Stress-induced protein S-glutathionylation and phosphorylation crosstalk in cardiac sarcomeric proteins - Impact on heart function

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Title: Stress-induced protein S-glutathionylation and phosphorylation crosstalk in cardiac sarcomeric proteins - impact on heart function.

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These authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation

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Abstract

Background: The interplay between oxidative stress and other signaling pathways in the contractile machinery regulation during cardiac stress and its consequences on cardiac function remains poorly understood. We evaluated the effect of the crosstalk between β-adrenergic and redox signaling on post-translational modifications of sarcomeric regulatory proteins, Myosin Binding Protein-C (MyBP-C) and Troponin I (TnI). **Methods and Results:** We mimicked *in vitro* high level of physiological cardiac stress by forcing rat hearts to produce high levels of oxidized glutathione. This led to MyBP-C S-glutathionylation associated with lower protein kinase A (PKA) dependent phosphorylations of MyBP-C and TnI, increased myofilament Ca^{2+} sensitivity, and decreased systolic and diastolic properties of the isolated perfused heart. Moderate physiological cardiac stress achieved *in vivo* with a single 35 min exercise (Low stress induced by exercise, LSE) increased TnI and cMyBP-C phosphorylations and improved cardiac function *in vivo* (echocardiography) and *ex-vivo* (isolated perfused heart). High stress induced by exercise (HSE) altered strongly oxidative stress markers and phosphorylations were unchanged despite increased PKA activity. HSE led to *in vivo* intrinsic cardiac dysfunction associated with myofilament Ca^{2+} sensitivity defects. To limit protein S-glutathionylation after HSE, we treated rats with N-acetylcysteine (NAC). NAC restored the ability of PKA to modulate myofilament Ca^{2+} sensitivity and prevented cardiac dysfunction observed in HSE animals. **Conclusion:** Under cardiac stress, adrenergic and oxidative signaling pathways work in concert to alter myofilament properties and are key regulators of cardiac function.

Introduction

Oxidative stress is common in many cardiac disorders including ischemia/reperfusion, diabetes, and hypertensive heart disease. Oxidative stress leads to impaired cardiac pump function due to changes in the expression or function of proteins that regulate the excitation contraction coupling. There is growing evidence that the cardiodepressant actions of reactive oxygen species (ROS) are not only mediated by myocardial injuries but also are attributable to subtler redox-dependent post-translational modifications of excitation contraction coupling proteins [1]. Each cardiac contraction is triggered by the release of calcium (Ca^{2+}) from the sarcoplasmic reticulum that binds on the troponin-tropomyosin regulatory complex, which activates subsequently the interaction between thick and thin filaments. Oxidative modifications of proteins involved in Ca^{2+} handling during excitation contraction coupling are largely described and include inhibitory SERCA2a redox modification [2] and oxidation as well as ROS-dependent hyperphosphorylation of the Ryanodine receptor [3]. Although the contractile machinery has been less studied, the sarcomere itself is also the target of oxidative modifications that further contribute to contractile dysfunctions in cardiac disease [4]. In the healthy heart, the role of such modifications to accommodate the difference in hemodynamic loads during rest and cardiac stress remains to be clarified and was the focus of the present study.

The contractile machinery is highly regulated by post-translational modifications of regulatory contractile proteins, such as Myosin Light Chain 2 (MLC2), Troponin I (TnI) and cardiac Myosin Binding Protein-C (cMyBP-C). These sarcomeric proteins are phosphorylated by ROS-sensitive enzymes [5] and can be directly impacted by ROS production [6]. Among redox-sensitive sarcomeric proteins, cMyBP-C is under the scope. Indeed, cMyBP-C is an important regulator of cardiac dynamics, a potential activator of the thin filaments that interacts with myosin [7] and titin [8] and contributes to the heart β-adrenergic response [9, 10]. cMyBP-C phosphorylation at Ser273, Ser282, and Ser302 is generally attributed to protein kinase A (PKA) and viewed as a mechanism that modulates the kinetic of thick-thin filament interactions and increases force generation [11]. In the last decade, cMyBP-C was shown to be a preferential target of S-glutathionylation, a reversible post-translational modification

between glutathione (GSH) and redox-sensitive cysteines of proteins activated during oxidative stress (see [12] for review). S-glutathionylation of cMyBP-C was identified first in a proteomic screen of cardiomyocytes stimulated *in vitro* with oxidized GSH or diamide a thiol selective oxidant [13]. The functional consequences of cMyBP-C S-glutathionylation were demonstrated later in the deoxycorticosterone acetate-salt hypertensive mouse model [14]. The level of cMyBP-C S-glutathionylation was correlated with diastolic dysfunction without apparent change in cellular Ca^{2+} fluxes [14]. The ROS associated with the disease induced S-glutathionylation of cMyBP-C, which slowed cross-bridge kinetics and increased Ca^{2+} sensitivity. Several cysteines of cMyBP-C can be S-glutathionylated *in vitro* and lead to an increase in myofilament Ca^{2+}-sensitivity [15, 16]. Previous experiments have found that every cardiac myofilament protein, except TnT and MLC2 has a cysteine that can be modified by oxidants [17, 18]. Among them all proteins are not S-glutathionylated. A high-molecular-weight protein, which could be titin has been identified as a likely target of S-glutathionylation following myocardial infarction [19]. Later a study showed clearly *in vitro* that titin can be S-glutathionylated [20]. Interestingly, an interaction between phosphorylation and S-glutathionylation of cMyBP-C was explicitly demonstrated *in vitro* [16]. Reduced site-specific cMyBP-C phosphorylation is accompanied by increased protein S-glutathionylation in ventricular tissue from patients with heart failure. The link between pathways and the functional consequences remains unclear in particular *in vivo*.

Therefore, the aim of the present study was to focus on the cardiac functional consequences of myofilament protein post-translational modifications induced by ROS or ROS-activated signaling enzymes. We particularly investigated cMyBP-C and cTnI. The β-adrenergic signaling and oxidative stress pathways were differentially activated by modulating in intensity and duration the physiological stress induced by exercise. We observed that depending on the stress duration, the cardiomyocyte contractile machinery is targeted by different post-translational modifications that differentially affect the cardiomyocyte contractile function *in vivo*.

Materials and Methods

Animal studies. Male Wistar rats (12-week-old, n=78; weight=361±4 g; Janvier, France) were housed with a 12-hour light-dark cycle and free access to water and food. All investigations conformed to the European Parliament Directive 2010/63/EU and were approved by the local ethics committee (Comité d'éthique pour l'expérimentation animale Languedoc-Roussillon, n° CEEA-00322.03). All rats were familiarized with the treadmill by running at 15 m/min for 15min daily for a week. Afterwards, to achieve different degrees of activation of the pathways investigated, some rats performed a bout of exercise at 20m/min (about 65% of the maximal aerobic velocity, MAV) for 35min (Low stress induced by exercise, LSE group) or until exhaustion for about 3 hours (186.7±14.6 min) to induce a high stress-induced by exercise (HSE) [21]. LSE and HSE animals were compared with non-runners (control group). Animals were regularly given access to water during the exercise protocol to avoid dehydration and hypovolemia. Other rats (n=12) were treated with 50mg/kg N-acetyl cystein (NAC) by i.p. injection 48 hours and 1 hour before HSE (HSE+NAC group). To study the modifications persisting after exercise, all experiments were performed after a recovery of 30min.

***In vivo* cardiac function analysis.** Rats were anesthetized with ketamine and xylazine (75mg/kg and 15mg/kg respectively, i.p.) for echocardiography (MyLab 30 ESAOTE), as previously described [22].

Isolated perfused hearts. Isolated hearts were retrogradely perfused and LV function evaluated all along the procedure, as described [23], in presence or not of 80µmol/L 1,3-bis-2chloroethyl-1-nitrourea (BCNU), a glutathione reductase inhibitor, for 5min (8), or with 0.1µM ISO for 5min (n=4).

Force measurements in permeabilized cardiomyocytes. Isometric force was measured in single cardiomyocytes permeabilized with 0.3% Triton X-100 for 5min.[22] The relationship between Ca^{2+}-activated force and internal Ca^{2+} concentration was measured at a SL of 2.3µm and fitted using a modified Hill equation. To mimic β-adrenergic stimulation, some permeabilized cardiomyocytes were

incubated before attachment with recombinant PKA catalytic subunit (Sigma Aldrich, France) at room temperature for 50min, as described [10].

Western blot analysis. Myocardial protein expression was studied, as previously described [5]. Briefly, frozen LV tissue was solubilized in non-reducing Laemmli buffer. Proteins were separated using SDS-PAGE electrophoresis and transferred onto nitrocellulose membranes (GE Healthcare). Membranes were blocked with the Odyssey Blocking Buffer in PBS (LI-COR Biosciences) and incubated overnight with primary antibodies (Supplemental TableS1). Bands were revealed and quantified with the Odyssey system (LI-COR Biosciences, Lincoln, Nebraska) after incubation with fluorescent secondary antibodies. Protein content was expressed relative to GAPDH or calsequestrin (CSQ) content.

Determination of glutathione levels and cMyBP-C S-glutathionylation. Levels of oxidized (GSSG) and reduced glutathione (GSH) in LV were measured using a Glutathione (GSSG/GSH) Detection Kit (ADI-900-160, EnzoLife Sciences, NY, USA), according to the manufacturer's instructions.
Non-reducing SDS-PAGE was used to determine cMyBP-C S-glutathionylation, following a previously described protocol.[15]

Malondialdehyde assay. As a marker of myocardial lipid peroxidation, Malondialdehyde (MDA) level was measured in cardiac tissue using the Lipid Peroxidation-MDA Assay Kit (ab118970, Abcam, Cambridge, Mass, United States), according to the manufacturer's instructions.

Determination of protein kinase A activity. PKA activity was determined by a non-radioactive protein kinase assay kit (PKA Kinase Activity Assay Kit, no. ab139435, Abcam, Cambridge, Mass, United States) according to the manufacturer's instructions.

Statistical analysis. Statistics were performed using StatView 5.0 (SAS Institute, Cary, NC). Data are presented as the mean±SEM. Differences were assessed with the one-way ANOVA when appropriate. When significant interactions were found, a Bonferroni *post hoc* test was applied with $p< 0.05$.

Results

S-glutathionylation affects post-translational modifications of contractile proteins and cardiac function in rats

Isolated control hearts were perfused with or without an inhibitor of glutathione reductase activity (BCNU). Glutathione reductase inhibition decreases the cellular reduced/oxidized glutathione ratio (GSH/GSSG), leading to protein S-glutathionylation [24]. Perfusion of isolated hearts with BCNU reduced markedly the level of free GSH as well as the GSH/GSSG ratio (Fig. S1) without impacting MDA (Fig S1B), used as a marker of lipid peroxidation. In line with altered GSH/GSSG ratio, BCNU increased the level of S-glutathionylated cMyBP-C (Fig. 1A). Regarding regulatory proteins of sarcomeric function, S-glutathionylation was specific to cMyBP-C and did not affect the other regulatory protein cTnI (Fig. 1B). Interestingly, the phosphorylation levels of cMyBP-C and TnI at PKA sites were decreased in particular for cMyBP-C (Fig. 1C and 1D). This was not due to a reduction of PKA activity with BCNU treatment since the level of PKA activity was not altered (Fig. 1E). Those post-translational modifications of cMyBP-C and TnI were associated with changes in the contractile machinery properties investigated in permeabilized cardiomyocytes. The relationship between the Ca^{2+}-activated tension and the amount of Ca^{2+} (pCa=-log[Ca^{2+}]) was altered in BCNU-treated myocytes compared with control cells (Fig. 1F). The maximal active tension was decreased by BCNU (Fig. 1F and 1H). The Ca^{2+} sensitivity of myofilaments, assessed as the pCa that develops half of the maximal tension (pCa_{50}), was higher in BCNU-treated myocytes than in controls (Fig. 1G and 1I). The diastolic stiffness of the myocytes was determined by measuring the level of passive tension developed when stretching the myocyte from the slack length (≈1.8µm) to 2.0 and 2.3µm of SL in absence of Ca^{2+}. Passive tension was not significantly altered by BCNU although a tendency to increase was observed (Fig. 1J). Increasing the amount of oxidized GSH altered the contractile properties of the whole heart evaluated *ex vivo* using a Langendorff isolated perfused heart model to control cardiac preload, afterload, heart rate and circulating factors. The LV diastolic properties were altered as indicated by the tendency to higher LV diastolic pressure (Fig. 1K) and significant lower relaxation velocity dP/dtmin (Fig. 1N) in BCNU-perfused hearts compared with untreated samples. Systolic function was also altered, as

indicated by the lower LV systolic pressure (Fig. 1L) and contraction velocity dP/dtmax (Fig. 1M). In conclusion, high oxidized GSH levels induce negative effects on both diastolic and systolic contractile properties, possibly via protein S-glutathionylation and decreased phosphorylation of regulatory contractile proteins.

Differential balance between β-adrenergic and oxidative pathways mediated by low and high stress-induced by exercise

We explored the physiological relevance of the interaction between protein S-glutathionylation and PKA-dependent phosphorylations in a context of acute physical exercise, known to activate both β-adrenergic and oxidative pathways [25]. Moderate physiological cardiac stress was achieved *in vivo* with a single 35min exercise (Low stress induced by exercise, LSE). For high stress induced by exercise (HSE), animals ran until exhaustion. The degree of β-adrenergic signaling pathway activation was determined by measuring the activity of its intracellular effector PKA (Fig. 2A). PKA activity was increased by 15% in LSE hearts and by 30% with HSE to a level reached when isolated control hearts were stimulated *ex vivo* with isoprenaline (ISO). The phosphorylation levels of cMyBP-C and TnI on PKA sites (Fig. 2B) were both increased with LSE compared with control hearts. The level of TnI phosphorylation after LSE was however lower when compared to hearts directly treated *ex vivo* with ISO. Interestingly the levels of cMyBP-C and TnI phosphorylation were similar to control hearts after HSE despite the increase in PKA activity (Fig. 2B).

The level of oxidative stress produced by cardiac stress was evaluated by several markers. A marker of lipid peroxidation, MDA, was increased significantly by 66% after LSE and by 240% with HSE compared with controls (Fig. 2C). ISO stimulated hearts had also a higher MDA content of 160%. Both LSE and HSE had no effect on irreversible post-translational modifications such as the protein carbonylation (Fig. S2B). We also measured the level of free GSH as well as the GSH/GSSG ratio (Fig. 2D and 2E), which reflect the degree of oxidative stress and altered cellular redox state. Both parameters were unchanged with LSE and significantly decreased with HSE or isoprenaline stimulation. This reflects an increase in oxidized glutathione content and decrease in free glutathione with HSE and isoprenaline stimulation. The level of S-glutathionylated cMyBP-C was increased in all conditions of

stress, LSE, HSE and ISO (Fig. 2F). Altogether, the results indicate that these stress protocols provide as expected differential levels of activation of β-adrenergic and oxidative pathways associated with different post-translational modifications of regulatory proteins.

Under these different stresses and modifications, myofilament contractility was unchanged by LSE and altered by HSE (Fig. 3A and 3B). The maximal active tension was decreased (Fig. 3A and 3C) and the Ca^{2+} sensitivity of myofilaments (pCa_{50}) increased in HSE myocytes compared with controls (Fig. 3B and 3D). Finally, the diastolic properties were altered, as indicated by the higher passive tension induced by HSE compared with control myocytes after a stretch to 2.3µm of SL in relaxing solution (Fig. 3E). LSE had modest impact on the whole heart contractility measured *ex vivo* in isolated perfused heart model. In such conditions, systolic parameters, such as the developed pressure (Fig. 3F) and $dP/dt_{max,}$ (Fig. 3G) were similar after LSE to control hearts and only relaxation velocity $dP/dt_{min,}$ tended to increase (Fig. 3H). Conversely, after HSE all systolic and diastolic parameters were reduced compared to control hearts (Fig. 3F to 3H). Finally, in another set of rats, cardiac contractile function was measured *in vivo* by echocardiography after a resting period of 30min under anesthesia (Fig. 3E). No difference of heart rate was observed between groups (supplemental Table S2). The left ventricular diastolic end diameter was increased after LSE and reduced after HSE compared with controls. The left systolic ventricular end diameter decreased only after HSE. Systolic function was increased only after LSE as indicated by the higher LV shortening fraction (Fig. 3I, Supplemental Table S2) and LV systolic wall motion velocity (Sm), evaluated by tissue Doppler imaging compared with controls (Supplemental Table S2). The diastolic anterior and posterior wall thicknesses were significantly increased in HSE compared with LSE, synonyms of reduced wall relaxation during diastole in HSE. Other indexes of diastolic function such as the E/A ratios of early diastolic and atrial transmitral inflow velocities (Fig. 3J) as well as the Em/Am ratios obtained by tissue Doppler imaging, were reduced only in HSE compared with controls and LSE (Fig. 3K). Altogether, the *in vivo* and *ex vivo* results indicate that in rats, LSE has no major effects on cardiac function and if there is one it is beneficial whereas HSE leads to *in vivo* intrinsic cardiac dysfunction associated with specific post-translational modifications such as reduced PKA-dependent phosphorylations and increased S-glutathionylated cMyBP-C.

Preventing redox modifications reduces the stress-induced by exercise

N-acetylcysteine (NAC), a cell-permeable cysteine precursor shown to replenish intracellular GSH levels [26], was administrated to rats to limit the activation of protein S-glutathionylation after HSE. MDA levels in HSE hearts were normalized to those of control rats (Fig. S2). Moreover the levels of free GSH as well as the GSH/GSSG ratio in HSE-NAC were significantly higher than HSE (Fig. 4A). NAC also prevented the increased level of S-glutathionylated cMyBP-C after HSE (Fig. 4B). Relative to controls, the levels of PKA-dependent phosphorylations were reduced for cMyBP-C and unchanged for TnI. In isolated hearts, systolic parameters such as the LV developed pressure tended to be higher in hearts from HSE+NAC rats compared with untreated HSE animals (Fig. 4C) and the difference reach significance regarding $dP/dt_{max,}$ (Fig. 4D). The impaired relaxation (dP/dt_{min}) after HSE was completely prevented by NAC (Fig. 4E). At the level of the contractile machinery, NAC pre-treatment had no effect on the maximal active tension (Fig. 4F) but prevented the changes induced by HSE on the passive tension and myofilament Ca^{2+} sensitivity (Fig. 4, G and H).

Incubation of permeabilized myocytes with the recombinant PKA catalytic subunit led to a reduction in the myofilament Ca^{2+} sensitivity (Fig. 4H) in control myocytes, as previously reported [10]. Conversely, myofilaments became insensitive to PKA after HSE. Similar results, as well as impaired relaxation, were previously obtained in transgenic mouse models in which TnI could not be phosphorylated by PKA [27] or cMyBP-C was absent [10, 28]. Interestingly, the myofilament sensitivity to PKA was maintained in the HSE+NAC group, as shown by the reduction in pCa_{50} upon incubation with recombinant PKA catalytic subunit (Fig. 4H). Altogether, the results indicate that HSE alters the contractile properties through a redox-dependent mechanism.

Discussion

In this study, we investigated the intrinsic contractile defects that persist in the myocardium after physiological cardiac stress induced by different levels of physical activity. We observed that following increased cardiac workload, the contractile machinery is the target of reversible post-translational modifications that affect the cardiac function *in vivo*. Specifically, moderate exercise induced persistent S-glutathionylation of the regulatory protein cMyBP-C and PKA-dependent phosphorylation of cMyBP-C and TnI that have positive effect on systolic cardiac function. On the other hand, higher cardiac stress induced by prolonged exercise led to cardiac dysfunction with persistent cMyBP-C S-glutathionylation that interfered on the PKA-dependent phosphorylation levels. Our study highlights a crosstalk between S-glutathionylation and phosphorylation of cardiac contractile regulatory proteins resulting in differential cardiac modulations (Fig. S3).

Intrinsic cardiac dysfunction after prolonged exhaustive exercise.

Acute high cardiac stress induced *in vivo* diastolic dysfunction with preserved systolic contractile function. This is in accordance with previous clinical [29] and experimental [21] studies showing that HSE induces transient early reduction of LV diastolic function with secondary altered systolic function following longer exercise [30]. Cardiac effects of prolonged exhaustive exercise have never been compared to the effects of a shorter exercise bout. We observed that moderate cardiac stress (LSE) increased systolic function. This beneficial effect on cardiac function disappears with prolonged stress (HSE) with the appearance of *in vivo* diastolic dysfunction and alteration of some systolic parameters function. The mechanisms underlying the decreased LV function after prolonged exhaustive exercise are unclear, but could involve in humans 1) diminution in blood volume due to dehydration that could reduce cardiac preload leading to decreased ventricular performance without direct alteration of cardiac contractility [31] or 2) intrinsic alterations independent of blood volume changes. Our investigation on isolated perfused hearts normalized loading conditions, heart rate and circulating factors between groups, and detected both systolic and diastolic dysfunctions in HSE. Such intrinsic systolic abnormalities were probably compensated *in vivo* by high levels of plasma epinephrine and norepinephrine previously

reported in similar conditions of HSE [21]. Consistent is the high level of PKA activity measured here in the myocardium after HSE to the same extent as observed with isoprenaline perfusion. *In vivo*, the heart is clearly under the regulation of the autonomic nervous system during exercise and recovery [32]. The complete recovery of the sympathovagal balance after the end of exercise may take up 48 hours [33] and is negatively impacted by the duration of exercise [34]. The β-adrenergic pathway plays a key role in the functional and molecular cardiac responses to exercise. However, the β-adrenergic pathway *per se* does not seem to be necessary to observe HSE-induced biochemical and functional modifications. In LSE (intermediate level of PKA activity) or Isoproterenol perfusion (high level of PKA activity but with short duration), some of the adaptations observed in HSE were present, but without cardiac dysfunction. Moreover, in conditions of severe adrenergic stress but preserved redox balance with NAC treatment (no oxidative stress and normal oxidized glutathione levels), deleterious effects of HSE were absent. Finally, pharmacological inhibition of glutathione reductase with BCNU mimicked the HSE-induced biochemical and functional modifications with no impact on PKA activity.

There are several potential mechanisms for diastolic dysfunction. One is increased diastolic Ca^{2+} resulting in a slowed ventricular relaxation and diastolic dysfunction. Alternatively, myofilament cross-bridge kinetics and response to Ca^{2+} are regulated and, if altered, could contribute to diastolic dysfunction ([35-37]). There is now various evidences that show clearly the link between myofilament S-glutationylation and impaired cardiac function. The group of Solaro has clearly showed in models of hypertension and familial hypertrophic myopathy [14, 38, 39] that S-glutationylation of cMyBP-C slowed cross-bridge cycling kinetics and increased Ca^{2+} sensitivity, which result is diastolic dysfunction. Those diastolic abnormalities occur with no change in the Ca^{2+} transients suggesting that the dysfunction occurs at the level of the sarcomere. Consistent with those previous findings, we evaluated the effect of HSE on cross-bridge cycling kinetics by measuring K*tr* after a release-restretch protocol. *Ktr* was significantly decreased after HSE consistent with a slower cross-bridge cycling kinetic (Fig. S4). Alternatively, diastolic changes of the heart and cardiomyocytes could be due to the giant elastic protein titin. The relationship between higher cellular stiffness and increased myofilament Ca^{2+} sensitivity observed after HSE is consistent with previous reports indicating that the level of titin-based passive tension can influence the myofilament Ca^{2+} sensitivity [40-42]. Additionally, S-glutathionylation has

been clearly shown *in vitro* to target titin [20]. We performed some exploratory experiments showing that S-glutathionylation of titin increases with our cardiac stress conditions LSE, HSE and after BCNU treatment (Fig S5). It is to note that no sign of titin degradation is observed in all conditions. S-glutathionylation of titin may not explain alone the higher passive tension after HSE since previous report showed *in vitro* a reduction of passive tension with S-glutathionylation of titin [20]. Changes in titin phosphorylation by PKA, PKC, PKG or CamKII are also expected to affect the level of passive tension [43, 44]. Muller, et al. reported that a single bout of 15 min exercise in rat increased cellular stiffness and altered PEVK titin phosphorylations probably mediated by PKCα[45]. Moreover, PKG pathway has been shown to be very important in increasing passive tension in heart failure with preserved ejection fraction [43, 44]. Thus, considering the complex regulation of titin by various redox post-translational modifications and the impact on both passive and active contractile properties, further studies are required to elucidate the exact participation of titin S-glutathionylation and phopshorylations in the contractile changes observed after cardiac stress.

Cardiac stress intensity and function

We propose from our experiments that β-adrenergic signaling pathway activation increased cardiac workload that induced oxidative stress. This consequently activated S-glutathionylation mechanisms that finally affected cardiac function. How to explain the different effects of cardiac stress in our study? We observed that both moderate and high cardiac stress increased S-glutathionylation of cMyBP-C. Considering that GSH can prevent damage to important cellular components caused by ROS, it is not surprising that S-glutathionylation is activated early [46]. Importantly, we did not detect any sign of major carbonylation, an irreversible redox modification, of contractile proteins (Fig. S2). This is consistent with previous studies suggesting that direct oxidative modifications of sarcomeric proteins lead to a decrease in force generation, whereas sarcomeric protein phosphorylation by ROS-activated enzymes decrease myofilament Ca^{2+} sensitivity, which is not observed here [4]. A main difference between stress conditions was that only moderate stress increased the level of cMyBP-C and TnI phosphorylation. Oxidative stress is a key intermediate in intense exercise-induced myocardium injury

[47]. In presence of NAC, HSE induced no persistent oxidative stress (normal MDA level), no S-glutathionylation and no PKA-dependent phosphorylation resulting in normal cardiac function. This is consistent with previous publications showing that restoring redox balance either by relieving NOS uncoupling in the DOCA-salt hypertensive mouse model [38] or using NAC in a familial hypertrophic cardiomyopathy mouse model [39] restored diastolic function in correlation with a reduction of S-glutathionylation of cMyBP-C.

Differences in the stimulus intensity and more importantly duration may explain different effects on the levels of cMyBP-C and TnI post-translational modifications and cardiac function. Firstly, oxidation can affect kinase activities. Hydrogen peroxide can activate type I PKA, which then translocates from the cytosol to the nuclear and myofilament compartments in cardiomyocytes in a cAMP-independent manner [48]. Similarly, PKA activity is enhanced in mild oxidizing conditions, or after short exposure to oxidants [49]. In contrast, longer or higher concentrations of the oxidant diamide inhibit PKA phosphorylation in the same conditions [49]. PKA is protected from oxidation in the inactive tetrameric state, but is susceptible to oxidation and S-glutathionylation upon activation [49]. The catalytic subunit can be protected against irreversible oxidation-induced inactivation by S-glutathionylation or by disulfide linkage to the regulatory subunit II of PKA. In our study, PKA activity remained increased by 15% with moderate exercise and by 30% with HSE to a level reached when isolated control hearts were stimulated *ex-vivo* with isoprenaline (Fig. 2). Thus, the reduced phosphorylation levels on PKA sites after HSE cannot be explained by a reduced kinase activity. However, it cannot be excluded that oxidation may modulate PKA phosphorylation via indirect routes by acting on phosphatases [49]. Secondly, S-glutathionylation itself could prevent phosphorylations on specific sites. Previous publications showed that several cysteines of cMyBP-C can be S-glutathionylated *in vitro* [15, 16]. Stathopoulou et al [16] showed that *in vitro* S-glutathionylation of Cys249, localized near the phosphorylation sites, attenuated phosphorylations by protein kinases (PKA and CaMKII). This may explain why after HSE, PKA-mediated phosphorylation of TnI and cMyBP-C was similar in control and HSE hearts despite higher PKA activity (Fig. 2A) and why myofilaments contractile properties were unchanged by recombinant PKA catalytic subunit incubation. Interestingly, similar myofilament insensitivity to PKA was observed previously in transgenic mouse models in which TnI [27] or cMyBP-

C [10] could not be phosphorylated on PKA sites. The BCNU experiments confirmed the direct link between increased GSSG and cMyBP-C S-glutathionylation amounts, the reduced phosphorylation of regulatory contractile proteins and the cardiac dysfunction with no impact on β-adrenergic signaling pathway (Figure 1E) and oxidative stress (Supplemental Figure 1B). Adaptive responses associated with environmental stressors are critical to cell survival. Under conditions when cellular redox and antioxidant defenses are overwhelmed, the selective oxidation of critical residues within selected protein sensors functions to down-regulate energy metabolism and the further generation of ROS. This may ensure minimal nonspecific cellular damage, allowing for rapid restoration of cellular function through repair of oxidized proteins.

Study limitation:

In our study, we used an antibody approach to detect S-glutathionylation and not an analytical approach. We thus analyzed only global S-glutathionylation status with the limitations of the detection. In particular, we cannot specifically indify the Cysteines modified and to which extent they are S-glutathionylated as a function of the duration/intensity of exercise/oxidative stress. The relative contribution of S-glutathionylation to reduce cMyBP-C PKA-dependent phosphorylation and to inhibit or prevent PKA activity on the effect of HSE on cardiac function needs to be investigated more precisely.

Only young male rats were tested in this study. Age- and gender-related factors could significantly influence cardiac response to exercise and subsequent cardiac remodeling. Experiments were performed 30 min after exercise and future studies should determine if such reversible changes could become irreversible when the exercise is repeated and lead to potential deleterious heart remodeling, as observed during heart failure.

Taking all these consideration together, our results pave the way: 1/ to further studies to investigate the pathophysiology and for instance propose various modalities of training to reduce these deleterious effects induced by strenuous exercise, and 2/ to pharmacological prevention of such deleterious effect of strenuous exercise. Indeed, activation of the glutathione system is easily available in daily practice (NAC) and above all, innocuity is well-established. Other candidates with anti-oxidative properties could be tested.

Conclusion:

In summary, we demonstrate the importance of both adrenergic and oxidative stress signaling pathways in regulating/dysregulating cardiac function. We show that both pathways work in concert to alter myofilament properties and cardiac function. Depending on the intensity/duration of the stress, we showed that dynamic regulations of the pathways with redox alterations can blunt the adrenergic signaling pathways and lead to cardiac dysfunction. Whether such reversible changes could become irreversible and constitute a key trigger of deleterious heart remodeling, as observed during heart failure remains to be investigated.

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Abbreviations Used

BCNU = 1,3-bis-2chloroethyl-1-nitrourea

β-AR = β- adrenergic receptors

Ca^{2+} = calcium

CSQ = calsequestrin

cAMP = cyclic adenosine monophosphate

DevP = Left ventricular developed pressure

dP/dtmin = minimal first derivative of left ventricular pressure

dP/dtmax = maximal first derivative of left ventricular pressure

E/A = ratio of early (E) diastolic and atrial (A) transmitral inflow velocities

Em/Am = ratio of early (Em) to late (Am) diastolic left ventricular wall motion

GAPDH = glyceraldehyde-3-phosphate dehydrogenase

GSH = glutathione

GSH/GSSG = ratio of reduced to oxidized glutathione

HSE = High stress induced by exercise

ISO = isoprenaline

Sm = left ventricular systolic wall motion velocity

LSE = Low stress induced by exercise

LV = Left ventricular

LVED = Left Ventricular end dimensions

MAV = maximal aerobic velocity

MDA = Malondialdehyde

MLC2 = Myosin Light Chain 2

MyBP-C = Myosin Binding Protein-C

NAC = N-acetylcysteine

NEM = N-ethylmaleimide

PKA = protein kinase A

ROS = reactive oxygen species

TnI = Troponin I

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Figure Legends:

Fig. 1. Effect of S-glutathionylation on cardiac function and post-translational modifications of contractile proteins. Isolated hearts were perfused or not with 80µM BCNU to block glutathione reductase activity. **(A)** Level of cMyBP-C S-glutathionylation (SG), (n=4 hearts/group at least in duplicate) **(B)** Representative SDS-PAGE analyses and quantification of cardiac troponin I immunoprecipitated from control hearts, BCNU-treated hearts and hearts that were subjected to low (LSE) and high (HSE) stress-induced by exercise do not reveal S-glutathionylation of TnI immunoblotted with anti-GSH antibody (green) and anti-TnI (red). PKA-dependent phosphorylation of cMyBP-C **(C)** and TnI **(D)** after BCNU treatment. (n=4 hearts/group in triplicate) **(E)** PKA activity (U/µg protein) in LV myocardium from control (Ctrl, n= 5) and control (BCNU, n=4) stimulated with 80µM BCNU. **(F)** The relationship between the tension generated by the myofilaments and the Ca^{2+} concentration expressed as pCa (=-log[Ca^{2+}]) was studied in permeabilized myocytes from Ctrl (n=16 cells from 4 hearts) and BCNU (n=16 cells from 4 hearts). The averaged absolute **(F)** and relative to maximal tension (**G**) tension-pCa curves were established. Effect of BCNU treatment on maximal active tension **(H)**, myofilament Ca^{2+} sensitivity (pCa_{50}) **(I)**, and passive tension **(J)** in permeabilized cardiomyocytes. **(F)** LV diastolic **(K)** and systolic **(L)** pressures and **(M)** dP/dt_{max} and **(N)** dP/dt_{min} were measured *ex vivo* in isolated perfused hearts after treatment or not with BCNU (n=4 hearts/group). *, $p<0.05$ BCNU vs Ctrl, ANOVA followed by Bonferroni's *post-hoc* tests.

Fig. 2. Effect of low (LSE) and high (HSE) stress-induced by exercise on markers of β-adrenergic and oxidative pathways. (A) PKA activity (U/µg protein) and **(B)** PKA-dependent phosphorylation of cMyBP-C and TnI were measured in LV myocardium from control (Ctrl, n= 5), LSE (35min exercise, n=4), HSE (3 hours exercise, n=4) and ISO-treated hearts (n=3). Levels of phosphorylated cMyBP-C at Ser282 were normalized to total cMyBP-C and phosphorylated TnI at Ser23/24 normalized to total TnI content (in duplicate); **(C** to **E)** Markers of oxidative stress measured in in LV myocardium from control (Ctrl, n= 5), LSE (n= 4), HSE (n=4) and ISO-treated hearts (n=3). **(C)** Measure of lipid peroxidation based on the malondialdehyde (MDA). **(D** and **E)** Redox Glutathione modulation indexed by the ratio between reduced glutathione (GSH) and oxidized glutathione (GSSG) and by the free glutathione

content. **(F)** Level of cMyBP-C S-glutathionylation (SG) increased significantly in LSE and HSE myocardium. Note that blots in B and F are composite images. *, p<0.05 *vs* Ctrl, ANOVA followed by Bonferroni's *post-hoc* tests.

Fig. 3. Effect of low (LSE) and high (HSE) stress-induced by exercise on cardiac function. (A) The averaged absolute and **(B)** relative to maximal tension-pCa curves were established from control (Ctrl; n=16 cells from 4 rats), LSE (n=15 cells from 4 rats) and HSE (n=16 cells from 4 rats) conditions. **(C** to **E)** indexes of myofilament contractility were measured: maximal active tension, myofilament Ca^{2+} sensitivity (pCa_{50}) and passive tension. *Ex vivo* measurement of LV developed pressure **(F)** and the peak rate variation of the first derivative of LV pressure rise and fall (dP/dt max, **G**) and fall (dP/dt min, **H**) in isolated perfused control (Ctrl, n=7), LSE rats (n=5) and HSE rats (n=9). **(I** to **K)** Echocardiographic measurements in controls (Ctrl) (n=6), LSE (n=5) and HSE (n=7) rats of fractional shortening, E/A wave ratio, and Em/Am velocity ratio. *, *p*<0.05 vs Ctrl, ANOVA followed by Bonferroni's *post-hoc* tests.

Fig. 4. Oxidative stress could be involved in cardiac dysfunction after high stress-induced by exercise. The antioxidant capacities of animals were increased by injecting 50 $mg.kg^{-1}$ of NAC, a non-specific permeant antioxidant, 48 hours and 1 hour before HSE (HSE+NAC). **(A)** Free glutathione content and ratio between reduced glutathione (GSH) and oxidized glutathione (GSSG) in the myocardium of HSE (n=4) and HSE rats treated with NAC (n=4) relative to control levels. **(B)** Levels of cMyBP-C S-glutathionylation (SG) and phosphor PKA cMyBP-C revealed by Western blotting normalized to total cMyBP-C level on the same membrane relative to control. Levels of phospho PKA cTnI normalized to total cTnI level (n=11 control hearts and 12 HSE and HSE+NAC hearts). **(C** to **E)** Effect of NAC treatment on LV developed pressure, and dP/dt_{max} and dP/dt_{min} measured *ex vivo* in isolated perfused hearts of HSE rats without NAC (n=9) and HSE rats with NAC (n=12) relative to control levels. **(F** to **H)** Effect of NAC treatment on myofilament contractile properties in permeabilized cardiomyocytes from HSE (n=16 cells from 4 hearts) and HSE-NAC (n=16 cells from 4 hearts). Expressed as the average delta relative to control conditions of maximal active tension **(F)** and passive tension **(G)**. *, p<0.05 *vs* Ctrl, ANOVA followed by Bonferroni's *post-hoc* tests. **(H)** Effect of PKA

stimulation of myocytes from control (n=16 cells from 4 hearts), HSE (n=16 cells from 4 hearts) and HSE+NAC myocytes (n=12 cells from 4 hearts). *, $p<0.05$ stress effect *vs* Ctrl within stimulated and non-stimulated PKA groups, †, $p<0.05$ PKA effect within the same group, ANOVA followed by Bonferroni‘s *post-hoc* test.

Figure 1

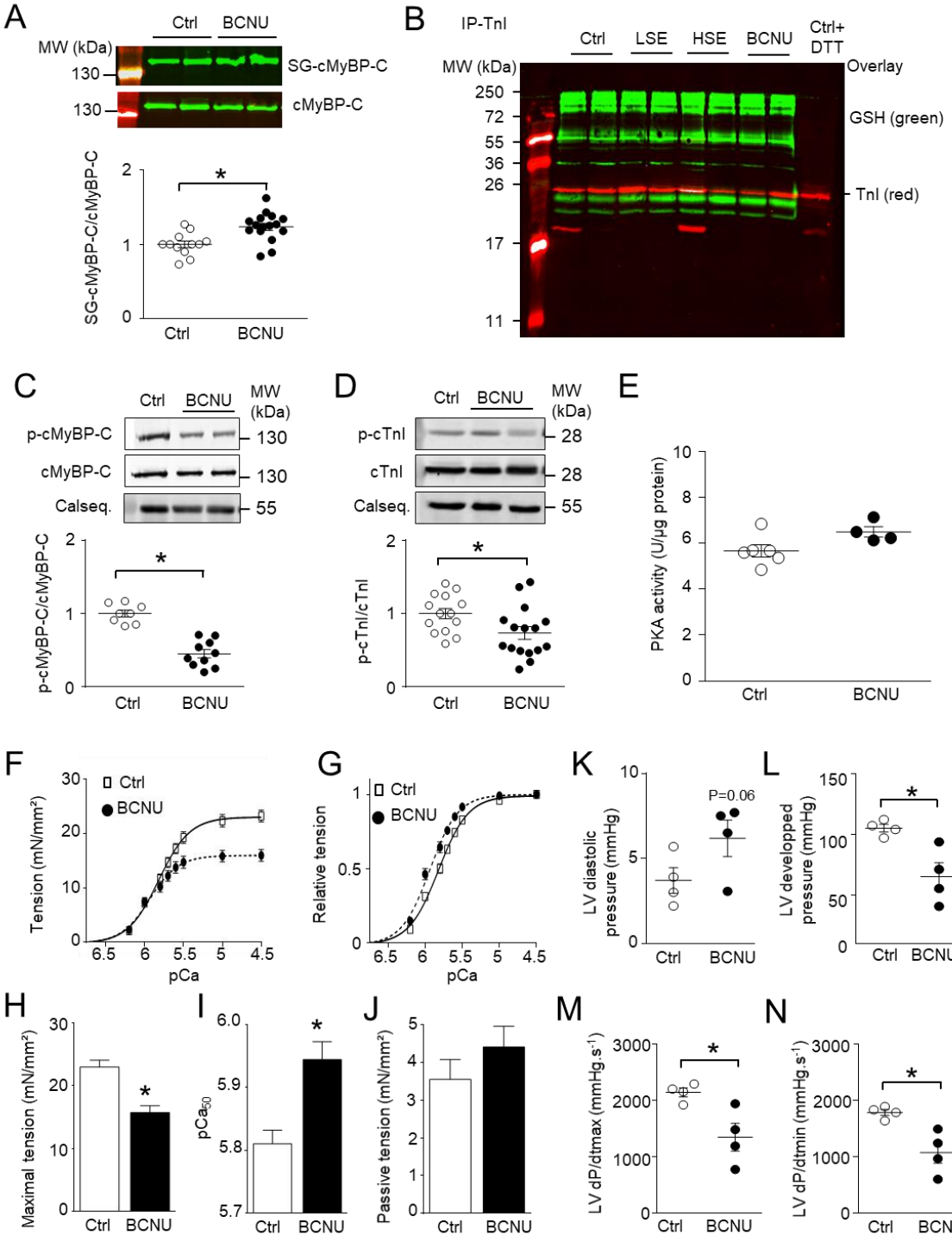
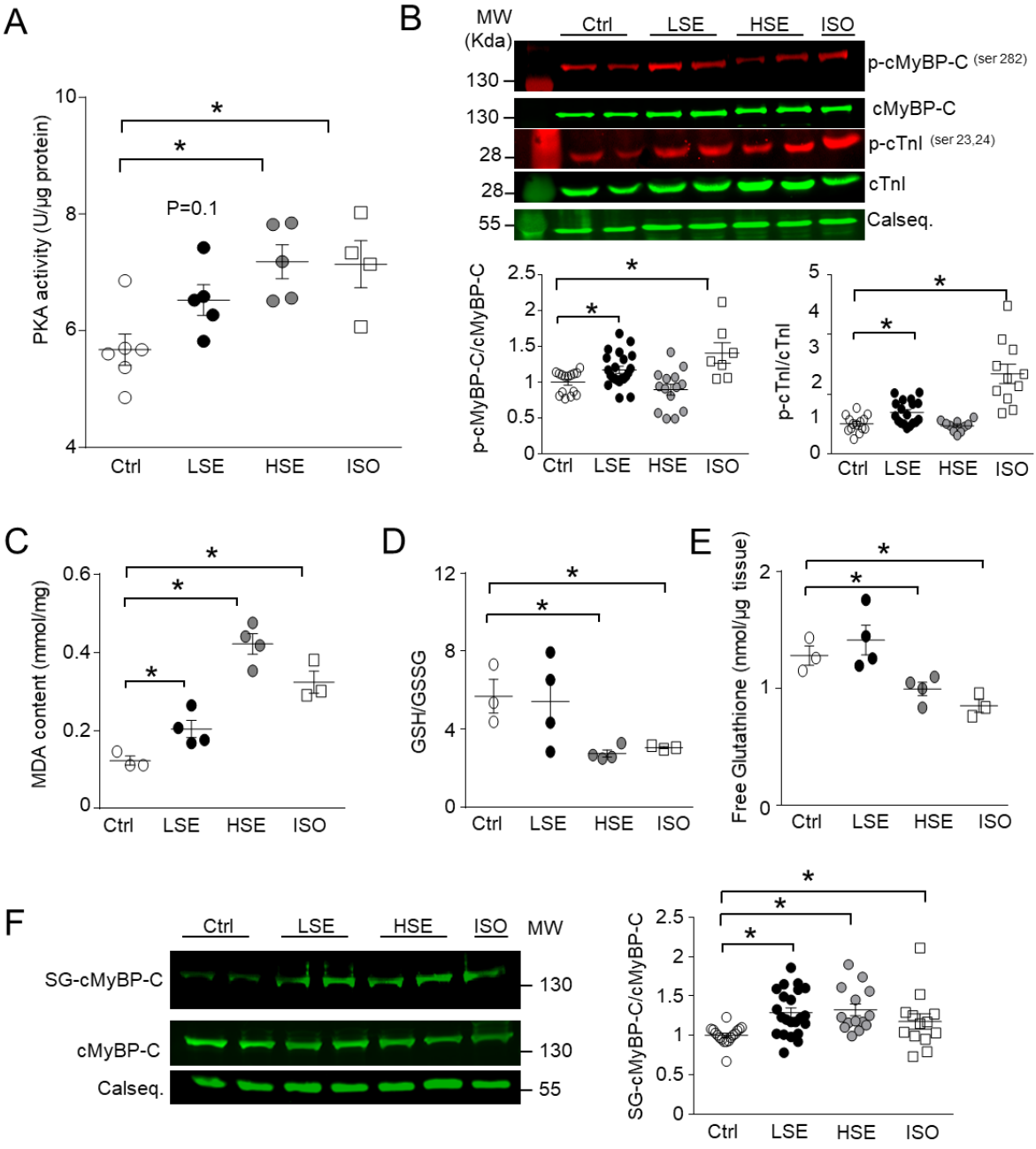

A
Ctrl
BCNU
MW (kDa)
130
SG-cMyBP-C
130
cMyBP-C
SG-cMyBP-C/cMyBP-C
2
1
0
*
Ctrl
BCNU
B
IP-TnI
Ctrl
LSE
HSE
BCNU
Ctrl+ DTT
MW (kDa)
250
72
55
36
26
17
11
Overlay
GSH (green)
TnI (red)
C
Ctrl
BCNU
MW (kDa)
p-cMyBP-C
130
cMyBP-C
130
Calseq.
55
p-cMyBP-C/cMyBP-C
2
1
0
*
Ctrl
BCNU
D
Ctrl
BCNU
MW (kDa)
p-cTnI
28
cTnI
28
Calseq.
55
p-cTnI/cTnI
2
1
0
*
Ctrl
BCNU
E
PKA activity (U/µg protein)
10
8
6
4
2
0
Ctrl
BCNU
F
Ctrl
BCNU
Tension (mN/mm²)
30
20
10
0
6.5
6
5.5
5
4.5
pCa
G
Ctrl
BCNU
Relative tension
1
0.5
0
6.5
6
5.5
5
4.5
pCa
K
LV diastolic pressure (mmHg)
10
5
0
P=0.06
Ctrl
BCNU
L
LV developped pressure (mmHg)
150
100
50
0
*
Ctrl
BCNU
H
Maximal tension (mN/mm²)
30
20
10
0
*
Ctrl
BCNU
I
pCa50
6.0
5.9
5.8
5.7
*
Ctrl
BCNU
J
Passive tension (mN/mm²)
5
4
3
2
1
0
Ctrl
BCNU
M
LV dP/dtmax (mmHg.s-1)
3000
2000
1000
0
*
Ctrl
BCNU
N
LV dP/dtmin (mmHg.s-1)
3000
2000
1000
0
*
Ctrl
BCNU

Figure 2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PKA activity (U/µg protein)
P=0.1
Ctrl
LSE
HSE
ISO
B
MW (Kda)
Ctrl
LSE
HSE
ISO
130
p-cMyBP-C (ser 282)
130
cMyBP-C
28
p-cTnI (ser 23,24)
28
cTnI
55
Calseq.
p-cMyBP-C/cMyBP-C
p-cTnI/cTnI
C
MDA content (mmol/mg)
D
GSH/GSSG
E
Free Glutathione (nmol/µg tissue)
F
SG-cMyBP-C
cMyBP-C
Calseq.
MW
130
130
55
SG-cMyBP-C/cMyBP-C

Figure 3

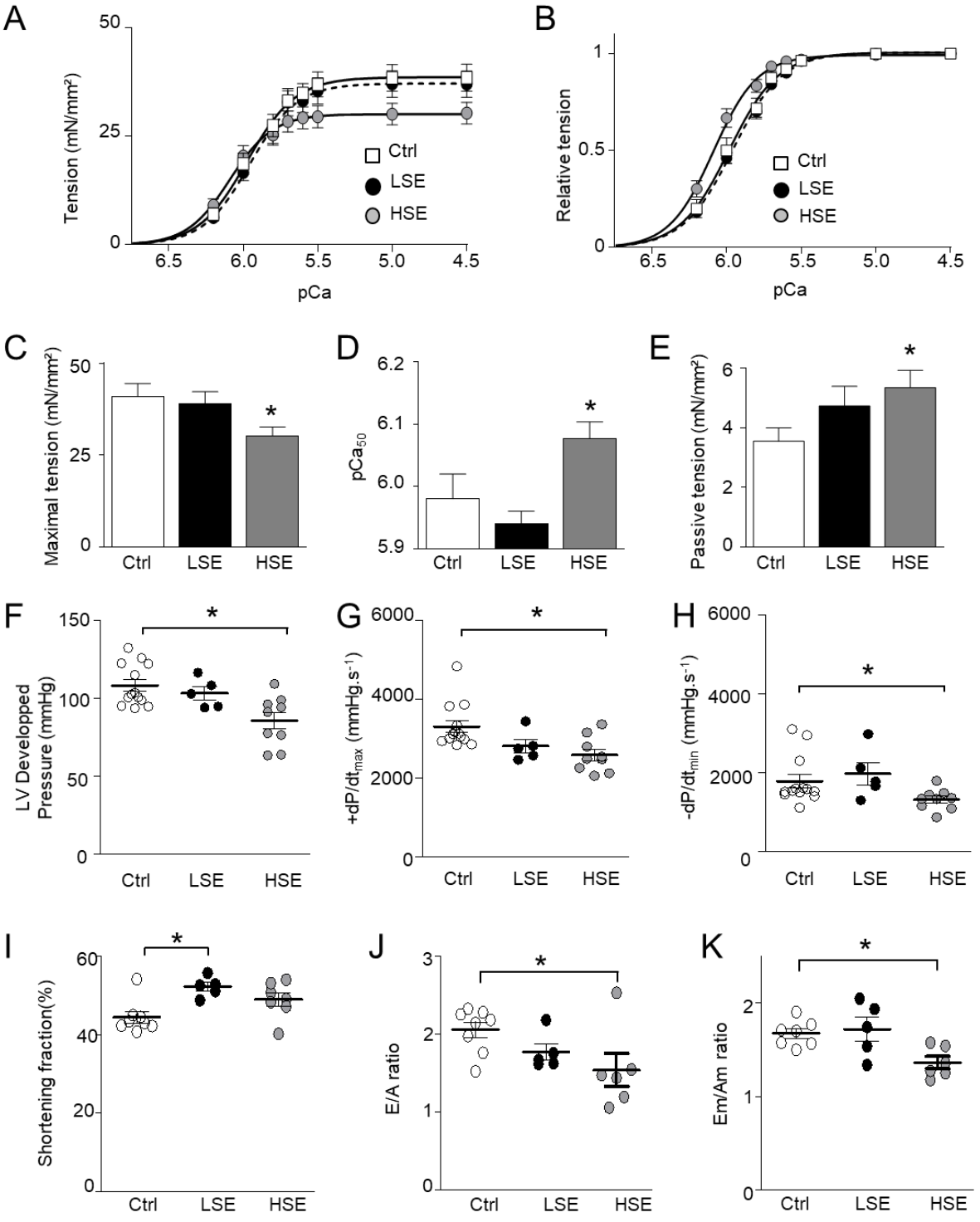
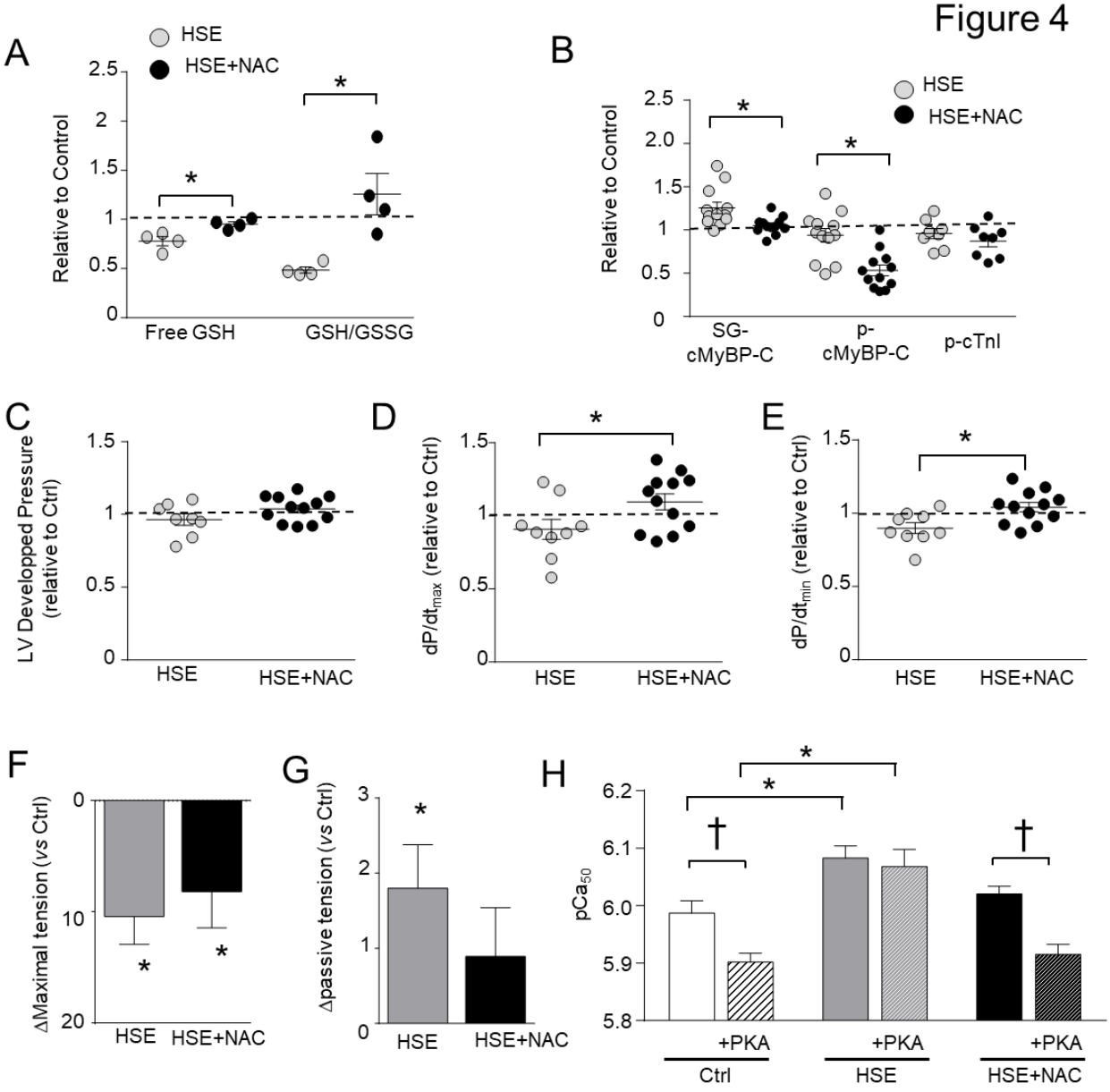

A
Tension (mN/mm²)
50
25
0
6.5
6.0
5.5
5.0
4.5
pCa
Ctrl
LSE
HSE
B
Relative tension
1
0.5
0
6.5
6.0
5.5
5.0
4.5
pCa
Ctrl
LSE
HSE
C
Maximal tension (mN/mm²)
50
25
0
Ctrl
LSE
HSE
*
D
pCa50
6.2
6.1
6.0
5.9
Ctrl
LSE
HSE
*
E
Passive tension (mN/mm²)
6
4
2
0
Ctrl
LSE
HSE
*
F
LV Developped Pressure (mmHg)
150
100
50
0
Ctrl
LSE
HSE
*
G
+dP/dtmax (mmHg.s-1)
6000
4000
2000
0
Ctrl
LSE
HSE
*
H
-dP/dtmin (mmHg.s-1)
6000
4000
2000
0
Ctrl
LSE
HSE
*
I
Shortening fraction(%)
60
40
20
0
Ctrl
LSE
HSE
*
J
E/A ratio
3
2
1
0
Ctrl
LSE
HSE
*
K
Em/Am ratio
2
1
0
Ctrl
LSE
HSE
*

Figure 4


A
HSE
HSE+NAC
Relative to Control
Free GSH
GSH/GSSG
B
HSE
HSE+NAC
Relative to Control
SG-cMyBP-C
p-cMyBP-C
p-cTnI
C
LV Developped Pressure (relative to Ctrl)
HSE
HSE+NAC
D
dP/dtmax (relative to Ctrl)
HSE
HSE+NAC
E
dP/dtmin (relative to Ctrl)
HSE
HSE+NAC
F
ΔMaximal tension (vs Ctrl)
HSE
HSE+NAC
G
Δpassive tension (vs Ctrl)
HSE
HSE+NAC
H
pCa50
+PKA
Ctrl
+PKA
HSE
+PKA
HSE+NAC

Supplemental Materials – R1

Stress-induced protein S-glutathionylation and phosphorylation crosstalk in cardiac sarcomeric proteins - impact on heart function

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Short Title: Oxidative stress in exercise-induced cardiac fatigue

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Supplementary information on Methods

Isolated perfused hearts. After anesthesia (100 mg/kg sodium pentobarbital, i.p.) and total loss of consciousness, rats were heparinized (1000 UI/kg, i.v.). The heart was rapidly removed and immersed in ice-cold Krebs solution. The aorta was cannulated for perfusion with oxygenated (95% O_2/5% CO_2) Krebs solution (118.3mmol/L NaCl, 25mmol/L $NaHCO_3$, 4.7mmol/L KCl, 1.2mmol/L $MgSO_4$, 1.2mmol/L KH_2PO_4, 11.1mmol/L glucose, 1.25mmol/L $CaCl_2$, pH=7.4, 37°C). The right atrium was excised. The atrioventricular node was crushed using fine forceps and the heart was paced at a rate of 300 beats/min with an electrical stimulator (Low voltage stimulator, BSL MP35 SS58L, 3V). A non-compliant balloon was inserted into the LV *via* the mitral valve and the balloon volume was adjusted to achieve a LV end diastolic pressure of 5mmHg. The heart was perfused at a constant pressure (80mmHg) and was allowed to stabilize for 30 min. To evaluate specifically the consequence of protein post-translational modifications, some control hearts (n=4) were perfused with 80µmol/L 1,3-bis-2chloroethyl-1-nitrourea (BCNU), a glutathione reductase inhibitor, for 5 min [1], or with 0.1µM ISO for 5 min (n=4). Throughout the procedure, the cardiac function parameters were recorded (MP35, BioPac System Inc) to calculate the developed pressure (DevP), the maximal (dP/dt_{max}) and minimal (dP/dt_{min}) first derivative of LV pressure. Finally, at the end of the experimental procedure, LVs were frozen quickly in liquid nitrogen and stored at -80°C for western blot analysis.

Cross-bridge kinetics:

Kinetic of tension redevelopment (k_{tr}) was measured by mechanically disrupting force-generating cross-bridges at either sub-maximal activating solution ($[Ca^{2+}]$=1.3µM) or at maximal calcium activation ($[Ca^{2+}]$=32µM). Cross-bridge disruption was induced by rapid release/restretch protocol [2, 3]. Briefly, cardiomyocytes were perfused with activating solution, when developed force reached steady state, a rapid (2 ms) release/restretch of 20% original cell length was applied. The cell was

shortly kept (20 ms) at the unload shortening prior to 100% restretch. Following the release/restretch step, force will drop to zero indicating that most cross-bridge have detached. After the restretch step, force rises exponentially up to initial maximal force with rate constant k_{tr}. For each cardiomyocyte, at a given SL, the above protocol was repeated 3 times and the average k_{tr} was estimated

Western blot analysis. Myocardial protein expression was studied, as previously described [4]. Briefly, LV tissue was quickly frozen in liquid nitrogen and solubilized in non-reducing Laemmli buffer (3% SDS, 50 mM Tris HCL pH 6.8, 8 M urea, 2 M thiourea, 1 mM EGTA, 1 mM EDTA, 10 mM Benzamidine, 0.2 mM Na3VO4, 50 mM NaF, 20 mM β-glycerophosphate). Proteins were separated using SDS-PAGE electrophoresis and were electrically transferred onto nitrocellulose membranes (GE Healthcare). Then, membranes were blocked with the Odyssey Blocking Buffer in PBS (LI-COR Biosciences) at room temperature for 45 min and incubated overnight with primary antibodies (Supplemental TableS1). Bands were revealed and quantified with the Odyssey system (LI-COR Biosciences, Lincoln, Nebraska) after incubation with fluorescent secondary antibodies (1:30,000 dilution, LI-COR Biosciences) at room temperature for 45 min. Protein content was expressed relative to GAPDH or calsequestrin (CSQ) content.

Determination of glutathione levels and cMyBP-C S-glutathionylation *(Fig S1).* As an index of myocardial lipid peroxidation, malondialdehyde (MDA) levels were measured in cardiac tissue using the Lipid Peroxidation (MDA) Assay Kit (ab118970, Abcam, Cambridge, Mass, United States), according to the manufacturer's instructions. Briefly, small LV pieces were homogenized in lysis buffer and directly centrifuged at 15,000 g at 4°C for 15 min. The resulting supernatants were used to measure MDA content by spectrofluorimetry.

Non-reducing SDS-PAGE was used to determine cMyBP-C and titin S-glutathionylation, following a previously described protocol.[5] Briefly, samples were solubilized in non-reducing Laemmli buffer (3% SDS, 50 mM Tris HCL pH 6.8, 8 M urea, 2 M thiourea, 1 mM EGTA, 1 mM EDTA, 10 mM Benzamidine, 0.2 mM Na3VO4, 50 mM NaF, 20 mM β-glycerophosphate) with 25mM N-ethylmaleimide (NEM) 45 min at room temperature. Proteins were resolved on 10% acrylamide SDS gel for MyBP-C and 3-8% SDS-polyacrylamide gradient gel and transferred onto nitrocellulose membranes (GE Healthcare). Membranes were blocked with the Odyssey Blocking Buffer in PBS (LI-COR Biosciences) at room temperature for 45 min and incubated with primary antibodies (anti-GSH, anti-cMyBP-C3, anti-C-terminus titin and anti-CSQ) overnight. Then, membranes were incubated with fluorescent secondary antibodies and revealed with the Odyssey system. For each sample, the glutathionylated band (140kDa) was normalized to the level of total cMyBP-C on the same gel.

Determination of glutathione levels (Fig S2). Levels of oxidized (GSSG) and reduced glutathione (GSH) in LV were measured using a Glutathione (GSSG/GSH) Detection Kit (ADI-900-160, EnzoLife Sciences, NY,USA), according to the manufacturer's instructions. Briefly, small LV pieces were homogenized in 5% meta-phosphoric acid (20µL/mg tissue) and directly centrifuged at 15,000 g at 4°C for 15 min. The resulting supernatants were used to measure total GSH. To measure GSSG levels, supernatants were incubated with 2M 4-vinylpyridine at room temperature for one hour.

Determination of protein kinase A activity. PKA activity was determined by a non-radioactive protein kinase assay kit (PKA Kinase Activity Assay Kit, no. ab139435, Abcam, Cambridge, Mass, United States) according to the manufacturer's instructions. Briefly, LV proteins were extracted in ice-cold lysis buffer (20 mM MOPS, 50 mM β-glycerolphosphate, 50 mM sodium fluoride, 1 mM Na_3VO_4, 5 mM EGTA, 2 mM EDTA, 1% NP40, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF, 10 µg/mL leupeptin). Protein lysates were centrifuged at 15,000 × g for 30 min at 4°C. Protein concentration was measured by the Pierce™ BCA Protein Assay Kit (catalog no. 23225, Thermo Fisher Scientific). Protein extracts

were incubated in a microplate precoated with substrate. The assay was developed with tetramethylbenzidine substrate and color development was stopped with acid stop solution. Absorbance measured at 450 nm in a microplate reader was divided by total protein (μg) per sample and the data represented as relative PKA activity.

References

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[5] Patel BG, Wilder T, Solaro RJ. Novel control of cardiac myofilament response to calcium by S-glutathionylation at specific sites of myosin binding protein C. Front Physiol. 2013;4:336.

Supplemental Table S1. Antibody details

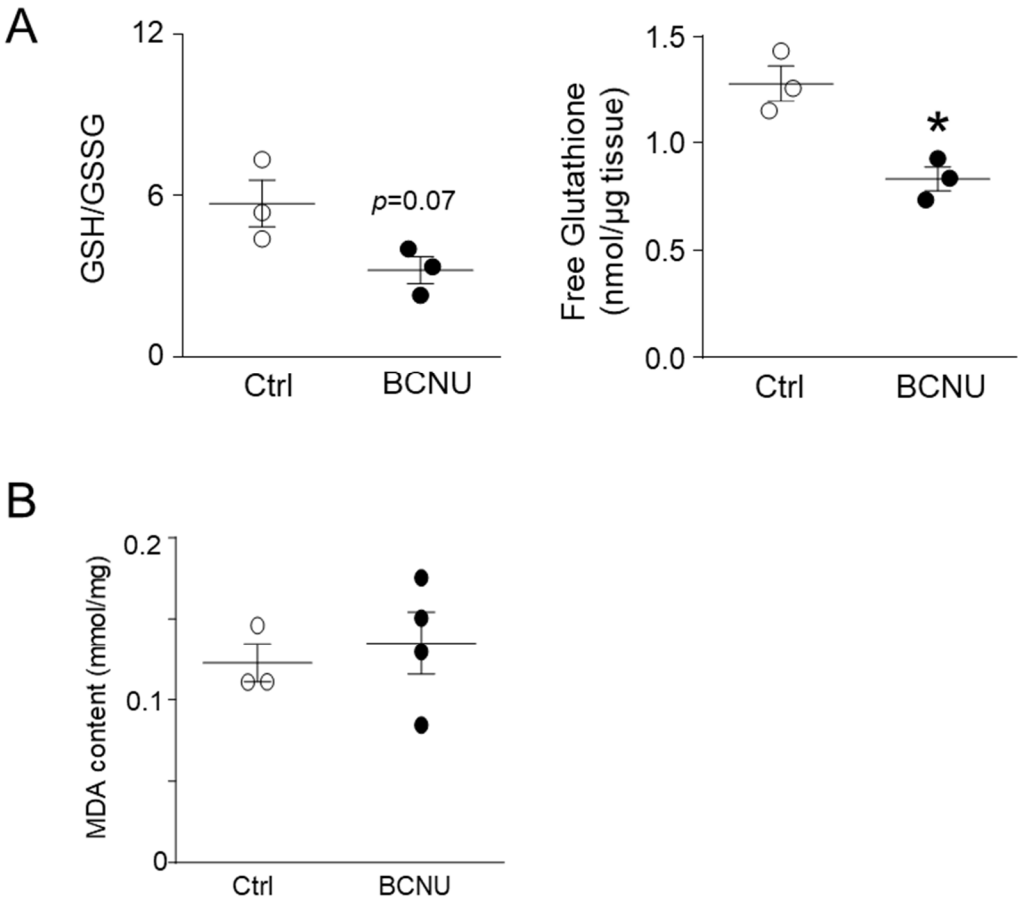
Antibody	Concentration	Source	Manufacturer
TnI	1 : 5,000	Mouse	Hytest (4T21)
TnI (p-Ser23/24)	1 : 10,000	Rabbit	Abcam (ab58545)
cMyBP-C3	1 : 1,000	Mouse	Santa Cruz Biotech.(sc-137181)
Titin (c-term)	1 : 500	Rabbit	S. Labeit gif ($X_{114-115}$)t
cMyBP-C3 (p-Ser282)	1 : 1,000	Rabbit	Enzo LS (ALX-215-057-R050)
GSH	1 : 1,000/250	Mouse	Virogen (101-A)
MLC2	1 : 4,000	Mouse	Enzo LS (F109-3E1)
MLC2 (pSer20)	1 : 2,000	Rabbit	Abcam (ab2480)
CSQ	1 : 5,000	Rabbit	Thermo Fisher (PA1-913)
GAPDH	1 : 60,000	Mouse	Abcam (ab8245)
DNP	1 : 300	Rabbit	Merck Millipore (S7150)

Supplemental Table S2: Echocardiographic measurements 30 min after low (LSE) or high (HSE) stress induced by exercise.

	Controls (N=8)	LSE (N=5)	HSE (N=7)
HR (bpm)	275 ± 10	301 ± 5	279 ± 8
LVEDd (mm)	7.57 ± 0.27	8.94 ± 0.23*	6.82 ± 0.29#
LVEDs (mm)	4.19 ± 0.15	4.26 ± 0.08	3.47 ± 0.18*,#
AWTd (mm)	1.37 ± 0.02	1.30 ± 0.04	1.47 ± 0.02#
PWTd (mm)	1.43 ± 0.02	1.34 ± 0.04	1.57 ± 0.04#
SF (%)	44.5 ± 1.4	52.2 ± 1.1*	49.0 ± 1.7
Sm (cm/s)	4.2 ± 0.3	5.4 ± 0.1*	4.1 ± 0.2
Em (cm/s)	6.3 ± 0.2	6.9 ± 0.3	4.5 ± 0.3*,#
Am (cm/s)	3.7 ± 0.2	4.1 ± 0.3	3.3 ± 0.2
Em/Am	1.67 ± 0.05	1.72 ± 0.12	1.36 ± 0.06*,#
E/Em	15.3 ± 1.0	15.0 ± 1.2	14.4 ± 0.9
E (cm/s)	95.2 ± 4.2	109.6 ± 6.9	66.9 ± 9.3*,#
A (cm/s)	47.9 ± 5.1	62.4 ± 4.7	43.5 ± 1.5#
E/A	2.05 ± 0.10	1.78 ± 0.10	1.54 ± 0.21*

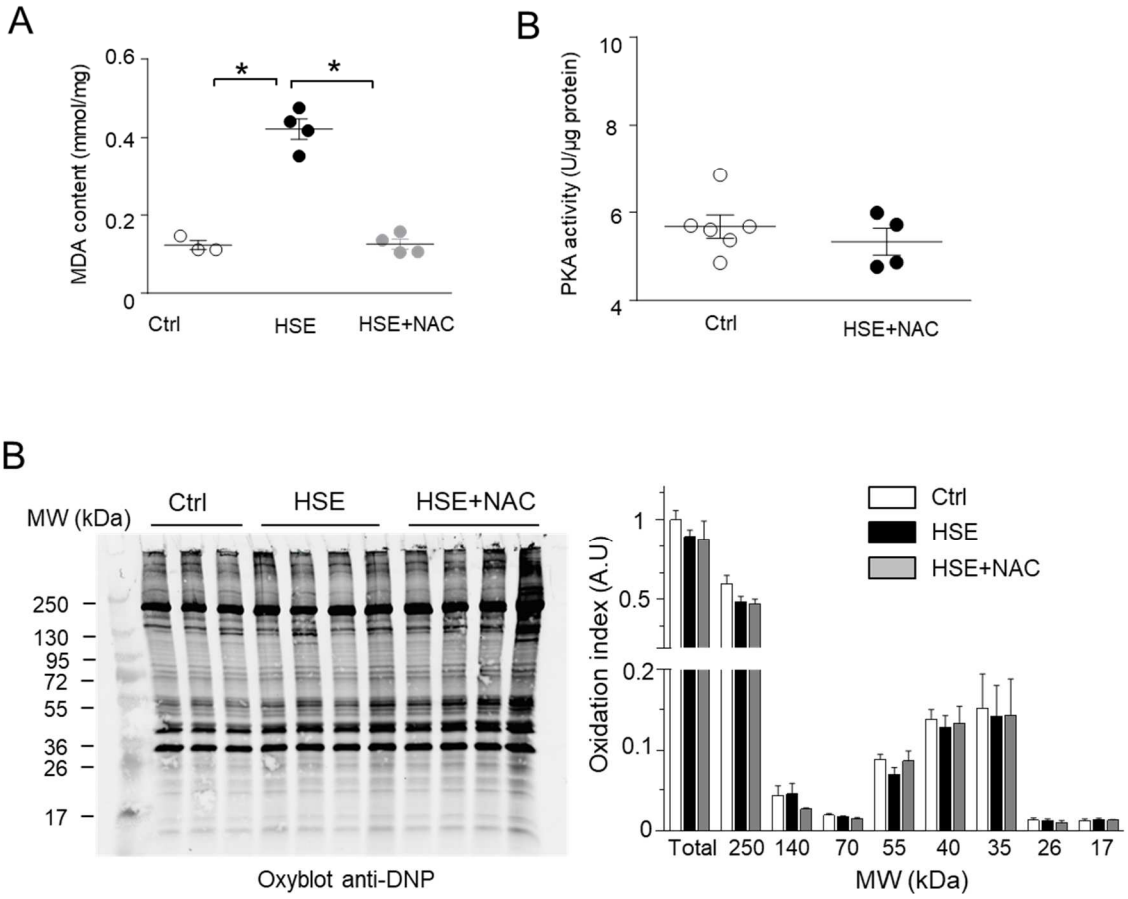
Results are presented in the mean±SEM. HR: heart rate; LVEDd: left ventricular end diameter in diastole; LVEDs: left ventricular end diameter in systole; AWTd: anterior wall thickness in diastole; PWTd: posterior wall thickness in diastole; SF: shortening fraction; Sm: systolic motion; Em: early diastolic motion; Am: late diastolic motion; E: early diastolic filling; A: late diastolic filling; *$p<0.05$ vs Ctrl, ANOVA followed by Bonferroni's *post-hoc* tests; #, $p<0.05$ vs LSE, ANOVA followed by Bonferroni's *post-hoc* tests.

Supplemental Figure 1



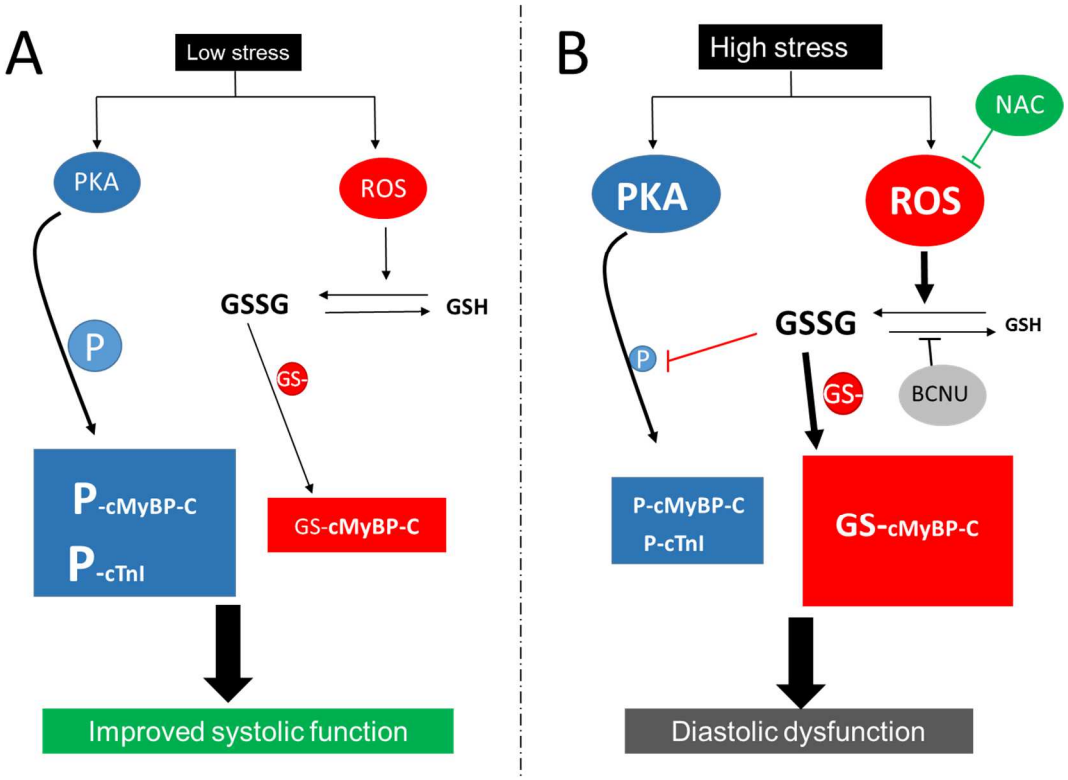
Supplemental Figure 1. Effect of BCNU on markers of oxidative stress measured in LV myocardium from control (Ctrl, n=3) and BCNU-treated hearts (n=3). **A)** Redox Glutathione modulation indexed by the ratio between reduced glutathione (GSH) and oxidized glutathione (GSSG) (left panel) and by the free glutathione content (right panel).**B) Malondialdehyde (MDA) content as an index of lipid peroxidation.** *, p<0.05 *vs* Ctrl, ANOVA followed by Bonferroni's *post-hoc* tests.

Supplemental Figure 2



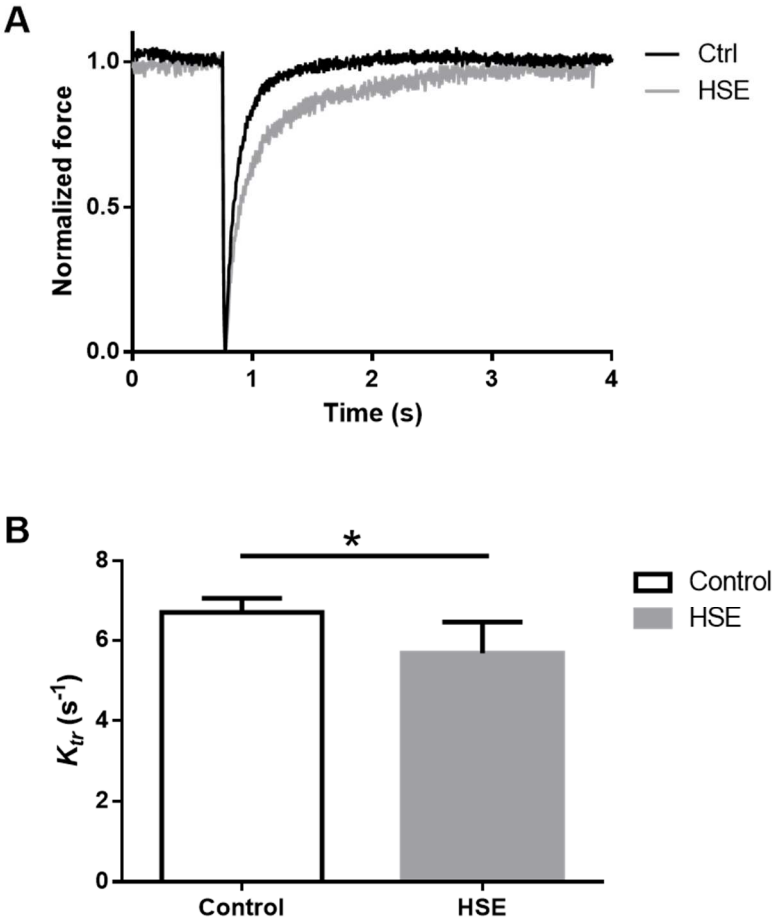
Supplemental Figure 2: Markers of oxidative stress after high stress-induced by exercise, effect of NAC pretreatment. The antioxidant capacities of animals were increased by injecting 50 mg.kg^{-1} of NAC, a non-specific permeant antioxidant, 48 hours and 1 hour before HSE (HSE+NAC). **A)** Measure of lipid peroxidation based on the malondialdehyde (MDA). **B)** PKA activity **C)** Carbonylation of sarcomeric proteins in LV myocardium of Ctrl (n=3 hearts), HSE (n=4 hearts), and HSE+NAC (n= 4 hearts) hearts. Total proteins extracted from LV myocardium were derivatized with DNP to detect protein carbonylation. The level of carbonylated proteins was revealed by immunoblotting with anti-DNP antibodies (left panel) and was normalized to TnI content on the same membrane. *, $p<0.05$ HSE vs Ctrl, †, $p<0.05$ HSE+NAC *vs* Ctrl, ANOVA followed by Bonferroni's *post-hoc* tests.

Supplemental Figure 3



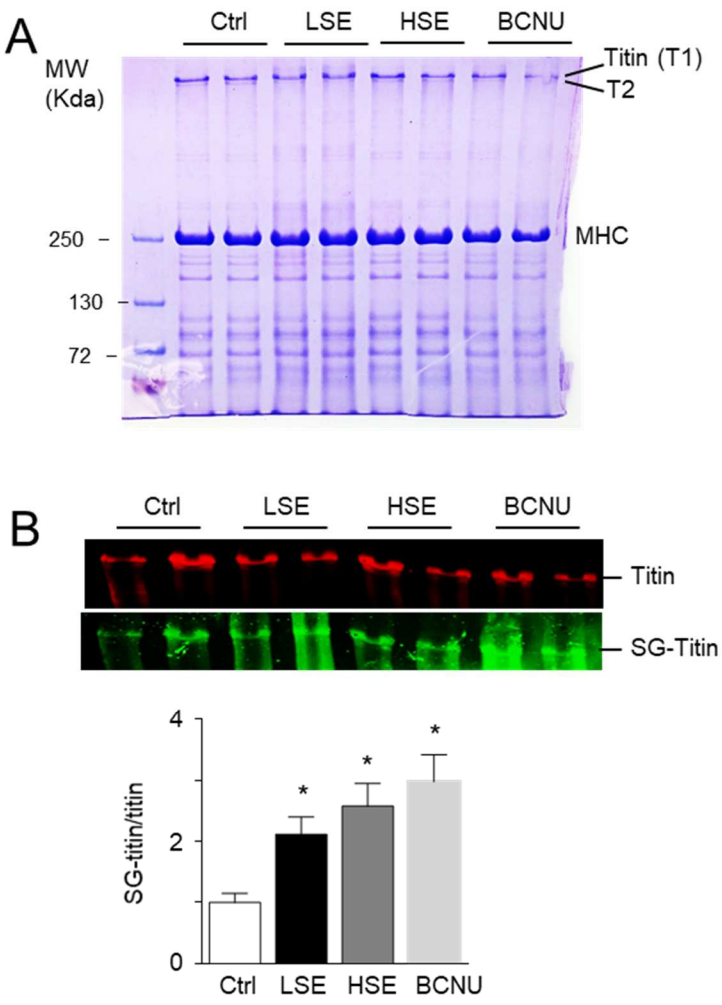
Supplemental Figure 3. Hypothetical impact of low (LSE) and high (HSE) stress-induced by exercise on cardiac function. (A) Low stress-induced by exercise increases in the heart PKA activity and ROS production leading to PKA-dependent phosphorylation and S-glutahionylation of the myofibrils, respectively. This improves systolic function. **(B) High stress-induced by exercise** further increases in the heart PKA activity and ROS production leading to high levels of S-glutahionylation that prevent PKA-dependent phosphorylation of the myofibrils. This induces diastolic dysfunction.

Supplemental Figure 4



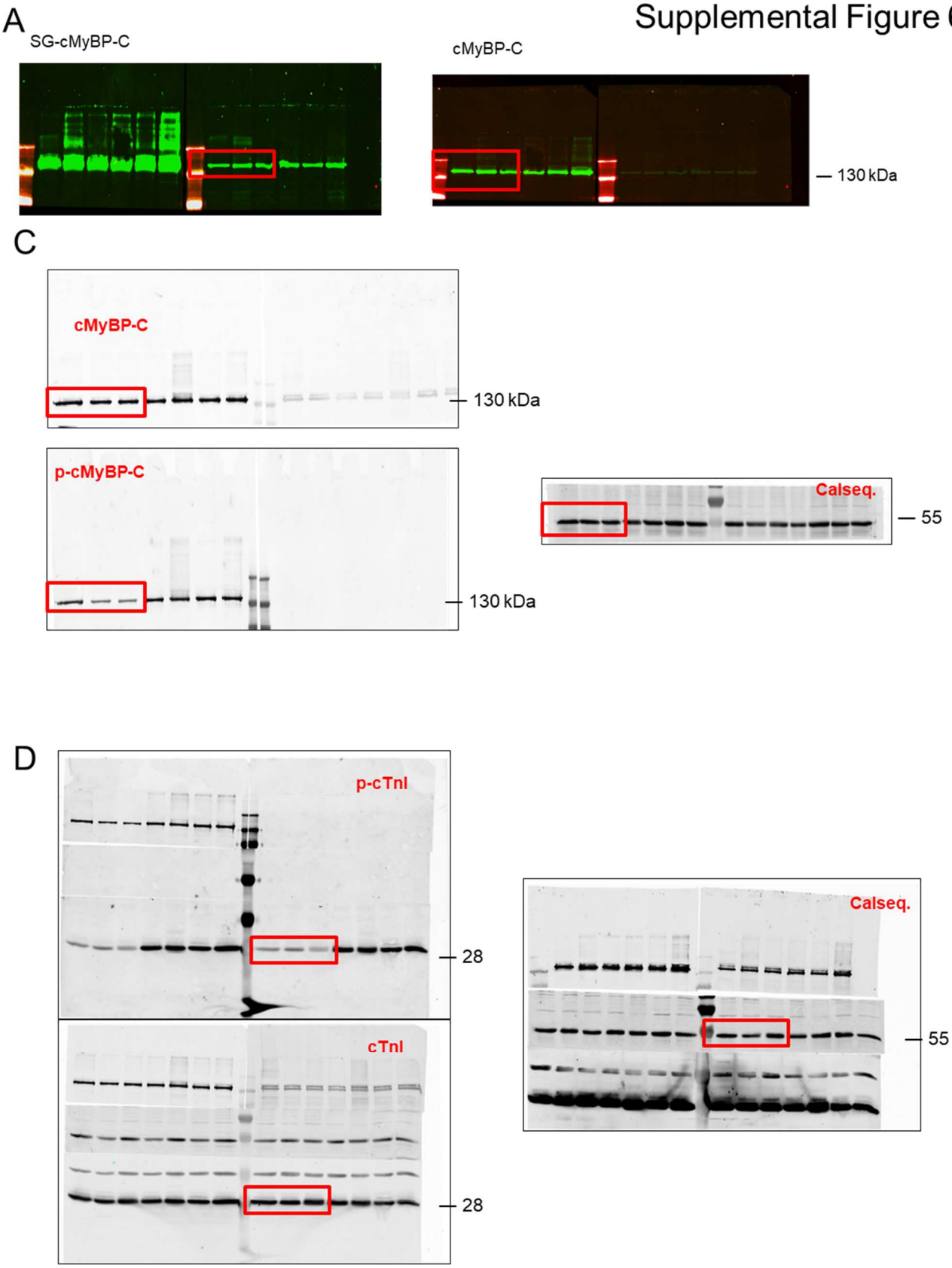
Supplemental Fig 4: **Effect of HSE on sarcomere function in skinned ventricular myocytes. A)** Representative traces of normalized force redevelopment in permeabilized myocytes from control (CTRL) and HSE myocytes. **B)** Rate of force redevelopment (ktr) was determined in activating solution (pCa 4.5) in permeabilized myocytes from control (n=4 animals; 16 myocytes/condition). * $P<0.05$ *vs.* Ctrl, ANOVA followed by Bonferroni's *post-hoc* tests..

Supplemental Figure 5



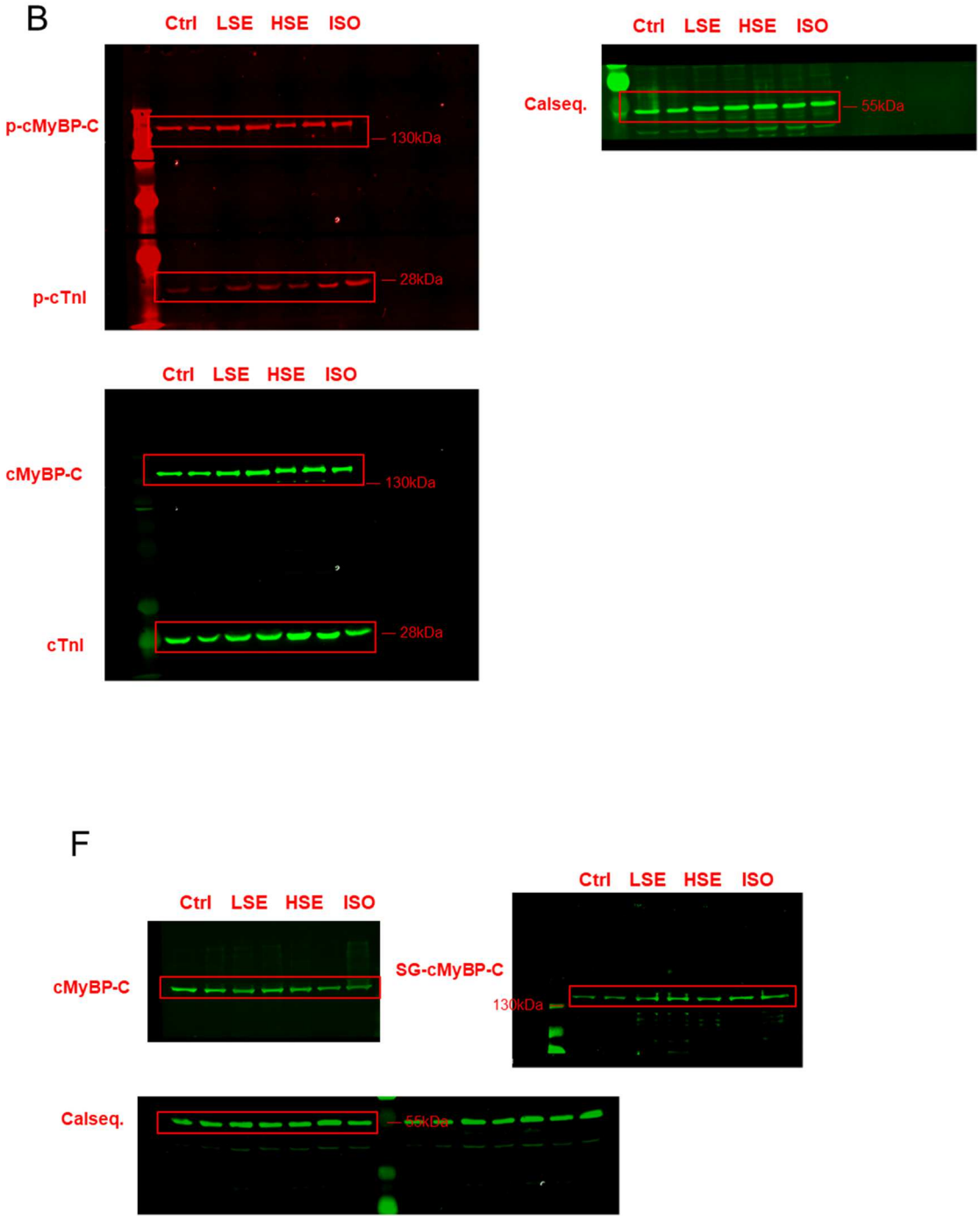
Supplemental Fig 5: **Effect of HSE on titin S-glutathionylation. A)** Representative coomassie 3-8% SDS-PAGE of control (Ctrl), LSE, HSE and BCNU treated myocardium. **B)** Western blot analysis of titin S-gluathionylation normalized by the level of titin content (C-terminus antibody) on the same band. (n=3 hearts/condition in duplicate). * $P<0.05$ *vs.* Ctrl, ANOVA followed by Bonferroni's *post-hoc* tests..

Supplemental Figure 6



Supplemental Figure 6. Whole Western blots of Figure 1A, 1C and 1D

Supplemental Figure 7



Supplemental Figure 7. Whole Western blots of Figure 2B and 2F