging assays [58]. Given the rapid reaction rates of HOCl with biological materials, however, much higher doses of L-ascorbic acid and thiols were required to effectively protect against the direct oxidative damage induced by HOCl. This latter suggests that inhibiting the generation of HOCl may be a better choice than scavenging HOCl after its generation, for amelioration of HOCl induced biological damage.

The distinct effect of the reducing agent DTT on F_{active} and $F_{passive}$ after MPO+H_2O_2 treatment found in this study might be explained by different modifications on the structural conformation or functional activity of the contractile and regulatory myofilament proteins. The precise nature of the redox-dependent functional changes upon H_2O_2 and MPO+H_2O_2 treatment is complex and determined also by the type and site of the induced post-translational modifications on individual proteins within the sarcomere [59]. SH residues of Cys can undergo both reversible and irreversible modifications. The reaction between the Cys thiolate anion and H_2O_2 results in formation of intra- or intermolecular disulfide bonds, which is reversible, but further oxidation can generate sulfinic or sulfonic acid, which are considered irreversible alterations [60]. The HOCl-induced protein carbonylation is thought

to be irreversible, while methionine oxidation can be reversed by MetSO-reductase [28] or can lead to an irreversible product (methionine-sulfone) [55].

The extent of overall SH oxidation observed after MPO treatment in this study was comparable to that in heart tissue slices exposed to high-dose HOCl [27]. There is biochemical evidence that oxidative modifications modulate the architecture of the myofilament protein actin [61] and myosin [62]. *In vitro* exposure of permeabilized human LV cardiomyocytes to the oxidative agent DTDP resulted in a decrease in maximal Ca^{2+}-activated force production with a parallel reduction in the SH content of actin and MLC-1 [26]. Consistent with this, in the present study H_2O_2 decreased the SH content of actin. However, despite the marked reduction in F_{active}, no additional decrease in this parameter was detected after MPO+H_2O_2 application, suggesting that SH oxidation may not be the main contributor to the MPO-evoked decrease in F_{active} under these experimental conditions. Moreover, formation of an actin and a Tm containing protein complex observed in this study is also unlikely to be responsible for the contractile changes observed in the cardiomyocytes after H_2O_2 and MPO+H_2O_2 administration. The possible functional consequences of the observed protein complexes require further examinations.

In a mouse model of experimental MI, we recently identified the increased carbonylation of actin and myosin heavy chain (MHC) in the infarcted area [2]. Similarly to MPO, *in vitro* Fenton-based myofilament carbonylation decreased pCa_{50}, irrespectively of the phosphorylation status of the myofilaments. Moreover, pCa_{50} correlated strongly with the myofilament carbonylation levels. In accord with this, a marked (3-fold) increase in carbonyl group formation in actin was observed after 1 mM, but not after 0.1 mM H_2O_2 treatment [25]. The application of H_2O_2 to cardiomyocytes at a concentration higher than 0.1 mM was hindered by its inhibitory effect on the activity of MPO [42]. 30 μM H_2O_2 lowered F_{active} in parallel with a slight, but significant increase in the carbonylation of actin and MyBP-C.

Similarly to SH oxidation, carbonylation of these myofilament proteins was not further affected by the addition of MPO, despite its noteworthy effects on cardiomyocyte active and passive force production. This implies that the physiological effects of MPO-catalyzed oxidative processes are independent of SH group oxidation or carbonylation of human myocardial proteins.

Oxidative modifications in the myocardium primarily have been considered to result in reduced force generation, as also demonstrated in the present study. However, recent evidence suggests a more complex picture. Reactive oxygen and nitrogen species can activate protective mechanisms and signaling pathways (redox regulation) [60] or even increase cardiac performance [63]. Mild oxidative stress induced S-nitrosylation at specific Cys residues was shown to be cardioprotective [64]. Subtle increases in ROS production may even enhance cardiac contractility under physiological conditions [65]. Indeed, certain oxidative myofilament modifications can lead to positive functional consequences, such as nitroxyl (HNO), a reactive nitrogen species related to nitric oxide, induces formation of actin-Tm heterodimers, which correlates with the increase in Ca^{2+} sensitivity and dimeric forms of MHC and MLC-1, which are associated with increased force generation [63]. HNO was also shown to increase maximum tension and Ca^{2+} sensitivity of trabeculae sarcomeres functioning *in situ* [66]. These results strongly suggest that the beneficial or deleterious functional outcome is likely dictated by the strength and the nature of the oxidizing agent and the redox milieu of the myofilament compartment.

Since isolation of cardiomyocytes and assessment of myofilament properties was performed on LV biopsies of unused donor hearts, possible changes in the phosphorylation and oxidative status of the myofilament proteins occurring before or during tissue sampling may have been interfered with the results of this study. In addition, activation of the β-adrenergic signaling and various oxidative pathways might also influence the baseline

mechanical and biochemical characteristics of the cardiomyocytes. We have checked the baseline functional parameters of the cells in the study and found no major differences in the cardiomyocyte mechanical properties. Moreover, the baseline myofilament SH contents were also similar in the LV samples used for the cardiomyocyte isolation. These observations are in line with those found in our previous study, in which the reducing agent DTT did not affect F_{active} and pCa_{50} of cardiomyocytes derived from human donor hearts [26].

In this study LV heart samples were frozen and their functional and biochemical properties were evaluated upon thawing. To validate the use of defrosted biopsy samples, in one of our previous studies [67] force recordings of cardiomyocytes isolated from a biopsy sample immediately after procurement were compared to those of cardiomyocytes isolated from a defrosted biopsy of the same patient. These force recordings yielded identical results. In addition, the extent of tissue heterogeneity was also addressed in previous studies using explanted hearts [68, 69] or surgically procured biopsies [70]. In these studies the variability of force measurements of cardiomyocytes isolated from different portions of the heart was always less than 5%.

It is also important to note that several additional MPO-sensitive processes, such as protein halogenation [71], protein nitration [72], Met oxidation, sulfonic acid generation (Cys), [73] or protein degradation [28], might be responsible for the observed functional alterations. Further studies are clearly required to elucidate the relative contributions of these processes to the overall pump function during human cardiac pathologies associated with elevated MPO levels.

Conclusion

MPO-derived oxidants contribute to myocardial contractile dysfunction by decreasing the cardiomyocyte force production and the myofilament Ca^{2+} sensitivity and increasing

$F_{passive}$ in human cardiomyocytes. These effects could be prevented by MPO inhibition and the antioxidant Met. The associated functional and biochemical alterations may provide a pharmacological tool for the prevention and/or reversion of MPO-induced contractile protein alterations, which could have therapeutic implications in cardiac pathologies characterized by elevated MPO levels.

Acknowledgments

This research was supported by grants from the European Commission (FP7-Health-2010; MEDIA-261409), and the Hungarian Scientific Research Fund (OTKA PD 108614 and OTKA K 109083), co-financed by the European Social Fund in the framework of TÁMOP 4.2.4. A/2-11-1-2012-0001 "National Excellence Program" and TÁMOP 4.2.2.A-11/1/KONV-2012-0045. We thank László Kardos MD for help in the statistical analyses. The monoclonal anti-tropomyosin antibody developed by Jim Jung-Ching Lin was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.

References

[1] Sugiyama, S.; Okada, Y.; Sukhova, G. K.; Virmani, R.; Heinecke, J. W.; Libby, P. Macrophage myeloperoxidase regulation by granulocyte macrophage colony-stimulating factor in human atherosclerosis and implications in acute coronary syndromes. *Am J Pathol* **158:**879-891; 2001.

[2] Balogh, A.; Santer, D.; Pasztor, E. T.; Toth, A.; Czuriga, D.; Podesser, B. K.; Trescher, K.; Jaquet, K.; Erdodi, F.; Edes, I.; Papp, Z. Myofilament protein carbonylation contributes to the contractile dysfunction in the infarcted LV region of mouse hearts. *Cardiovasc Res* **101:**108-119; 2014.

[3] Gutteridge, J. M.; Halliwell, B. Free radicals and antioxidants in the year 2000. A historical look to the future. *Ann N Y Acad Sci* **899:**136-147; 2000.

[4] Heymes, C.; Bendall, J. K.; Ratajczak, P.; Cave, A. C.; Samuel, J. L.; Hasenfuss, G.; Shah, A. M. Increased myocardial NADPH oxidase activity in human heart failure. *J Am Coll Cardiol* **41:**2164-2171; 2003.

[5] Paulus, W. J.; Tschope, C. A novel paradigm for heart failure with preserved ejection fraction: comorbidities drive myocardial dysfunction and remodeling through coronary microvascular endothelial inflammation. *J Am Coll Cardiol* **62:**263-271; 2013.

[6] Nicholls, S. J.; Hazen, S. L. Myeloperoxidase and cardiovascular disease. *Arterioscler Thromb Vasc Biol* **25:**1102-1111; 2005.

[7] Klebanoff, S. J. Myeloperoxidase: contribution to the microbicidal activity of intact leukocytes. *Science* **169:**1095-1097; 1970.

[8] Brennan, M. L.; Penn, M. S.; Van Lente, F.; Nambi, V.; Shishehbor, M. H.; Aviles, R. J.; Goormastic, M.; Pepoy, M. L.; McErlean, E. S.; Topol, E. J.; Nissen, S. E.; Hazen, S. L. Prognostic value of myeloperoxidase in patients with chest pain. *N Engl J Med* **349:**1595-1604; 2003.

[9] Mocatta, T. J.; Pilbrow, A. P.; Cameron, V. A.; Senthilmohan, R.; Frampton, C. M.; Richards, A. M.; Winterbourn, C. C. Plasma concentrations of myeloperoxidase predict mortality after myocardial infarction. *J Am Coll Cardiol* **49:**1993-2000; 2007.

[10] Vasilyev, N.; Williams, T.; Brennan, M. L.; Unzek, S.; Zhou, X.; Heinecke, J. W.; Spitz, D. R.; Topol, E. J.; Hazen, S. L.; Penn, M. S. Myeloperoxidase-generated oxidants modulate left ventricular remodeling but not infarct size after myocardial infarction. *Circulation* **112:**2812-2820; 2005.

[11] Hazen, S. L.; Heinecke, J. W. 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. *J Clin Invest* **99:**2075-2081; 1997.

[12] Shao, B.; Oda, M. N.; Oram, J. F.; Heinecke, J. W. Myeloperoxidase: an inflammatory enzyme for generating dysfunctional high density lipoprotein. *Curr Opin Cardiol* **21:**322-328; 2006.

[13] Abu-Soud, H. M.; Hazen, S. L. Nitric oxide is a physiological substrate for mammalian peroxidases. *J Biol Chem* **275:**37524-37532; 2000.

[14] Loria, V.; Dato, I.; Graziani, F.; Biasucci, L. M. Myeloperoxidase: a new biomarker of inflammation in ischemic heart disease and acute coronary syndromes. *Mediators Inflamm* **2008:**135625; 2008.

[15] Rudolph, V.; Rudolph, T. K.; Hennings, J. C.; Blankenberg, S.; Schnabel, R.; Steven, D.; Haddad, M.; Knittel, K.; Wende, S.; Wenzel, J.; Munzel, T.; Heitzer, T.; Meinertz, T.; Hubner, C.; Baldus, S. Activation of polymorphonuclear neutrophils in patients with impaired left ventricular function. *Free Radic Biol Med* **43:**1189-1196; 2007.

[16] Tang, W. H.; Tong, W.; Troughton, R. W.; Martin, M. G.; Shrestha, K.; Borowski, A.; Jasper, S.; Hazen, S. L.; Klein, A. L. Prognostic value and echocardiographic determinants of

plasma myeloperoxidase levels in chronic heart failure. *J Am Coll Cardiol* **49:**2364-2370; 2007.

[17] Sinning, C.; Schnabel, R.; Peacock, W. F.; Blankenberg, S. Up-and-coming markers: myeloperoxidase, a novel biomarker test for heart failure and acute coronary syndrome application? *Congest Heart Fail* **14:**46-48; 2008.

[18] Podrez, E. A.; Abu-Soud, H. M.; Hazen, S. L. Myeloperoxidase-generated oxidants and atherosclerosis. *Free Radic Biol Med* **28:**1717-1725; 2000.

[19] Heinecke, J. W. Oxidative stress: new approaches to diagnosis and prognosis in atherosclerosis. *Am J Cardiol* **91:**12A-16A; 2003.

[20] Zhang, R.; Brennan, M. L.; Shen, Z.; MacPherson, J. C.; Schmitt, D.; Molenda, C. E.; Hazen, S. L. Myeloperoxidase functions as a major enzymatic catalyst for initiation of lipid peroxidation at sites of inflammation. *J Biol Chem* **277:**46116-46122; 2002.

[21] Klebanoff, S. J. Reactive nitrogen intermediates and antimicrobial activity: role of nitrite. *Free Radic Biol Med* **14:**351-360; 1993.

[22] Hansson, M.; Olsson, I.; Nauseef, W. M. Biosynthesis, processing, and sorting of human myeloperoxidase. *Arch Biochem Biophys* **445:**214-224; 2006.

[23] Cai, H.; Griendling, K. K.; Harrison, D. G. The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases. *Trends Pharmacol Sci* **24:**471-478; 2003.

[24] Harrison, D.; Griendling, K. K.; Landmesser, U.; Hornig, B.; Drexler, H. Role of oxidative stress in atherosclerosis. *Am J Cardiol* **91:**7A-11A; 2003.

[25] Canton, M.; Neverova, I.; Menabo, R.; Van Eyk, J.; Di Lisa, F. Evidence of myofibrillar protein oxidation induced by postischemic reperfusion in isolated rat hearts. *Am J Physiol Heart Circ Physiol* **286:**H870-877; 2004.

[26] Hertelendi, Z.; Toth, A.; Borbely, A.; Galajda, Z.; van der Velden, J.; Stienen, G. J.; Edes, I.; Papp, Z. Oxidation of myofilament protein sulfhydryl groups reduces the contractile

force and its Ca2+ sensitivity in human cardiomyocytes. *Antioxid Redox Signal* **10:**1175-1184; 2008.

[27] Fliss, H. Oxidation of proteins in rat heart and lungs by polymorphonuclear leukocyte oxidants. *Mol Cell Biochem* **84:**177-188; 1988.

[28] Hawkins, C. L.; Pattison, D. I.; Davies, M. J. Hypochlorite-induced oxidation of amino acids, peptides and proteins. *Amino Acids* **25:**259-274; 2003.

[29] Winterbourn, C. C. Biological reactivity and biomarkers of the neutrophil oxidant, hypochlorous acid. *Toxicology* **181-182:**223-227; 2002.

[30] Favero, T. G.; Colter, D.; Hooper, P. F.; Abramson, J. J. Hypochlorous acid inhibits Ca(2+)-ATPase from skeletal muscle sarcoplasmic reticulum. *J Appl Physiol* **84:**425-430; 1998.

[31] Kuroda, M.; Kaminishi, T.; Uchida, K.; Miyazawa, K.; Tomoike, H.; Doi, K. Ca2+ increase and pH decrease induced by hypochlorous acid in single quiescent myocytes isolated from rat ventricles. *Jpn J Physiol* **45:**619-630; 1995.

[32] Eley, D. W.; Korecky, B.; Fliss, H.; Desilets, M. Calcium homeostasis in rabbit ventricular myocytes. Disruption by hypochlorous acid and restoration by dithiothreitol. *Circ Res* **69:**1132-1138; 1991.

[33] Przygodzki, T.; Lapshina, E.; Zavodnik, I.; Sokal, A.; Bryszewska, M. 2,3-Butanedione monoxime does not protect cardiomyocytes under oxidative stress. *Cell Biochem Funct* **24:**413-418; 2006.

[34] Lacy, F.; O'Connor, D. T.; Schmid-Schonbein, G. W. Plasma hydrogen peroxide production in hypertensives and normotensive subjects at genetic risk of hypertension. *J Hypertens* **16:**291-303; 1998.

[35] Hampton, M. B.; Kettle, A. J.; Winterbourn, C. C. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* **92:**3007-3017; 1998.

[36] Liu, X.; Zweier, J. L. A real-time electrochemical technique for measurement of cellular hydrogen peroxide generation and consumption: evaluation in human polymorphonuclear leukocytes. *Free Radic Biol Med* **31:**894-901; 2001.

[37] Mutze, S.; Hebling, U.; Stremmel, W.; Wang, J.; Arnhold, J.; Pantopoulos, K.; Mueller, S. Myeloperoxidase-derived hypochlorous acid antagonizes the oxidative stress-mediated activation of iron regulatory protein 1. *J Biol Chem* **278:**40542-40549; 2003.

[38] Souza, C. E.; Maitra, D.; Saed, G. M.; Diamond, M. P.; Moura, A. A.; Pennathur, S.; Abu-Soud, H. M. Hypochlorous acid-induced heme degradation from lactoperoxidase as a novel mechanism of free iron release and tissue injury in inflammatory diseases. *PLoS One* **6:**e27641; 2011.

[39] Spickett, C. M.; Jerlich, A.; Panasenko, O. M.; Arnhold, J.; Pitt, A. R.; Stelmaszynska, T.; Schaur, R. J. The reactions of hypochlorous acid, the reactive oxygen species produced by myeloperoxidase, with lipids. *Acta Biochim Pol* **47:**889-899; 2000.

[40] Zhang, C.; Patel, R.; Eiserich, J. P.; Zhou, F.; Kelpke, S.; Ma, W.; Parks, D. A.; Darley-Usmar, V.; White, C. R. Endothelial dysfunction is induced by proinflammatory oxidant hypochlorous acid. *Am J Physiol Heart Circ Physiol* **281:**H1469-1475; 2001.

[41] Nakamura, T. Y.; Goda, K.; Okamoto, T.; Kishi, T.; Nakamura, T.; Goshima, K. Contractile and morphological impairment of cultured fetal mouse myocytes induced by oxygen radicals and oxidants. Correlation with intracellular Ca2+ concentration. *Circ Res* **73:**758-770; 1993.

[42] Malle, E.; Furtmuller, P. G.; Sattler, W.; Obinger, C. Myeloperoxidase: a target for new drug development? *Br J Pharmacol* **152:**838-854; 2007.

[43] Borbely, A.; Toth, A.; Edes, I.; Virag, L.; Papp, J. G.; Varro, A.; Paulus, W. J.; van der Velden, J.; Stienen, G. J.; Papp, Z. Peroxynitrite-induced alpha-actinin nitration and contractile alterations in isolated human myocardial cells. *Cardiovasc Res* **67:**225-233; 2005.

[44] Fabiato, A.; Fabiato, F. Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J Physiol (Paris)* **75:**463-505; 1979.

[45] Canton, M.; Menazza, S.; Sheeran, F. L.; Polverino de Laureto, P.; Di Lisa, F.; Pepe, S. Oxidation of myofibrillar proteins in human heart failure. *J Am Coll Cardiol* **57:**300-309; 2011.

[46] Vinten-Johansen, J. Involvement of neutrophils in the pathogenesis of lethal myocardial reperfusion injury. *Cardiovasc Res* **61:**481-497; 2004.

[47] Miller, D. J.; MacFarlane, N. G. Intracellular effects of free radicals and reactive oxygen species in cardiac muscle. *J Hum Hypertens* **9:**465-473; 1995.

[48] MacFarlane, N. G.; Miller, D. J. Effects of the reactive oxygen species hypochlorous acid and hydrogen peroxide on force production and calcium sensitivity of rat cardiac myofilaments. *Pflugers Arch* **428:**561-568; 1994.

[49] van Heerebeek, L.; Franssen, C. P.; Hamdani, N.; Verheugt, F. W.; Somsen, G. A.; Paulus, W. J. Molecular and cellular basis for diastolic dysfunction. *Curr Heart Fail Rep* **9:**293-302; 2012.

[50] LeWinter, M. M.; Granzier, H. Cardiac titin: a multifunctional giant. *Circulation* **121:**2137-2145; 2010.

[51] Borbely, A.; van Heerebeek, L.; Paulus, W. J. Transcriptional and posttranslational modifications of titin: implications for diastole. *Circ Res* **104:**12-14; 2009.

[52] Grutzner, A.; Garcia-Manyes, S.; Kotter, S.; Badilla, C. L.; Fernandez, J. M.; Linke, W. A. Modulation of titin-based stiffness by disulfide bonding in the cardiac titin N2-B unique sequence. *Biophys J* **97:**825-834; 2009.

[53] MacFarlane, N. G.; Miller, D. J. Depression of peak force without altering calcium sensitivity by the superoxide anion in chemically skinned cardiac muscle of rat. *Circ Res* **70:**1217-1224; 1992.

[54] Szuchman-Sapir, A. J.; Pattison, D. I.; Ellis, N. A.; Hawkins, C. L.; Davies, M. J.; Witting, P. K. Hypochlorous acid oxidizes methionine and tryptophan residues in myoglobin. *Free Radic Biol Med* **45:**789-798; 2008.

[55] Vogt, W. Oxidation of methionyl residues in proteins: tools, targets, and reversal. *Free Radic Biol Med* **18:**93-105; 1995.

[56] Oien, D. B.; Canello, T.; Gabizon, R.; Gasset, M.; Lundquist, B. L.; Burns, J. M.; Moskovitz, J. Detection of oxidized methionine in selected proteins, cellular extracts and blood serums by novel anti-methionine sulfoxide antibodies. *Arch Biochem Biophys* **485:**35-40; 2009.

[57] Dalle-Donne, I.; Rossi, R.; Giustarini, D.; Gagliano, N.; Di Simplicio, P.; Colombo, R.; Milzani, A. Methionine oxidation as a major cause of the functional impairment of oxidized actin. *Free Radic Biol Med* **32:**927-937; 2002.

[58] Zhongwei Liu, Y. Y., Suhua Wang, Wei-Yi Ong, Choon Nam Ong, Dejian Huang. Assaying myeloperoxidase inhibitors and hypochlorous acid scavengers in HL60 cell line using quantum dots. *American Journal of Biomedical Sciences* **5:**140-153; 2013.

[59] Steinberg, S. F. Oxidative stress and sarcomeric proteins. *Circ Res* **112:**393-405; 2013.

[60] Chung, H. S.; Wang, S. B.; Venkatraman, V.; Murray, C. I.; Van Eyk, J. E. Cysteine oxidative posttranslational modifications: emerging regulation in the cardiovascular system. *Circ Res* **112:**382-392; 2013.

[61] Milzani, A.; DalleDonne, I.; Colombo, R. Prolonged oxidative stress on actin. *Arch Biochem Biophys* **339:**267-274; 1997.

[62] Wilson, G. J.; dos Remedios, C. G.; Stephenson, D. G.; Williams, D. A. Effects of sulphydryl modification on skinned rat skeletal muscle fibres using 5,5'-dithiobis(2-nitrobenzoic acid). *J Physiol* **437:**409-430; 1991.

[63] Gao, W. D.; Murray, C. I.; Tian, Y.; Zhong, X.; DuMond, J. F.; Shen, X.; Stanley, B. A.; Foster, D. B.; Wink, D. A.; King, S. B.; Van Eyk, J. E.; Paolocci, N. Nitroxyl-mediated disulfide bond formation between cardiac myofilament cysteines enhances contractile function. *Circ Res* **111:**1002-1011; 2012.

[64] Kohr, M. J.; Evangelista, A. M.; Ferlito, M.; Steenbergen, C.; Murphy, E. S-nitrosylation of TRIM72 at cysteine 144 is critical for protection against oxidation-induced protein degradation and cell death. *J Mol Cell Cardiol* **69:**67-74; 2014.

[65] Kubin, A. M.; Skoumal, R.; Tavi, P.; Konyi, A.; Perjes, A.; Leskinen, H.; Ruskoaho, H.; Szokodi, I. Role of reactive oxygen species in the regulation of cardiac contractility. *J Mol Cell Cardiol* **50:**884-893; 2011.

[66] Dai, T.; Tian, Y.; Tocchetti, C. G.; Katori, T.; Murphy, A. M.; Kass, D. A.; Paolocci, N.; Gao, W. D. Nitroxyl increases force development in rat cardiac muscle. *J Physiol* **580:**951-960; 2007.

[67] Borbely, A.; van der Velden, J.; Papp, Z.; Bronzwaer, J. G.; Edes, I.; Stienen, G. J.; Paulus, W. J. Cardiomyocyte stiffness in diastolic heart failure. *Circulation* **111:**774-781; 2005.

[68] van Der Velden, J.; Klein, L. J.; Zaremba, R.; Boontje, N. M.; Huybregts, M. A.; Stooker, W.; Eijsman, L.; de Jong, J. W.; Visser, C. A.; Visser, F. C.; Stienen, G. J. Effects of calcium, inorganic phosphate, and pH on isometric force in single skinned cardiomyocytes from donor and failing human hearts. *Circulation* **104:**1140-1146; 2001.

[69] van der Velden, J.; Papp, Z.; Zaremba, R.; Boontje, N. M.; de Jong, J. W.; Owen, V. J.; Burton, P. B.; Goldmann, P.; Jaquet, K.; Stienen, G. J. Increased Ca2+-sensitivity of the

contractile apparatus in end-stage human heart failure results from altered phosphorylation of contractile proteins. *Cardiovasc Res* **57:**37-47; 2003.

[70] van der Velden, J.; Klein, L. J.; van der Bijl, M.; Huybregts, M. A.; Stooker, W.; Witkop, J.; Eijsman, L.; Visser, C. A.; Visser, F. C.; Stienen, G. J. Isometric tension development and its calcium sensitivity in skinned myocyte-sized preparations from different regions of the human heart. *Cardiovasc Res* **42:**706-719; 1999.

[71] Davies, M. J. Myeloperoxidase-derived oxidation: mechanisms of biological damage and its prevention. *J Clin Biochem Nutr* **48:**8-19; 2011.

[72] Yan, Z.; Liang, F.; Guo, L.; Wang, J.; Wang, X. L.; Cheng, X. L.; Ma, X. L.; Liu, H. R. Myeloperoxidase increased cardiomyocyte protein nitration in mice subjected to nonlethal mechanical trauma. *Biochem Biophys Res Commun* **393:**531-535; 2010.

[73] Hawkins, C. L.; Morgan, P. E.; Davies, M. J. Quantification of protein modification by oxidants. *Free Radic Biol Med* **46:**965-988; 2009.

Figure captions

FIG. 1. Myeloperoxidase (MPO) and hydrogen peroxide (H_2O_2) impair the force generation of human permeabilized cardiomyocytes. (A) A single cardiomyocyte (isolated from a human left ventricle myocardium) mounted between a sensitive force transducer and an electromagnetic motor. **(B)** Original force recordings of maximal Ca^{2+}-activated active (F_{active}) and Ca^{2+}-independent passive ($F_{passive}$) force components before (left panel) and after MPO+H_2O_2 treatment (right panel) at pCa (i.e $-\log_{10}[Ca^{2+}]$) 4.75 and pCa 9.0, respectively. MPO + H_2O_2 were applied in Iso for 15 min. **(C)** pCa-force relationships determined before and after H_2O_2 or MPO+H_2O_2 treatments (number of cardiomyocytes, *n=7* and *12*, respectively). Force levels are expressed relative to the values measured before the treatments. (* vs. Before H_2O_2, # vs. Before MPO+H_2O_2, & vs. After H_2O_2; $^{*,\#,\&}p<0.05$) **(D)** Changes in $F_{passive}$ measured in the presence of Iso and after sequential applications of H_2O_2 or MPO+H_2O_2. **(E)** Significant rightward shift (i.e. decrease in the Ca^{2+} sensitivity of force production (pCa_{50})) in the normalized pCa-force relationships in response to MPO+H_2O_2, but no change after H_2O_2 treatment **(F)**. (Data are expressed as mean±SEM.)

FIG. 2. Similar effects of the MPO inhibitor (MPO-I), but distinct actions of methionine (Met) on the chlorinating and peroxidase activities of myeloperoxidase (MPO). Met inhibits the chlorinating **(A)**, but not the peroxidase **(B)** activity of MPO. Values are expressed relative to the MPO activity measured in the presence of Iso and hydrogen peroxide (H_2O_2). (Data are expressed as mean±SEM, $^{*}p<0.05$).

FIG. 3. The myeloperoxidase inhibitor (MPO-I) and methionine (Met) prevent the MPO-induced changes in isometric force production of human cardiomyocytes. Maximal (pCa 4.75) Ca^{2+}-dependent active (F_{active}) **(A)** and Ca^{2+}-independent (pCa 9) passive

($F_{passive}$) force **(B)** in left ventricular cardiomyocytes treated in isolating solution (Iso) supplemented with hydrogen peroxide (H_2O_2) or myeloperoxidase (MPO)+H_2O_2, MPO-I or Met. Forces are expressed relative to the values measured before the subsequent treatments. The MPO-I **(C)** and Met **(D)** prevent the MPO-evoked rightward shift in the normalized pCa-force relationships. Dashed lines indicate force-pCa relationships determined in Iso. **(E)** Changes in the Ca^{2+} sensitivity of force production (pCa_{50}) upon H_2O_2, MPO+H_2O_2, MPO-I or Met treatments. (Data are expressed as mean±SEM, *p<0.05)

FIG. 4. Myeloperoxidase (MPO) and hydrogen peroxide (H_2O_2) similarly alter sulfhydryl (SH) group oxidation in myofilament proteins. (A) SH group oxidation in a cardiomyocyte suspension treated in isolating solution (Iso) supplemented with H_2O_2 and MPO (Ellman's reaction). **(B-E)** Representative examples of SH content determination in actin **(B)**, myosin-binding protein C (MyBP-C) **(C)**, N2BA **(D, E)** and N2B **(D, F)** titin isoforms after H_2O_2 or MPO+H_2O_2 treatments through use of a protein biotinylation assay. T2 indicates the titin degradation product. Samples exposed to dithiodipyridine (DTDP, 2.5 mM, for 2 min) were used as positive control. Total protein amount was determined with the Sypro Ruby Protein Blot Stain. Values are expressed relative to the SH group content determined in Iso (time control). (Data are expressed as mean±SEM, *p<0.05 *vs.* Iso.)

FIG. 5. No additional disulfide cross-bridge formation after hydrogen-peroxide (H_2O_2) and myeloperoxidase (MPO) treatment. Left ventricular myocardial samples solubilized in non-reducing (-β-mercaptoethanol (β-ME)) or reducing (+β-ME) sample buffers and probed with anti-tropomyosin (Tm) (left panel) and anti-actin (right panel) antibodies after immunoblotting. (Protein amount was determined with the Sypro Ruby Protein Blot Stain, MW - molecular weight.)

FIG. 6. Myeloperoxidase (MPO) and hydrogen peroxide (H_2O_2) increase the carbonylation of actin and myosin-binding protein C (MyBP-C), but not that of titin. Representative examples and measurement of carbonyl group formation in actin **(A)**, MyBP-C **(B)**, N2BA **(C, D)** and N2B **(C, E)** titin isoforms treated with isolating solution (Iso) supplemented with H_2O_2 or MPO+H_2O_2. Left ventricular myocardial samples treated with Fenton reagent ($FeSO_4$, H_2O_2 and ascorbic acid) served as positive control. Protein carbonylation is expressed as carbonylation index (CI) (CI=1, carbonyl group content measured in Iso). Total protein amount was determined with the Sypro Ruby Protein Blot Stain. (Data are expressed as mean±SEM, *p<0.05)