SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Effects of oxidative stress, acute stress and female hormones on cardiomyocyte contractile function

by Judit Kalász

Supervisor: Attila Borbély, MD, PhD

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Supervisor: Attila Borbély, MD, PhD

Kálmán Laki Doctoral School, University of Debrecen

Head of the **Examination Committee**:	Péter Antal-Szalmás, MD, PhD, DSc
Members of the Examination Committee:	Róbert Halmosi, MD, PhD, DSc
	Péter Bay, PhD, DSc

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Head of the **Defense Committee**:	Péter Antal-Szalmás, MD, PhD, DSc
Reviewers:	Márta Julianna Sárközy, MD, PhD
	Róbert Pórszász, MD, PhD
Members of the Defense Committee:	Róbert Halmosi, MD, PhD, DSc
	Péter Bay, PhD, DSc

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1. Introduction and background

Myocardial infarction (MI), ischemic heart disease, and heart failure are responsible for a significant proportion of cardiovascular deaths in the developed countries. During the development of these diseases, several structural and functional alterations occur in the myocardium, which together could lead to impaired pump function of the heart. Exploring the processes underlying myocardial dysfunction is essential, as a better understanding of these can lead to the development of drugs with new mechanism of action, consequently, to a reduction in cardiovascular mortality. During the development of cardiovascular diseases oxidative damage to the myofilament proteins and phosphorylation changes mediated by the β-adrenergic signaling pathway also contribute to cardiac systolic and diastolic dysfunction.

Epidemiological studies have shown marked differences between women and men in the incidence of cardiovascular diseases and the distribution of its risk factors. The latter can be attributed in part to the protective effects of female sex hormones. In addition to traditional risk factors, emotional or physical stress may also play a role in the development of cardiovascular diseases by activating the β-adrenergic pathway. A more detailed assessment of these factors would greatly contribute to a better understanding of the role of female sex hormones in the development of cardiovascular pathologies.

1.1. The role and significance of oxidative stress in cardiovascular diseases

1.1.1. Generation of reactive oxygen species (ROS)

Oxidative stress is when the amount of reactive oxygen (ROS) and reactive nitrogen species (RNS) produced in cells and tissues exceeds the body's antioxidant capacity, so the latter is unable to neutralize the oxidative potential of ROS/RNS leading to damage of different molecules (proteins, lipids, nucleic acids). ROS is generated via the imperfect reduction of molecular oxygen in mitochondria (electron transport chain complex 1, NADH dehydrogenase and complex 3, ubisemiquinone and reduced cytochrome-b), in inflammatory

processes by NAD(P)H-oxidase (Nox) in phagocytes, by biotransformation in the liver (cytochrome p450) and also non-enzymatically (ionizing radiation, photolysis, nitrogen oxides and ozone). In myocardial cells, these reactive intermediates are produced mainly by the Nox2 and 4 and xanthine oxidase (XO). During the formation of ROS, superoxide ($O_2^{\bullet -}$), a weaker free radical is generated from oxygen by the addition of an electron, which forms hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 is a stable and membrane-permeable molecule whose *in vivo* concentration is highly dependent on its rate of formation and degradation. The neutralization of H_2O_2 is performed by two enzyme systems in the body: at high H_2O_2 concentrations, the catalase enzyme, while at low H_2O_2 concentrations, the glutathione peroxidase enzyme reduces H_2O_2 to water. If not neutralized, H_2O_2 reacts with metal ions (iron, copper) to form a hydroxyl radical ($^{\bullet}OH$) in the Fenton reaction by adding an electron, or to form hypochlorous acid (HOCl) as a co-substrate for the enzyme myeloperoxidase (MPO). The former has high reactivity and an extremely short lifespan (10^{-9} s), the latter serves as an effector of MPO. During RNS formation, nitric oxide free radical ($^{\bullet}NO$) is formed from molecular oxygen by NOS. $^{\bullet}NO$ uses O_2 to form nitrogen dioxide (NO_2) or superoxide to form peroxynitrite ($ONOO^-$).

1.1.2. Oxidative stress in cardiovascular diseases

Oxidative stress plays a central role in inflammatory cardiovascular conditions: hypertension, atherosclerosis, ischemia-reperfusion (I/R) injury, MI, left ventricular (LV) remodeling, and heart failure. Under physiological conditions, ROS are involved in the regulation of vascular function. In the progression of atherosclerosis, ROS produced by endothelial cells, macrophages, and vascular smooth muscle cells oxidizes low-density lipoprotein (LDL) cause endothelial dysfunction and destabilize atherosclerotic plaques. The decreased blood supply resulting from plaque rupture causes an oxygen deficiency in the affected myocardial tissue, leading to MI and I/R injury. The phenomenon of severe oxidative damage to myocardial

tissue during reperfusion following ischaemia is known as the *oxygen paradox*. This is due, on the one hand, to the large amount of XO and Nox2 and Nox4 upregulation accumulated in myocardial cells during ischemia, which form free radicals during reperfusion using oxygen. Although the influx of neutrophils into the oxygen-deficient area during ischemia is slow, rapid accumulation of ROS released from neutrophil granulocytes during reperfusion can be observed, which directly damages myocardial cells. Following myocardial cell death observed in MI, a recurrent inflammatory reaction with leukocyte influx occurs during scar tissue formation in the affected myocardium. However, excessive inflammatory processes can lead to a pathological overshot, remodeling and heart failure. During LV remodeling, ROS activate growth signaling pathways, leading to hypertrophy, activating matrix metalloproteinases (MMPs), and thus contributing to LV dilation by inducing collagen degradation. In heart failure, ROS leads to Ca^{2+} overload of myocardial cells by influencing the function of Ca^{2+}-ATPases, thereby leading to contractile dysfunction and the development of endothelial dysfunction, interstitial fibrosis, and apoptosis.

1.1.3. Oxidative protein damage in the contractile system of the myocardium

ROS are able to reduce the force-generating capacity of the contractile system, thus causing contractile dysfunction not only indirectly (disruption of Ca^{2+} homeostasis, activation of kinases) but also directly by impairing the function of myofilament proteins. The effects of H_2O_2 on the myocardium can be divided into two groups: 1. direct protein oxidation, in which the oxidation of the sulfhydryl (SH) groups and/or carbonylation of proteins (actin, myosin light chain-1 (MLC-1) takes place; 2. indirect effects, when H_2O_2 activates protein kinases as a secondary messenger (redox signaling, protein kinase C delta (PKCδ), troponin I (TnI) phosphorylation). H_2O_2 was shown to cause LV dysfunction in a concentration-dependent manner and induces disulfide bridge formation in actin and tropomyosin in rat hearts.

Oxidative damage to the contractile system occurs at the level of sarcomomers by modification of certain amino acids. Oxidation of *methionine (Met)* produces methionine sulfoxide (MetSO), which can be converted back to the native amino acid by MetSO reductase. Based on these, the surface Met side chains of proteins are considered endogenous antioxidants in the body, as effective free radical scavengers. Due to the terminal SH group of the amino acid cysteine (Cis), it is also extremely sensitive to oxidative damage, which can lead to the formation of intra- or intermolecular disulfide bridges (RS-SR') with or without the unstable sulfenic acid (R-S-OH) intermediate. Sulfenic acid is converted to sulfinic acid ($R\text{-}SO_2H$) and then to sulfonic acid ($R\text{-}SO_3H$) by additional ROS. Reaction with oxidized glutathione produces glutathionylated thiol (RS-SG). S-glutathionylation and disulfide formation are reversible, while sulfonic acid formation is an irreversible process. In contrast, recent research has confirmed that mild oxidative stress may also play a cardioprotective (S-nitrosylation of proteins (SNO)) or regulatory (cysteines that act as "redox switches" sensing and initiating a response) role.

Increased oxidative stress results in *carbonylation* of myocardial proteins. By direct oxidation, carbonyl groups can be formed on the amino acid side chains of lysine, arginine, proline and threonine in proteins. The metal catalyzed reaction (Fenton reaction - in the presence of iron, *in vivo*) produces aldehydes and ketones. In addition, by reacting with carbonyl radicals formed on lipids, carbohydrates, and advanced glycation end-products, carbonyl derivatives can also be formed on cysteine, lysine, and histidine amino acids in a secondary reaction.

Tyrosine amino acids can be nitrated and chlorinated by oxidative action. 3-chlorothyrosine is a specific marker of MPO function, while nitrotyrosine can also be formed during MPO and $ONOO^-$ reactions. Upon the effect of MPO, the tyrosine amino acid can also

be oxidized to a tyrosyl radical, and then two tyrosyl radicals combine to form dithyrosine to form protein crosslinks.

1.2. Myeloperoxidase enzyme (MPO)

1.2.1. Characteristics of MPO, chlorination and peroxidase activity

MPO (EC 1.11.2.2) is a member of the hemperoxidase enzyme family. During their operation, the members of the enzyme family acquire a strong oxidizing capacity by using H_2O_2, which functions as a cosubstrate, with which they participate as an effector in the fight against pathogens. MPO is the only member of the enzyme family to increase the oxidative potential of H_2O_2 to form hypochlorous acid (HOCl) by oxidizing chloride ion (Cl^-).

MPO, located in the azurophilic granules of leukocytes, is secreted after leukocyte activation and is involved in the innate immune response. It is found mainly in neutrophil granulocytes, but is also present in monocytes as well as in some types of tissue macrophages. In addition to its bactericid role in the natural immune response, in inflammatory processes outside of the phagosomes, the ROS created by the MPO can also damage the body's tissues, protein, lipid and DNA structures.

The MPO enzyme is a 144 kDa molecular weight tetramer composed of two identical subunits connected by a disulfide bridge. During its operation, MPO forms ROS through its halogenation (chlorination) and peroxidase cycles. Since the two-electron reduction of the enzyme in *halogenation* cycle produces most of the highly reactive HOCl *in vivo* and under experimental conditions, this activity of MPO is referred as chlorination activity. During two single electron reduction of the enzyme in *peroxidase activity*, reactive nitrogen intermediates, tyrosyl radicals and dithyrosine are formed. MPO function can be inhibited by the MPO inhibitor (MPO-I) 4-aminobenzhydrazide (ABAH).

1.2.2. Significance of MPO in cardiovascular diseases

It is known that people with complete or partial MPO deficiency (1 in 2000-4000 Caucasians) are less likely to develop CV diseases. MPO enzyme levels increase with age and are higher in women than in men.

Recent research suggests that MPO is a promising biomarker in CV diseases. An association between plasma levels of MPO and mortality of MI has been shown, and MPO has also been identified as an inflammatory biomarker in ischemic heart disease and acute coronary syndrome (ACS). MPO reduces vasodilation by the consumption of nitric oxide and its presence can also be detected in the atherosclerotic plaque, where it can oxidize LDL. Plasma concentrations of MPO do not differ in ischemic and non-ischemic cardiomyopathy, suggesting an independent role for the enzyme in the development of LV dysfunction. Therefore, inhibition of MPO may be a promising therapeutic target for the prevention and/or reduction of reperfusion injury during revascularization.

1.2.3. The main effector of myeloperoxidase is hypochlorous acid (HOCl)

The reaction of H_2O_2 with chloride ion produces highly reactive HOCl. HOCl, formed during the chlorination activity of MPO, is known as a strong oxidizing agent, and despite its short lifetime, its high reactivity, membrane permeability, and broad target spectrum make it the strongest oxidant of leukocytes. The concentration of HOCl generated by MPO is in the order of micromolar, however, it can reach millimolar range in inflammatory tissue. HOCl shows high reactivity with the amino acid side chains of proteins. The HOCl-induced posttranslational protein modifications include the formation of disulfides from Cis amino acids via the sulfenyl chloride intermediate as well as the formation of carbonyl groups during the conversion of chloramines to aldehydes. By chlorinating the amino acid Tyr, it produces 3-chlorothyrosine, a specific marker of HOCl-induced oxidative protein damage. Due to its

high reactivity to methionine, the amino acid is known as a HOCl scavenger and an endogenous antioxidant.

1.3. The role of the giant protein titin in the sarcomere

Titin is the largest protein in the sarcomere, acting as a molecular spring responsible for the formation of the calcium-independent passive force ($F_{passive}$) of the myocardial cells. Titin spans through half of the sarcomere, extending from the Z disc to the M band, ensuring a high organization of the sarcomere. The giant protein is involved in several signal transduction processes and, as a mechanosensor, is able to detect force changes during diastolic elongation and systolic contraction. In mammalian myocardium, there are two isoforms of titin: the longer and more flexible N2BA (~3.2–3.7 MDa) and the shorter and stiffer N2B (~3.3 MDa), which can be put in a ratio of 35:65 in healthy human myocardium. In rodent hearts, however, the shorter and stiffer N2B isoform presents almost exclusively. Changes in the ratio of N2BA and N2B titin isoforms (titin isoform change) affect the passive tension of myocardial cells. The stiffness of titin is also affected by posttranslational modifications, as the protein has phosphorylation sites that affect passive cardiomyocyte tension (protein kinase A, G, Cα (PKA, PKG, PKCα), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)). Oxidative damage to titin may also play an important role in the regulation of $F_{passive}$: disulfide bridges formed by oxidation in the N2Bus region increase caridomycyte resting tension.

1.4. The role of female gender and stress in cardiovascular diseases

1.4.1 The protective role of female sex hormones

Sex does play an important role in the development, on the effectiveness of the applied therapy, as well as on the mortality of CV diseases. Women before menopause are less likely to develop CV disease: ischemic heart disease may be delayed by 10-15 years due to a more favorable lipid profile, lower incidence of LV hypertrophy and remodeling, and lower I/R injury was reported. These observations suggest a cardioprotective effect of female sex

hormones (estrogen and progesterone), however, hormone replacement therapy in clinical practice has not improved outcomes or even increased CV risk.

1.4.2 The role of estrogen and progesterone in the cardiovascular system

Estrogen is a steroid hormone that acts through its nuclear receptors (ERα and ERβ) and its membrane receptor (GPR30). Estrogen receptors are present in myocardial cells, fibroblasts, endothelial and vascular smooth muscle cells in both women and men. Upregulation of estrogen receptors has been shown in hypertrophy and heart failure. Estrogen has an inhibitory effect on the renin-angiotensin system (RAAS) and lowers blood pressure. In addition, by influencing NOS expression, it promotes NO-mediated vasodilation and reduces Ca^{2+} overload in cells during I/R injury. Its anti-inflammatory and antioxidant effects are manifested in the reduction of ROS production and apoptosis.

Progesterone is also produced in the adrenal glands at levels equal to or greater than those produced by the ovaries. During stress, being a precursor to cortisol, adrenal progesterone production and secretion are increased by adrenocorticotropic hormone (ACTH) stimulation regardless of gender and estrogen. Progesterone activates genomic and non-genomic responses through nuclear receptors (PR-A and PR-B) and membrane-bound receptors (mPRα-ε). It inhibits the I/R injury by inhibiting the inflammatory response and has an antiarrhythmic effect by accelerating repolarization. Progesterone is able to inhibit the apoptosis of myocardial cells, mediates antiproliferative effects on vascular smooth muscle cells, causes vasodilation by increasing NO levels after NOS activation, thus lowering blood pressure.

1.4.3 The role of stress in cardiovascular disease

In addition to known CV risk factors, psychosocial stress also has a detrimental effect on the CV system. One manifestation of this is Takotsubo cardiomyopathy (TCM), which develops primarily in postmenopausal women following an intense emotional or physical stress. In

TCM, a substantial increase in catecholamine levels results in apical hypokinesis and balloon-like dilation of the LV, which is thought to be due to the switching of β2-adrenergic receptors from Gs to Gi protein and the consequent decrease in myocardial cell contractility.

The body's stress response is mediated through the hypothalamic-pituitary-adrenal (HPA) axis. First, adrenal catecholamine secretion and sympathetic nervous system activation occur, followed by glucocorticoid secretion within minutes. Glucocorticoids increase the responsiveness of the CV system to sympathetic activation. Activation of the sympathetic nervous system through β-adrenergic signaling patways plays a central role in the regulation of contractile function and Ca^{2+} sensitivity of myocardial cells. PKA-mediated phosphorylation of contractile proteins (myosin-binding protein C (MyBP-C), troponin-I (TnI), titin) affects the contractile function of myocardial cells.

2. Aims

2.1. Investigation of the mechanism of action of myeloperoxidase enzyme (MPO) involved in the development of myocardial infarction and ischemia reperfusion injury

In an *in vitro* model system using human left ventricular myocardial samples, we aimed to investigate

- the effects of MPO and its substrate, hydrogen peroxide, on the contractile function of demembranated cardiomyocytes
- oxidative myofilament protein modifications due to peroxidase and chlorination activity of MPO (sulhydryl (SH) group oxidation and carbonylation)
- reversibility and possible prevention of MPO-induced functional and biochemical alterations (DTT, MPO-I, Met).

2.2. Investigation of the effects of stress and ovariectomy on contractile function of myocardial cells

In a female as well as in an ovariectomized female rat model, to investigate

- whether the lack of female sex hormones and acute stress affect the force genaration of left ventricular isolated myocardial cells
- posttranslational myofilament protein modifications wich may explain the changes in the contractile function of left ventricular myocardial cells.

3. Materials and methods

3.1 Myocardial samples

3.1.1 Human left ventricular myocardial samples

Left ventricular myocardial samples were obtained from four unused human donor hearts (a 41- and 46-year-old woman and a 53- and 56-year-old male). After removal, the hearts were placed in cardioplegic solution and stored at -80 °C until quick freezing in liquid nitrogen until use. Our experiments on human myocardial samples were performed in accordance with the guidelines of the Declaration of Helsinki, with the approval of the Hungarian Ministry of Health (license number: No. 323-8 / 2005-1018EKU) and the Ethics Committee of University of Debrecen.

3.1.2 Acute stress and ovariectomy in a rat model

The animal model was developed at the Institute of Medical Biology, Faculty of Medicine of the J.J. Strossmayer University using 32 female Sprague-Dawley rats. Half of the animals (n = 16) underwent ovariectomy (OVX) at 12 weeks of age. During the operation, part of the ovaries and fallopian tubes were removed from the anesthetized animals. The remaining 16 age-matched, non-operated female rats served as controls (Control). At 28 weeks of age, 8 Control and 8 OVX animals were subjected to acute stress protocol (Control-S and OVX-S groups): the animals were kept in a cold room at 4 °C for 1 hour in a metal cylinder of a diameter that restricted their free movement (cold restrained stress). The animals were then immediately anesthetized using a combination of isoflurane inhalation and intramuscular ketamine (30 mg/kg) injection. Following blood collection, the heart was excised, the left ventricle was detached, placed in liquid nitrogen, and stored at -80 °C. The experimental protocol was approved by the Ethics Committee of the Osijek Medical University. The samples were transported on the basis of the existing sample transport agreement between the Osijek Medical University and the University of Debrecen (DETTI/22-3/2013).

3.2 Measurement of isometric force on dememranated left ventricular cardiomyocytes

During the isolation of myocardial cells, pieces of frozen left ventricular myocardial samples were thawed and then broken in an isolation solution containing protease inhibitors using a mechanical tissue homogenizer. Myocardial cells were then permeabilized using an isolation solution supplemented with 0.5% Triton-X-100 detergent. During force measurements, the myocardial cells were fixed with a silicone adhesive to a sensitive force transducer and an electromagnetic motor, and the average sarcomere length of the preparations was adjusted to 2.3 μm. The force parameters were determined in activating and relaxing solutions with pCa values (negative logarithm of Ca^{2+} concentration (-log10 [Ca^{2+}])) of 4.5 and 9, respectively. Isometric force was measured by moving myocardial cells from the relaxing solution to the activating solution. After force development, the length of the preparation was reduced to 80% (by 20%) of the original length by the electromagnetic motor, and after 2 ms the original cell length was restored ("release-restretch"). The force fell from the maximum value to zero (total force (F_{total}) and then rebuilt in the presence of Ca^{2+}. The Ca^{2+}-independent, passive force component ($F_{passive}$) in the relaxing solution was determined using a similar but longer (8 s) maneuver. By subtracting $F_{passive}$ from F_{total} the Ca^{2+}-dependent active force of the myocardial cell can be calculated (F_{active}). When F_{active} measured by submaximal Ca^{2+} concentrations is plotted against the force obtained in the activating solution (pCa 4.75) a sigmoidal curve can be generated. By fitting this curve, the Ca^{2+} sensitivity of the contractile machinery can be determined, which can be characterized by the Ca^{2+} concentration required to generate the half of the maximum force (pCa_{50}).

3.2.1 Investigation of the effect of the myeloperoxidase enzyme on the force generation of human myocardial cells

To study the functional effects of MPO, F_{active}, $F_{passive}$, and pCa_{50} were determined for MPO substrate H_2O_2 (30 μM, 15 min), MPO+H_2O_2 (MPO, 8 U/L), MPO+H_2O_2+MPO-I (4-

aminobenzhydrazide, 50 μM) and MPO+H_2O_2+Met (10 mM) treatments. The reversibility of MPO+H_2O_2-induced effects was tested using the reducing agent dithiothreitol (DTT, 10 mM, 30 min). The effects of the different treatments on F_{active} and $F_{passive}$ were expressed as a percentage of the values measured before the treatments. Force values were compared to those obtained in the isolation solution (15 min) (time control). 5-12 cardiomyocytes from 3-4 hearts were used for each treatment.

3.2.2 The effect of acute stress and ovariectomy on myocardial cell force generation

Similar to the experiments on human myocardium, F_{active}, $F_{passive}$, and pCa_{50} were measured in control, stressed control (Control-S), ovariectomized (OVX), and stressed ovariectomized (OVX-S) animal groups. 10-10 cardiomyocytes per group were examined.

3.3 Measurement of MPO chlorination and peroxidase activity

Measurement of MPO chlorination and peroxidase activity was performed using special, commercially available kits. During the operation of the assay for chlorinating activity, MPO-generated hypochlorite (OCl^-) converts the non-fluorescent substrate to fluorescein with a strong fluorescence, whereas in the assay for peroxidase activity, MPO converts nonfluorescent ADHP to fluorescent resorufin. The increase in fluorescence intensity was detected at different wavelengths. Fluorescence intensity values were fitted by linear regression prior to saturation, and the slope of the function was used to determine MPO activity. The value measured in the absence of MPO was considered as a background, and the enzyme activity was normalized to this. The values of MPO+H_2O_2 activity were considered to be 100%, and the effect of MPO-I and Met was compared to this values.

3.4 Measurement of serum estrogen, progesterone, glucose and cholesterol levels

In an ovariectomized rat model, serum estradiol and progesterone levels were determined using a competitive chemiluminescent immunoassay. The estradiol range was 4.9 pg/mL to 4.3 ng/mL and the progesterone level ranged from 29.9 pg/mL to 60.1 ng/mL. Serum glucose

levels were measured by an enzymatic photometric, and serum cholesterol was determined by enzymatic colorimetric method.

3.5 Determination of protein SH oxidation, carbonylation and phosphorylation

3.5.1 Determination of SH content of myofilament proteins by Ellman reaction

SH-oxidation of myofilament proteins by MPO was examined by the Ellman reaction. Dememranated left ventricular human cardiomyocytes were incubated for 15 min in isolating solution (Iso) (time control) or treated with Iso containing 30 μM H_2O_2 and 38 U/L MPO+H_2O_2, respectively. After washing steps, the samples were incubated in Ellman's reagent, which reacts with the SH group of the proteins to form yellow 2-nitro-5-thiobenzoic acid (NTB). The absorbance of NTB was measured at 412 nm. The absorbance values of N-acetyl-L-cysteine (NAC) standard series with a known SH-content measured after incubation with Ellman's reagent were used as a calibration curve (exponential fitting). The SH content of the samples was given per 1 mg of lyophilized myocardial homogenate, considering the SH content of the time control (Iso) samples as 100%.

3.5.2 Determination of protein oxidation by SH group biotinylation method

During SH-biotinylation, dememranated myocardial cells were isolated and then treated with isolation solution containing H_2O_2 or MPO+H_2O_2. Samples treated with the oxidizing agent dithiodipyridine (DTDP) served as a positive control. The SH groups of myofilamentary proteins were labeled with biotin and, after washing, solubilized in sample buffer. After centrifugation, the protein concentration from the supernatant was determined by dot-blot method. During SDS-polyacrylamide gel electrophoresis (SDS-PAGE), 0.5% agarose-strengthened, 2%, 4%, 10%, 15% polyacrylamide gels and 4-15% gradient gels were used for separation of myofilament proteins. After transfer to a nitrocellulose membrane, the amount of protein was determined with a fluorescent protein dye (Sypro Ruby Protein Blot Stain).

Biotin-labeled SH groups were detected with horseradish peroxidase-conjugated streptavidin, and after washing the signals were visualized by enhanced chemiluminescent method (ECL).

3.5.3 Protein carbonylation assay

Human left ventricular myocardial samples were treated with H_2O_2 and MPO. Samples treated with Fenton's reagent were used as positive controls. Samples were dissolved in sample buffer, and after centrifugation, the carbonyl groups were derivatized using the supernatant. The protein content of the derivatized and neutralized samples was determined by using a dot-blot method. Proteins were separated by gel electrophoresis, transferred to a nitrocellulose membrane, and the exact amount of protein was determined by Sypro Ruby protein blot stain. Subsequently, a primary and a secondary antibody recognizing the derivatized carbonyl groups were used as specified by the manufacturer. Carbonylated proteins were visualized by the ECL method. Protein carbonylation determined by the OxyBlot™ method was normalized to the amount of protein, and then the degree of carbonylation was given as a carbonylation index. (CI=1 was the carbonylation state of the so-called time control samples).

3.5.4 Determination of phosphorylation status of myocardial proteins

Myocardial cells obtained from control and ovariectomized rats were solubilized in sample buffer, centrifuged, and protein concentration was determined from the supernatant by dot-blot method. Titin, myosin-binding protein C (MyBP-C), and troponin I (TnI) were separated in gels, and the phosphorylation status of the proteins was assayed with ProQ® Diamond phosphoprotein gel stain, and protein quantification was performed with Coomassie blue stain. Protein phosphorylation levels were normalized to protein content and expressed as a percentage of an internal control.

3.6 Data analysis and statistics

The force generation of demembranated cardiomyocytes was measured using a personalized force measurement system (National Instruments). Excel (Microsoft, 2007) and GraphPad Prism 5.0 were used to evaluate and represent the results.

To determine the intensity of chemiluminescent (antibodies), fluorescent (Sypro Ruby-protein, ProQ Diamond-phosphorylation), and Coomassie signals, the area under the curve was measured using ImageJ and Magic Plot programs.

Data are presented as mean±SEM in the figures and the text. For statistical analysis, analysis of variance (ANOVA, Bonferroni, and Dunnet posthoc test) or multilevel mixed-effect linear regression analysis was used to compare pCa-force relationships with an unpaired and paired t-test. Relationships between protein phosphorylation, sex hormones, and myocardial cell function were determined by linear regression. A difference was considered statistically significant if p value was less than 0.05.

4. Results

4.1 Investigation of the effect of myeloperoxidase enzyme (MPO)

4.1.1 Investigation of the functional effects of MPO on human left ventricular cardiomyocytes

Prior to the experiments with MPO, we aimed to determine a concentration of H_2O_2 that already has an independent functional effect, but this effect is enhanced in the presence of MPO. Of the concentrations used during the incubations, the 30 μM H_2O_2 solution significantly reduced the active force of the myocardial cells, so we used this dose of H_2O_2 in our further experiments.

4.1.2 MPO+H_2O_2 treatment impairs the contractile function of human myocardial cells

When MPO (8 U/l) and H_2O_2 (30 μM) were co-administered (15 min), there was a significant decrease in the maximal calcium-dependent active force (F_{active}, pCa 4.75) (57.7 ± 4.1% of the pre-treatment value, n=12) and a significant increase in the calcium-independent passive force component ($F_{passive}$, pCa 9.0) (179.6±14.6% of the untreated myocardial cells, n=12) of the demembranated human left ventricular myocytes were observed. During incubation of the cardiomyocytes in isolation solution (Iso) (15 min, time control), only a slight decrease in F_{active} occured (to 89.0±1.6% of the pre-treatment value). When force values measured in the activating solutions with submaximal Ca^{2+} concentrations before and after the treatments were compared to the maximum force values (F_{active}, pCa 4.75) before the treatments (relative force-pCa correlation), then the force decrease after MPO+H_2O_2 was significantly higher than that in the presence of H_2O_2. The increase in $F_{passive}$ induced by MPO+H_2O_2 was also significantly higher than the increase in $F_{passive}$ observed after H_2O_2 treatment (79.6±14.6% vs. 23.9±7.4%, $p<0.001$).

When calcium sensitivity (pCa_{50}) of the contractile protein system was determined, the force values measured during the activations with submaximal Ca^{2+} concentrations were normalized to the maximum force assesesed in the activating solution (pCa 4.75) before and

after treatments (normalized force). After MPO+H_2O_2 treatment, a significant rightward shift of the normalized force-pCa relationship, i.e. a decrease in pCa_{50}, was observed (pCa_{50} 5.83±0.02 vs. 5.66± 0.02, p <0.001, n=12). In contrast, H_2O_2 treatment did not affect pCa_{50} (5.85±0.05 vs. 5.82±0.03, p=0.55, n=7). The difference between the F_{active}, $F_{passive}$ and pCa_{50} values of myocardial cells before treatment was 5.4%, 5.5% and 0.9%, respectively. Neither H_2O_2 nor MPO+H_2O_2 treatment resulted in a change in the cross striation pattern of the myocardial cells under the light microscope.

4.1.3 MPO inhibitor (MPO-I) and methionine (Met) prevent, dithiothreitol (DTT) partially reverses MPO-induced contractile dysfunction

The possible prevention of adverse functional effects of MPO+H_2O_2 treatment on myocardial cells was investigated using MPO-I (50 μM) and the antioxidant Met (10 mM) and the reducing agent DTT (10 mM). Both MPO-I and Met prevented the F_{active} reduction due to MPO (MPO: 57.7±4.1%, MPO-I: 80.0±5.3%, Met: 80.1±3.6 %, p<0.001 vs. MPO, n=5-6) and the increase in $F_{passive}$ (MPO: 179.6±14.6%, MPO-I: 147.7±6.1% and Met: 139.9±8.7%, p<0.05 vs. MPO, n=5-6). The F_{active} and $F_{passive}$ measured in the presence of MPO-I and Met were comparable to the force values determined after H_2O_2 treatment. In the presence of MPO-I and Met, a shift of the calcium sensitivity curve to the right (decrease in pCa_{50}) could not be observed (MPO-I: 5.88±0.07 vs. MPO: 5.66±0.02, p<0.05 and Met: 5.81±0.04 vs. MPO: 5.66±0.02, p<0.001, n=5-6).

The reducing agent DTT reversed the MPO-induced increase in $F_{passive}$ ($\Delta F_{passive}$ MPO: 89.3±27.3% vs. DTT: 19.7±10.4%, p<0.05, n=6), however, DTT could not prevent the decrease in F_{active} (MPO: 57.3±6.4% vs. DTT: 43.8±5.1%, p=NS, n=6) and pCa_{50} (5.66 ± 0.03 vs. 5.69 ±0.03, p=NS, n = 6) after MPO treatment.

4.1.4 Met inhibits the chlorination activity but not the peroxidase activity of MPO

To investigate the biochemical mechanisms underlying the functional effects of MPO, we determined the chlorination and peroxidase activity of the enzyme in the presence of MPO-I and Met. MPO-I inhibited both the chlorination and peroxidase activity of the enzyme (to 0.3±0.2% and 10.4±6% of baseline activity, respectively, $p<0.001$, n=4). In contrast, Met only reduced the chlorination activity of MPO (to 2.3±1.3%, $p<0.001$, n=4), with no effect on the peroxidase activity of the enzyme (78.4±8.6%, p=NS, n=4).

4.1.5 Investigation of the MPO-induced oxidative protein alterations

In our biochemical experiments, we aimed to investigate how oxidants generated during the action of MPO alter myocardial proteins. To this end, changes in SH group content and carbonylation of myofilamentary proteins were studied after MPO treatment.

4.1.6 Effect of MPO+H_2O_2 treatment on SH content of myofilament proteins

Compared to the amount of SH groups determined in the myocardial cells incubated in Iso, a small but significant decrease in the total SH group content was observed during H_2O_2 and combined MPO+H_2O_2 treatment (90.4±1.5%, $p<0.05$, n=3 and 86.7±4%, respectively, $p<0.01$, n=3).

An attempt was also made to identify proteins undergoing SH oxidation. H_2O_2 and MPO+H_2O_2 treatment significantly reduced actin SH content (to 75.9±7.1%, $p<0.01$, n=4, and to 84.2±4.4%, $p<0.05$, n=9) relative to the SH content of the samples treated in Iso. In contrast, neither the SH content of MyBP-C nor that of the more flexible (N2BA) and stiff (N2B) titin isoforms change significantly after the treatments. SH oxidation can also form intermolecular disulfide bridges between proteins, leading to the formation of high molecular weight complexes. Using a Western immunoblot technique, a protein complex with a molecular weight of nearly 90 kDa, containing tropomyosin and actin, was detected in a non-reducing environment, but its intensity decreased with H_2O_2 or MPO+H_2O_2 treatment.

In our experiments, we also examined the amount of differently oxidized forms of titin dissolved in non-reducing and reducing solutions. Increasing the concentration of DTDP significantly reduced the content of supernatant titin obtained by denaturation in non-reducing buffer, while the amount of titin remaining in the pellet increased. Thus, the supernatant/pellet ratio was significantly reduced for both the N2B and N2BA titin isoforms. However, the MPO+H_2O_2 treatment did not affect the titin supernatant/pellet ratio compared to the initial (Iso) sample.

4.1.7 Effect of MPO on carbonylation of myocardial proteins

H_2O_2 treatment showed a small but significant increase in the content of the actin carbonyl group (carbonylation index (CI)=1.1±0.046, $p<0.05$, n=5). However, this value did not change after the addition of MPO (CI=1.1±0.052, p=0.9 vs. H_2O_2, n=11). Similar to actin, a small but significant increase in MyBP-C protein carbonylation was measured after both H_2O_2 (CI=1.5±0.2, $p<0.05$, n=2) and MPO+H_2O_2 treatment (CI=1.4±0.2, n=4, $p<0.05$ vs. time control). The carbonylation of the N2BA and N2B isoforms of the giant protein titin did not change after H_2O_2 and MPO+H_2O_2 treatment (N2BA-H_2O_2: CI=0.9±0.2, p=0.47 vs. Iso and MPO: 1.0±0.2, p=0.68 vs. Iso, N2B-H_2O_2: CI=1.0±0.1, p=0.36 vs. Iso and MPO: CI=0.9±0.1, p=0.64 vs. Iso).

4.2 Effects of ovariectomy and acute stress on contractile function of rat left ventricular myocytes

4.2.1 Effect of ovariectomy and stress on physical and laboratory parameters of the experimental animals

The body weight as well as the heart weight of the rats were significantly higher in the OVX-S group, however, the heart-body weight ratio did not differ between the animal groups. Serum glucose as well as cholesterol values were similar in the four groups of animals.

4.2.2 Effects of ovariectomy and stress on serum estrogen and progesterone levels

Serum levels of female sex hormones were determined in Control and OVX rats. In the study, estrogen levels in OVX animals did not reach the lower detection limit (4.9 pg/mL), indicating a successful ovariectomy. Significantly higher (more than three-fold) serum progesterone levels were measured in Control-S (35.6±4.8 ng/mL) and OVX-S (21.9±4.0 ng/mL) rats compared to Control (10±2.9 ng/mL) and OVX (2.8±0.5 ng/mL) animals (n=8/group, $p<0.001$ Control-S vs. Control; $p<0.01$ OVX-S vs. OVX). The increase in progesterone levels (25.6 ng/mL and 19.1 ng/mL, respectively) was similar in the Control-S and OVX-S groups after acute stress.

4.2.3 Ovariectomy affects the contractile function of myocardial cells

F_{active} was found to be significantly higher in OVX and OVX-S animals compared to Control and Control-S animals (25.9±3.4 kN/m^2 and 26.3±3.0 kN/m^2 vs. 16.4±1.2 kN/m^2 and 14.4±0.9 kN/m^2, $p<0.05$, n=10). In contrast, acute stress did not affect F_{active} in either the Control or OVX animal groups. The decrease in pCa_{50} under stress was not found to be statistically significant. Neither ovariectomy nor stress affected the values of $F_{passive}$ or nHill coefficient. The cross-sections of the myocardial cells used in OVX and OVX-S animals were significantly smaller. The sarcomere length-force relationship was maintained in all four groups of animals, and both F_{active} and $F_{passive}$ increased as a result of stretching.

4.2.4 Effect of ovariectomy and stress on the composition and phosphorylation state of myofilamentary proteins

No significant difference in myofilamentary protein composition was found between the Control and OVX groups. Phosphorylation of TnI was significantly higher in OVX animals compared to the Control group (153.0±14.4% vs. 98.5±4%, $p<0.001$), but this did not correlate with pCa_{50} measured on the cardiomyocytes ($r=0.268$, $p=0.73$). The TnI phosphorylation measured in the OVX-S group (113.1±3.6%) was comparable to the values

determined in the Control and Control-S groups. Phosphorylation of MyBP-C was significantly higher in OVX animals compared to the Control group (167.7±7% vs. 131.4±5.5%, $p<0.001$), however, no further significant increase was observed in OVX-S animals. (182.9±6.2%). Consistent with the unchanged $F_{passive}$ values observed in the functional measurements, we found no difference in titin phosphorylation between the four groups of animals.

4.2.5 Relationship between cardiomyocyte contractile function, phosphorylation of myocardial proteins and levels of female sex hormones

Comparing the functional parameters of the cardiomycytes with the values of protein phosphorylation, we found a significant correlation between F_{active} and MyBPC phosphorylation ($r=0.986$, $p<0.05$). However, titin phosphorylation showed no correlation with $F_{passive}$ as determined on the myocardial cells ($r=0.14$, $p=0.86$). There was a significant inverse correlation between serum progesterone levels and pCa_{50} ($r=0.96$, $p<0.05$).

5. Discussion

5.1 Effect of myeloperoxidase enzyme (MPO) on the mechanical and biochemical properties of human myocardial cells

During myocardial inflammation and I/R injury, an increase in oxidative stress and development of contractile dysfunction have been observed. In our measurements, MPO+H_2O_2 treatment significantly reduced the active force and calcium sensitivity of the cardiomyocytes. The effect of H_2O_2 alone was concentration dependent, however, the significant detrimental effect of MPO+H_2O_2 treatment on active force may be due to HOCl, the strong oxidant formed in the reaction. When examined with light microscopy during MPO+H_2O_2 treatment, the cross striation pattern of myocardial cells did not change, however, it is important to note that electron microscopy confirmed HOCl-induced damage to the myofilament network. Calcium-independent passive force was slightly increased by H_2O_2, while it was significantly increased by H_2O_2+MPO. These results are consistent with the previous observation that low H_2O_2 concentration (10 μM) did not increase passive force in skinned rat trabeculae, whereas treatment with HOCl caused a significant increase in the passive tension. The giant sarcomere protein, titin, has been shown to play a key role in the generation of the passive tension of demembranated myocardial cells by acting as a molecular spring. Cardiomyocyte passive force may change due to titin isoform shift as well as posttranslational modifications (phosphorylation, SH oxidation, and presumably carbonylation). In the present study, biochemical methods failed to detect SH oxidation and carbonylation of titin after H_2O_2+MPO treatment, however, in our functional measurements, DTT significantly reduced H_2O_2+MPO-induced growth in cardiomyocyte $F_{passive}$. The reversibility of the MPO effect with a reducing agent suggests its oxidative nature. H_2O_2+MPO treatment showed a significant decrease in calcium sensitivity, which contradicts the results of a previous study in which an increase in pCa_{50} was observed after HOCl

treatment. The different results may be due to several factors: in our experiments, for example, the amount of HOCl produced is unknown, so the two experimental setups may differ in the dose used, moreover, treatment of permeabilized myocytes and trabeculae is also difficult to compare. H_2O_2 treatment had no effect on the calcium sensitivity of the cardiomyocyets, so we hypothesize that it causes structural rather than regulatory changes at the level of myofilament proteins, even by reducing the number of force-generating cross-bridges.

In the recent years, the role of MPO has been demonstrated in a number of CV diseases (e.g. I/R injury, acute MI, HFpEF). Determining serum levels of MPO as a biomarker may be useful in diagnosing these conditions, and inhibition of the enzyme may play a key role in the treatment of these conditions. Despite the potential negative immunological consequences of MPO inhibition, MPO inhibitors are currently undergoing in clinical trials for the treatment of a number of CV diseases (myocardial infarction, HFpEF).

The MPO-I we used, 4-aminobenzhydrazide, was able to counteract the detrimental effects of MPO on the myocardial cells. Like MPO inhibitor, Met was also able to prevent MPO from exerting its functional effects, suggesting that the chlorination activity of MPO may be responsible for the observed alterations. The latter finding was also confirmed by the MPO activity assay. Based on previous studies, it is known that oxidation of physiologically relevant Met side chains can impair protein function, which has also been shown for myocardial proteins (e.g. actin). With the MPO inhibitor and Met, we successfully prevented the oxidative effects of MPO at two points. However, the reducing agent DTT was only partially able to reverse the MPO-induced mechanical changes: it reduced the passive force to baseline, but did not affect the active force and pCa_{50}. Due to the variety of oxidative effects mediated by MPO, it is likely that in our case, several different reactions resulted in different posttranslational modifications to myocardial proteins. In our experiments, we found a

significant decrease in SH content in the case of actin, however, the extent of this did not increase with MPO, thereby this alone does not explain the decrease in cardiomyocyte active force.

In a mouse model of myocardial infarction previously used by our group, the decrease in calcium sensitivity was associated with carbonylation of actin and MHC, while high concentrations of H_2O_2 induced carbonylation of actin. In the present study, we observed a small increase in the carbonylation index for actin and MyBP-C after 30 μM H_2O_2 treatment. As no further increase in this parameter was observed with MPO treatment, the MPO-induced functional effects could not be explained by SH oxidation or carbonylation of a specific myocardial protein.

Possible crosslinking between myocardial proteins was also examined in myocardial samples dissolved in non-reducing buffer. A protein complex reacting with antibodies to actin and Tm was identified under non-reducing conditions, which amount decreased with MPO treatment. This phenomenon may be due to the fact that the solubility of the protein decreased as a result of the treatment, so that less of it could get into the gel when dissolved in non-reducing buffer.

Other protein modifications (eg chlorination, nitration, Met oxidation, sulfonic acid formation, protein degradation) may be involved in mediating the effect of MPO on myocardial cells, which were not covered by our experiments.

In our present study, MPO impaired the contractile function of myocardial cells. Numerous studies have been already conducted on the harmful effects of oxidative stress, but newer evidence supports a much more complex picture. Indeed, it has been shown that mild oxidative stress can even be cardioprotective and even improve the performance of the contractile apparatus. Overall, the effect of oxidative agents on myocardium is highly dependent on their strength and nature, as well as the redox environment of myofilaments.

5.2 Effects of acute stress and female sex hormones on contractile function of myocardial cells and phosphorylation of myofilament proteins

In different animal models, the type and duration of stress applied and the mode of application (acute or chronic) can greatly influence the extent of the responses. Because the effects of chronic stress are difficult to study due to the desensitization that occurs, in our present animal model we examined the effects of acute stress. The acute stress protocol used in our experiment (cold with immobilization) occurs relatively rare in the everyday life, but it also causes an increase in ACTH and corticosteroid levels. In our animal model a significant increase in the serum progesterone levels was observed in both the stressed Control and OVX animal group, moreover, its extent was also fully comparable with the hormone levels measured in the literature. The cardiovascular effects of progesterone are more likely linked with the activation of the non-genomic pathways. The involvement of these rapid signal transduction pathways in the progesterone response raises the potential effects of acute stress-induced increases in progesterone levels on the contractile function and/or regulation of myocardial cells.

Excessive catecholamine release under stress leads to activation of PKA via the β-adrenergic signaling pathway. PKA-mediated phosphorylation of TnI affects contractile function through decreased calcium sensitivity of the contractile machinery. In the present study only a small, non-significant decrease in calcium sensitivity was observed in the stressed Control-S and OVX-S animal groups, but this was not associated with changes in the phosphorylation status of TnI. The conflicting results may also be explained by the non-specific kinase and phosphorylation site staining method we used.

In our experiments, we found a significant correlation between serum progesterone levels and pCa_{50} values measured in the myocardial cells. This suggests that the small, non-significant decrease in Ca^{2+} sensitivity observed in Control-S and OVX-S animals was caused

by emotional stress. However, based on our observation, it is not clear whether Ca^{2+} sensitivity was affected predominantly by the stress-induced progesterone or the stress-induced activation of the β-adrenergic pathway.

Stimulation of the β-adrenergic signaling pathway following ovariectomy, the consequent increase in cAMP concentration and PKA activation leads to phosphorylation of myofilamentary proteins. Phosphorylation of the MyBP-C protein accelerates the kinetics of actin-myosin cross-linking and increases the maximal Ca^{2+}-activated force. Consistent with this observation, we also observed significant growth in F_{active} in OVX animals. In addition, phosphorylation of MyBP-C showed a significant correlation with F_{active}. Based on our results, the increase in F_{active} of cardiomyocytes isolated from OVX animals may be caused by MyBP-C phosphorylation due to the β1-adrenegic stimulation induced by ovariectomy.

In our work, in accordance with the data in the literature, we did not find any differences between the groups of animals either in the $F_{passive}$ or in the phosphorylation state of its main regulator protein, titin.

In parallel with the continuously increasing recognition of takotsubo cardiomyopathy in clinical practice, there is a desperate need for a more detailed understanding of the acute and late effects of stress and female sex hormones on myocardial contractile function, and for development of adequate, specific therapies.

Original observations of the doctoral thesis

Based on the results of the doctoral thesis the following original statements can be made:

1. MPO impairs calcium-dependent isometric force generation under *in vitro* experimental conditions, increases calcium-independent passive force, and decreases calcium sensitivity of force production.
2. The functional effect induced by MPO can be prevented by using MPO inhibitor and the antioxidant methionine, while the increase in passive force can be reversed by reducing agent.
3. SH-oxidation of actin and carbonylation of actin and MyBP-C increases with H_2O_2 and H_2O_2+MPO treatment.
4. The functional effects induced by MPO are mediated by the chlorination activity of MPO.
5. Ovariectomy increased the force generation of myocardial cells, which was confirmed to be parallel with an increase in MyBP-C phosphorylation.
6. Ovariectomy had no effect on the passive force and calcium sensitivity (pCa_{50}) of isolated cardiomyocytes, however, after acute stress, a marked decrease in pCa_{50} was observed. The latter shows inverse correlation with the elevated serum progesterone levels upon acute stress.

6. Summary

Oxidative stress and acute stress have been showed to play an important role in the pathogenesis of several cardiovascular pathologies (myocardial infarction, ischemia/reperfusion injury, heart failure). Among multiple pivotial effects they also induce contractile dysfunction by posttranslational modifications of myofilament proteins.

In our experiments the *MPO-derived oxidants* contributed to myocardial contractile dysfunction *in vitro* by decreasing the cardiomyocyte force production and the myofilament Ca^{2+} sensitivity and increasing the passive force component in human cardiomyocytes. These functional alterations could be induced by SH group oxidation, however, seemed to be independent from carbonylation of myofilamentary proteins. The deleterious effects of MPO could be prevented by MPO inhibition and the antioxidant Met. The identified functional and biochemical alterations may provide a pharmacological tool for the prevention and/or reversion of MPO-induced contractile dysfunction and myofilament protein alterations, which could have therapeutic implications in cardiac pathologies characterized by elevated MPO levels. In our other study, single acute stress significantly increasd serum progesterone levels in both non- ovariectomized and ovariectomized rats. The lack of estrogen alters contractile function of left vetricular isolated cardiomyocytes by increasing Ca^{2+}-activated force production through myosin-binding protein C phosphorylation. Parallel with the stress-induced increase in the serum progesterone level, a decrease in the myofilament Ca^{2+}-sensitivity could be observed. The latter might be a protective response of the body to the emotional stress.

The exact mechanisms underlying the detrimental effects of oxidants and the protective effects of female hormones in the pathogenesis of the above-mentioned cardiac pathologies remains unanswered. Better understanding of the functional consequences of the specific myofilament protein modifications may lead to the development of novel therapeutic approches for cardiovascular diseases.

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List of publications

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List of publications related to the dissertation

1. **Kalász, J.**, Pásztorné Tóth, E., Fagyas, M., Balogh, Á., Tóth, A., Csató, V., Édes, I., Papp, Z., Borbély, A.: Myeloperoxidase impairs the contractile function in isolated human cardiomyocytes.
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