Short Communication

Exercise-induced alterations of myocardial sarcomere dynamics are associated with hypophosphorylation of cardiac troponin I

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Although the knowledge of sports cardiology advanced significantly in the recent years, the molecular mechanisms by which exercise training augments cardiac performance is poorly understood. Here we aimed at determining left ventricular (LV) myocardial sarcomeric protein modifications in a rat model of exercise training and detraining. Young male Wistar rats were divided into exercised (Ex) and control (Co) groups. Trained rats swam 200 min/day for 12 weeks. Detrained (DEx) and control (DCo) rats remained sedentary for 8 weeks after completion of the 12-week-long protocol. Ca^{2+}-regulated active force production (F_{active}), its Ca^{2+}-sensitivity (pCa_{50}) and Ca^{2+}-independent passive tension ($F_{passive}$) were determined in isolated permeabilized cardiomyocytes and phosphorylation levels of sarcomeric proteins were assayed by biochemical methods. Means of maximal Ca^{2+}-activated isometric force (F_{max}) and pCa_{50} values were higher ($p < 0.05$) in the Ex group (28.0 $\pm$ 1.4 kN/m^2 and 5.91 $\pm$ 0.03, respectively, mean $\pm$ SEM) than those in the Co group (15.8 $\pm$ 0.8 kN/m^2 and 5.81 $\pm$ 0.03, respectively). $F_{passive}$ did not differ between these two groups. The level of cardiac troponin I (cTnI) phosphorylation decreased upon exercise (from 1.00 $\pm$ 0.02 to 0.66 $\pm$ 0.06, $p < 0.05$; in relative units). Site specific phosphorylation assays revealed cTnI hypophosphorylations at the protein kinase A (PKA)-specific Ser-22/23 sites and at the protein kinase C (PKC)-specific Thr-143 site. Mechanical and biochemical parameters of the DEx and DCo groups did not differ from each other following the detraining period. Exercise-induced hypertrophy is associated with reversible increases in Ca^{2+}-dependent force production and its Ca^{2+}-sensitivity in LV cardiomyocytes, which can be associated with changes in cTnI phosphorylation.

Keywords

Athlete's heart; Isolated cardiomyocyte; Active force; Cardiac troponin I

1. Introduction

The complex morphological and functional aspects of remodeling evoked by long-term exercise training is called athlete's heart [1]. Exercise training-induced cardiac hypertrophy involves improved systolic and diastolic ventricular functions [2, 3]. Results of cellular electrophysiological studies (conducted in isolated cardiomyocytes with intact cell membranes) implicated characteristic alterations in intracellular Ca^{2+} transients and an increase in the Ca^{2+}-sensitivity of myofilament force production, and therefore provided plausible explanations for the observed improvements in LV pump function [4, 5].

Alterations in myofilament protein phosphorylations (e.g., cTnI, myosin binding protein-C (cMyBP-C) and titin) have been formerly linked to ventricular systolic and diastolic dysfunctions in cardiac pathological conditions [6–9]. Of note, the troponin protein complex (composed of cTnI, troponin T and troponin C) is central in the regulation of the cardiac contractile protein machinery, and hence its molecular alterations may well impact cardiomyocyte force production during physiological hypertrophy as well [10]. Within the troponin complex cTnI is of particular interest, as this protein holds several phosphorylation sites (e.g., for protein kinase A (PKA) and protein kinase C (PKC)), and phosphorylation of cTnI can mediate a range of distinct contractile responses [7, 9, 11]. Nevertheless, in athlete's heart Ca^{2+}-regulated and Ca^{2+}-independent myofilament characteristics and the role of myofilament protein phosphorylation remains obscure [3–5, 12, 13].

Here we studied exercise-induced cardiac LV remodeling and its reversion following detraining in a rodent model that involved long-term swim training [3, 12]. To limit the number of potential confounding variables, we compared hypothetical relationships between cardiomyocyte contractile function and phosphorylations of cTnI, cMyBP-C and titin in animals of identical age-sex groups with or without exercise [14]. Permeabilized cardiomyocytes aided the detailed characterization of sarcomere dynamics and site-specific phosphorylation assays allowed the recognition of changes in the phosphorylation level of cTnI. In this context, phosphorylation of the PKA-specific Ser-22/23 sites or the PKC-specific

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Ser-43 and Thr-143 sites reflect hypothetical alterations in β-adrenergic or angiotensin II dependent signaling processes.

2. Materials and methods

2.1 Study design, experimental groups, training and detraining protocols

Young male Wistar rats (Toxi-Coop, Dunakeszi, Hungary) (n = 36, m = 200–225 g) were housed in standard cages at 22 $\pm$ 2 °C with natural day and night exchange. Standard laboratory chow and tap water were available *ad libitum*.

After acclimation, thirty-six rats were divided into control (Co, n = 9), exercised groups (Ex, n = 9), detrained control (DCo, n = 9) and detrained exercised (DEx, n = 9) groups. To induce physiological hypertrophy, Ex and DEx rats underwent a 12-week-long swim training program and were compared to their counterparts (Co and DCo rats) as described before [3]. To investigate reversibility, DEx and DCo rats remained sedentary for 8 weeks after the 12-week long protocol as documented previously [12]. Rats were euthanized after completion of the *in vivo* experiments. Subsequently LV myocardium samples were collected, snap-frozen and stored at –80 °C.

2.2 Isometric force measurements in permeabilized cardiomyocytes

LV tissue samples were mechanically disrupted in isolating solution (ISO, (1 mM $MgCl_2$, 100 mM KCl, 2 mM EGTA, 4 mM ATP, 10 mM imidazole; pH 7.0, 0.5 mM phenylmethylsulfonyl fluoride, 40 μM leupeptin and 10 μM E-64, all from Sigma-Aldrich, St. Louis, MO, USA) and thereafter permeabilization was performed with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA), detergent for 5 min, as described elsewhere [6]. Briefly, single permeabilized cardiomyocytes were mounted with silicone adhesive (DAP 100% all-purpose silicone sealant; Baltimore, MD, USA) to two stainless steel insect needles, which were connected to a sensitive force transducer (SensoNor, Horten, Norway) and to an electromagnetic high-speed length controller (Aurora Scientific Inc., Aurora, ON, Canada) in ISO at 15 °C. The average sarcomere length was adjusted to 2.3 μm. The contractile protein machinery was activated by transferring the cardiomyocyte from a relaxing (containing 37.11 mM KCl, 10 mM BES, 6.41 mM $MgCl_2$, 7 mM EGTA, 6.94 mM Na_2ATP, 15 mM Na_2CrP, 40 μM leupeptin, 10 μM E64; pH 7.2) to an activating solution (containing 37.34 mM KCl, 10 mM BES, 6.24 mM $MgCl_2$, 7 mM CaEGTA, 6.99 mM Na_2ATP, 15 mM Na_2CrP, 40 μM leupeptin, 10 μM E64; pH 7.2). The Ca^{2+} concentrations ([Ca^{2+}]) expressed in pCa units refer to -lg[Ca^{2+}]. The pCa of the activating and relaxing solutions was 4.75 and 9.0, respectively. When a steady force level had been reached, a rapid release-restretch maneuver (30 ms) was applied to determine the baseline of the force generation and hence the Ca^{2+}-activated total force (F_{total}). Fitting of the force re-development phase to a single exponential following the release-restretch maneuver allowed the characterization of the maximal turnover rate of actin–myosin cross-bridges (rate constant of force re-development in the presence of saturating [Ca^{2+}]). About 6 s after the onset of force re-development, the Ca^{2+}-independent passive tension ($F_{passive}$) was measured by shortening to 80% of the original preparation length at pCa 9.0 for 8 s. The active force (F_{active}) was calculated as a difference of the F_{total} and $F_{passive}$. Maximal activation at pCa 4.75 was used to determine the maximal Ca^{2+}-activated isometric force (F_{max}), while activations with intermediate [Ca^{2+}] (pCa 5.4–7.0) yielded the pCa–isometric force relationship. Isometric forces at submaximal [Ca^{2+}] normalized to F_{max} were plotted and then fitted to a modified Hill-equation (Origin 6.0, Microcal Software, Northampton, MA, USA) and to determine the Ca^{2+}-sensitivity of force production (pCa_{50}). Original forces of every individual cell were normalized to cardiomyocyte cross sectional-area, calculated from the width and height of the cardiomyocytes. Force values were expressed in kN/m^2 units.

2.3 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting

Cardiomyocytes were solubilized in sample buffer (containing 8 M urea, 2 M thiourea, 3% (w/v) sodium dodecyl sulfate (SDS), 75 mM DTT, 50 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, bromophenol blue, 40 μM leupeptin and 10 μM E-64, Sigma-Aldrich, St. Louis, MO, USA) followed by 45 min vortexing. After centrifugation (16,000 g for 5 min at 24 °C), protein amount of the supernatant was determined by the dot-blot technique using bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) standard, and the concentration of the samples was set to 2 mg/mL. Agarose-strengthened 2% SDS-polyacrilamide gels were used to separate N2B titin. Total phosphorylation status of titin protein was assessed by Pro-Q® Diamond phosphoprotein staining (Invitrogen, Eugene, OR, USA) according to the manufacturer's protocol, while total amount of protein was stained by Coomassie blue (Reanal, Budapest, Hungary).

Western immunoblotting was applied to assess site-specific phosphorylation status of cTnI. Separation of cTnI and cMyBP-C was carried out in 4% and 12% polyacrilamide gels, respectively. After PAGE and protein blotting procedure, the membranes were blocked with 2% BSA diluted in PBS containing 0.1% (v/v) Tween 20 (PBST, Sigma-Aldrich, St. Louis, MO, USA) for 30 min, then cTnI phosphorylation-sensitive antibodies were used to determine the levels of PKA- and PKC-dependent cTnI (Ser-23/24 (1:1000), Ser-43 (1:500) and Thr-143 (1:500), Abcam, Cambridge, UK) phosphorylation. The signal was detected with a peroxidase-conjugated anti-rabbit IgG secondary antibody (1:300) (Sigma-Aldrich, St. Louis, MO, USA) in nitrocellulose membranes. Total protein amounts were visualized with super sensitive membrane staining (UD-GenoMed, Debrecen, Hungary). Chemiluminescence (ECL) signals of site-specific phosphorylation of cTnI and cMyBP-C were normalized to a Western immunoblot stain.

2.4 Data analysis and statistics

Cardiomyocyte force generation was measured with a custom-built system (utilizing the DAQ platform produced by National Instruments, Austin, TX, USA) and recorded by a custom-built LabVIEW (National Instruments) module. Results were evaluated in GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). The number of experiments in each group varied between seven and twelve from three or four different hearts. Western immunoblot assays were performed in triplicates. Intensities of protein bands were quantified by determining the area under the intensity curves by a Gaussian fit using ImageJ (National Institutes of Health, Bethesda, MD, USA) and Magic Plot 3.0.1 (Magicplot Systems, Saint Petersburg, Russia) softwares.

Graphs were created in GraphPad Prism 6.0 software. Differences between groups were calculated by Student's *t*-test. Group descriptions were based on the mean $\pm$ SEM values. Statistical significance was accepted at $p < 0.05$. Each protein band of exercised and detrained exercised groups were normalized to the values of control and detrained control groups.

3. Results

3.1 Reversible increases in Ca^{2+}-activated force generation and its Ca^{2+}-sensitivity in LV cardiomyocytes from exercised-induced hearts

Ca^{2+}-activated force (F_{active}) development was followed in permeabilized cardiomyocytes isolated from LV tissue samples *in vitro* at different Ca^{2+} concentrations (pCa: from 4.75 to 7.0). Mean values of F_{active} (incl. F_{max}) were significantly higher following exercise training (Ex group) than those of controls (Co group) between pCa 6.2 and 4.75. Following detraining (DEx group) F_{active} was similar as in the control (DCo) group at all pCa values (Fig. 1A, Table 1). Ca^{2+}-sensitivity of force production (pCa_{50}) was significantly higher in the Ex group than in the Co group. Mean pCa_{50} values did not differ after detraining (Fig. 1B, Table 1). Passive tension ($F_{passive}$) of cardiomyocytes and the maximal actin-myosin cross-bridge cycling rate ($k_{tr,max}$) were similar in the Co and Ex groups. Moreover, no significant differences between $F_{passive}$ and $k_{tr,max}$ values were observed in the detrained groups either (Table 1).

3.2 Changes in sarcomeric protein phosphorylations

Following exercise training, overall phosphorylation level of cTnI decreased markedly in LV cardiomyocytes. However, this difference disappeared after detraining. Overall phosphorylation levels of cMyBP-C and titin (not shown) were similar in permeabilized LV cardiomyocytes in all experimental groups (Fig. 2A, Table 1). To elucidate the molecular background of increased F_{max} and pCa_{50} values of exercised animals, site-specific phosphorylation assays were included for cTnI. Hypophosphorylation both at the PKA-specific Ser-22/23 and at the PKC-specific Thr-143 sites of cTnI were observed in the Ex groups. The phosphorylation levels of cTnI at the PKC-specific Ser-43 site did not differ between the Co and Ex groups. No differences in cTnI phosphorylation at all investigated phosphorylation sites between the detrained (DCo) and control (DEx) groups were observed (Fig. 2B, Table 1).

4. Discussion

Exercise-induced cardiac hypertrophy, improvements in systolic and diastolic function and their reversions have been formerly documented for this animal model by our group [3, 12]. In the present study we characterized myocardial sarcomere dynamics and sarcomeric protein alterations in LV cardiomyocytes in detail. Major novelty of the present study is the apparent association between exercise-induced increases in F_{active}, pCa_{50} of LV cardiomyocytes and hypophosphorylation of cTnI. All above parameters became indistinguishable from those of the control (DEx) group following detraining suggestive for a close relationship between the changes in cardiomyocyte mechanics and the investigated myofilament protein alteration.

Myocardial contractility is a major determinant of systolic function, that depends mainly on Ca^{2+}-regulated myofilaments. Animal models in sports cardiology furnished with evidence for increases in LV stroke volume and stroke work in athlete's heart, that were also observed in the hearts of our swim-trained rodents [3]. Moreover, an increase in the Ca^{2+}-sensitivity of force production was also implicated in experimental studies [4, 13]. Here we confirm the increase in Ca^{2+}-sensitivity of force production and extend it by the recognition of an almost doubled F_{max} level in LV cardiomyocytes of animals underwent prolonged training.

The impact of physical deconditioning on exercise-induced cardiac hypertrophy has also been intensively investigated. Although with some uncertainties, most literature data support complete reversibility of exercise-induced alterations after cessation of training, which can be utilized to distinguish it from pathological hypertrophy [12, 15]. Accordingly, the complete reversion of exercise-induced changes in sarcomere dynamics and myofilament protein alterations reflect a physiological type of LV hypertrophy in our model.

Exercise activates the sympathetic nervous system, β_1-adrenergic receptors and PKA to enhance cardiomyocyte contractility and relaxation. Site-specific cTnI-Ser-22/23 phosphorylation has been formerly associated with PKA activation and with decreases in Ca^{2+}-sensitivity of force production [7]. In end-stage heart failure (when contractility is impaired), a reduction in PKA activity results in decreased Ser-22/23 phosphorylation in association with increased myofilament Ca^{2+}-sensitivity [8]. Interestingly, our present data show that the increased Ca^{2+}-sensitivity of force production might also be related to cTnI hypophosphorylation at the Ser-22/23 sites. cMyBP-C phosphorylation was not altered by exercise in LV cardiomyocytes in our model, although this protein had been also considered as a target for PKA in a murine model of exercise training [13]. PKA activity has been studied in a recent study on our experimental

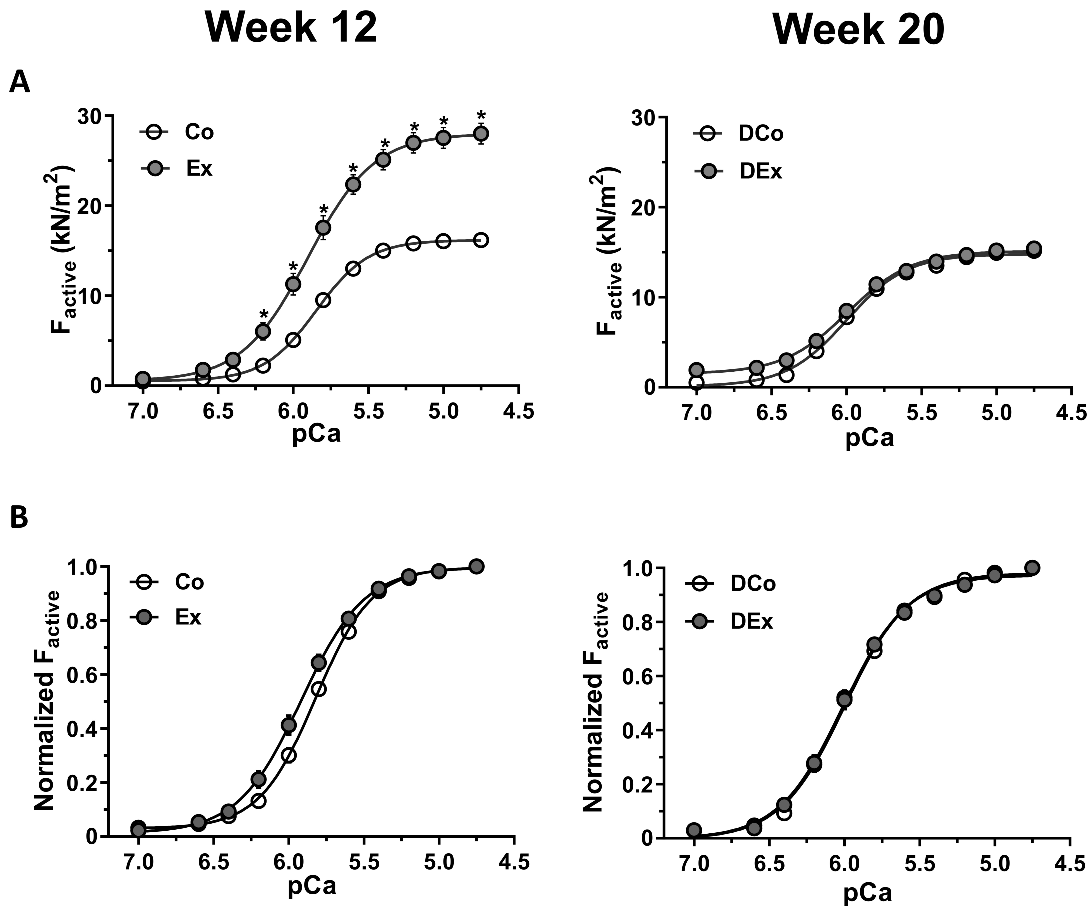

Fig. 1. Force production in permeabilized LV cardiomyocyte-sized preparations following exercise training (week 12) and detraining (week 20). (A) F_{active} values were measured during repeated contractures by activating solutions with different Ca^{2+} concentrations (pCa: between 4.75 and 7.0) in order to construct pCa-force relationships. F_{max} refers to F_{active} at the highest Ca^{2+} concentration (pCa 4.75). (B) F_{active} was normalized to F_{max} in each cell to illustrate differences in the Ca^{2+}-sensitivity of force production. pCa = -lg[Ca^{2+}] where [Ca^{2+}] is the molar concentration of Ca^{2+}. Data are mean ± SEM. Co: control group, Ex: exercised group, DCo: detrained control group, DEx: detrained exercised group. *$p < 0.05$ vs. the control (Co) group. Numerical values are given in Table 1.

Table 1. Characteristics of LV cardiomyocytes following exercise training (week 12) and detraining (week 20).

	Week 12		Week 20	
	Co (n = 11)	Ex (n = 12)	DCo (n = 11)	DEx (n = 9)
F_{max} (kN/m^2)	15.78 ± 0.84	28.02 ± 1.42*	15.17 ± 0.46	15.48 ± 0.71
pCa_{50}	5.81 ± 0.03	5.91 ± 0.03*	5.99 ± 0.02	6.00 ± 0.02
$F_{passive}$ (kN/m^2)	1.49 ± 0.19	1.75 ± 0.12	1.70 ± 0.21	1.50 ± 0.17
$k_{tr,max}$ (1/s)	3.69 ± 0.21	4.26 ± 0.19	3.64 ± 0.23	4.16 ± 0.25
cTnI-P (rel.)	1.00 ± 0.02	0.66 ± 0.06*	1.00 ± 0.03	0.91 ± 0.11
cMyBP-C-P (rel.)	1.00 ± 0.06	1.11 ± 0.06	1.00 ± 0.09	0.88 ± 0.08
Titin-P (rel.)	1.00 ± 0.02	0.91 ± 0.04	1.02 ± 0.04	0.99 ± 0.04
$cTnI^{Ser-22/23-P}$ (rel.)	1.00 ± 0.07	0.76 ± 0.01*	1.00 ± 0.04	0.98 ± 0.08
$cTnI^{Thr-143-P}$ (rel.)	1.00 ± 0.03	0.77 ± 0.05*	1.00 ± 0.02	1.02 ± 0.06
$cTnI^{Ser-43-P}$ (rel.)	1.00 ± 0.06	0.99 ± 0.03	1.00 ± 0.01	1.03 ± 0.07

Values are mean ± SEM in absolute or relative (rel.) units. The number of animals (n) in the different experimental groups is given in brackets. *$p < 0.05$ vs. the control (Co) group.

model [16]. Results of this investigation suggested that PKA activity is not reduced in trained hearts, but its intracellular target proteins (e.g., phospholamban, SERCA) are phosphorylated in an uneven fashion. By combining those data with the ones presented here, we postulate that PKA may translocate primarily to the sarcoplasmic reticulum rather than to the myofilament compartment in exercise trained hearts.

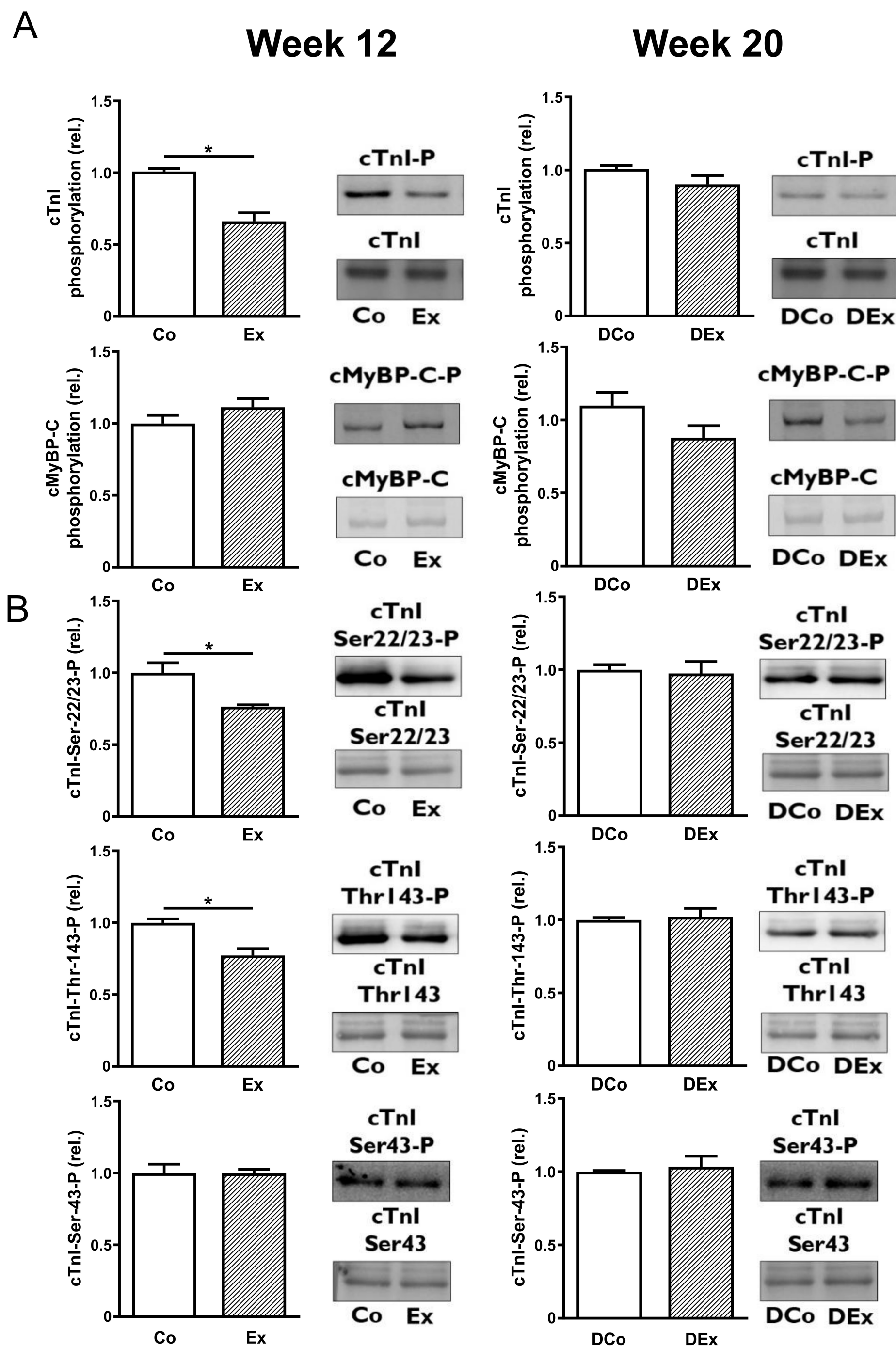

Fig. 2. Phosphorylation levels of sarcomeric proteins of LV cardiomyocytes following exercise training (week 12) and detraining (week 20). (A) Overall phosphorylation levels of cardiac troponin I (cTnI) and cardiac myosin binding protein-C (cMyBP-C) in LV cardiomyocytes in control (Co), exercised (Ex), detrained control (DCo), and detrained exercised (DEx) rats (from left to right, representative results). Pro-Q® Diamond staining was used to detect overall phosphorylation levels of cTnI (upper panel) and cMyBP-C (lower panel). Total protein amounts were assessed by Coomassie-blue staining. Phosphorylation levels of myofilament proteins were normalized to protein amounts and expressed in relative units. (B) Site-specific phosphorylation levels of cTnI. cTnI phosphorylation levels of the Ser-22/23, Thr-143 and Ser-43 residues (from top to bottom) were determined by Western immunoblotting in LV cardiomyocytes. The upper bands reflect the phosphorylation status of proteins and the lower bands indicate total protein amounts. Bar graphs illustrate mean ± SEM, *$p < 0.05$ vs. the control group (Co). Numerical values are given in Table 1.

Here we also investigated posttranslational modifications at PKC specific cTnI sites (Ser-43 and Thr-143), that might affect cardiac contractility, F_{max} and pCa_{50}. There is evidence that PKC-mediated phosphorylation of cTnI can lead to a reduction in F_{max} in the mammalian myocardium and that the consequence of PKC-mediated myofilament phos-

phorylation can result in an impairment of contractility *in vivo* [9]. Exercise training did not alter cTnI phosphorylation at the Ser-43 site, however it decreased it at the Thr-143 site. Interpretation of these effects is not straightforward, and hence, further studies are required to verify if hypophosphorylation of the cTnI Thr-143 site can account, at least in part, for the massive increase in F_{max} as observed here [7, 9, 11].

Taken together, the observed changes in cTnI phosphorylation, F_{max} and pCa_{50} are in line with the improved inotropic state of athlete's heart, whereby cardiomyocyte systolic force production is augmented by the increased Ca^{2+}-responsiveness of myofilament proteins. Nevertheless, our data cannot exclude the possible involvement of additional changes in the sarcomeric protein machinery and cardiomyocyte Ca^{2+} cycling [16].

In contrast to pathological conditions, physiological myocardial hypertrophy is associated with preserved or even enhanced diastolic function [15]. In this study we found that $F_{passive}$ and $k_{tr,max}$ did not change in exercised animals, suggesting that these sarcomeric characteristics did not affect LV diastolic performance. Titin, the giant protein of the sarcomere is mainly involved in the determination of ventricular diastolic function. Our results showed no alteration in titin phosphorylation in physiological hypertrophy, which is in line with unchanged ventricular stiffness and $F_{passive}$ during functional measurements [3].

In summary, this work implicates a close relationship between increased F_{max}, pCa_{50} of left ventricular cardiomyocytes and reduced cTnI phosphorylation levels during physiological myocardial hypertrophy.

Abbreviations

Ca^{2+}, calcium ion; [Ca^{2+}], calcium ion concentration; cTnI, cardiac troponin I; DTT, dithiothreitol; ECL, enhanced chemiluminescence; F_{active}, active force; $F_{passive}$, passive tension; F_{max}, maximum Ca^{2+}-activated force level; F_{total}, total force level; ISO, isolating solution; LV, left ventricle; pCa, -lg of calcium ion concentration; pCa_{50}, -lg of calcium ion concentration at half-maximal isometric force production; N2B, stiff titin isoform; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SL, sarcomere length.

Author contributions

BB, AO and LM performed the experiments and analyzed the data; AO, TR, BB and ZP conceived and designed the experiments; AT, BM, AO and ZP wrote the paper. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures were approved by the Ethical Committee of Hungary for Animal Experimentation (permission number: PEI/001/2374-4/2015). All animals received humane care in compliance with the "Principles of Laboratory Animal Care", formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, Revised 1996).

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Conflict of interest

The authors declare no conflict of interest. Zoltán Papp is serving as one of the Guest editors of this journal. We declare that Zoltán Papp had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Peter Kokkinos.

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