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Long-term intense exercise training is associated with benign myocardial remodeling in the right ventricle in an experimental model of athlete's heart

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Abstract:	Purpose Research projects have focused on exercise-induced alterations of the right ventricle (RV) of the heart, because the exercise-associated disproportionate load on the RV might lead to pathological consequences, such as interstital fibrosis, chamber dilation or pro-arrhytmic remodelling. We aimed at providing a complex characterization of RV alterations induced by regular exercise training in a rat model of athlete's heart. Methods Young, adult rats were divided into control (Co) and exercised (Ex) groups. Exercised rats swam 200min/day for 12 weeks. In vivo cardiac electrophysiological study and in vitro force measurements on isolated permeabilized RV cardiomyocytes were performed to investigate electrical and functional alterations, respectively. Molecular biological and histological investigations were carried out.

Results Exercise training was associated with mild increased RV hypertrophy (cardiomyocyte diameter: 12.5±0.1μm Co vs. 13.7±0.2μm Ex, $p<0.05$) and corresponding hyperphosphorylation of protein kinase B (Akt). Absence of pathological remodelling was revealed by unchanged pro-fibrotic and pro-apoptotic markers. We found increased maximal force development (12.1±1.0kN/m2 Co vs. 16.7±1.1 kN/m2 Ex, $p<0.05$) and improved calcium sensitivity in the cardiomyocytes of exercised animals. Sarcomere protein investigations revealed marked overall and site-specific (Ser22/23, Ser43 and Thr143) hypophosphorylation of troponinI. We found prolonged QT interval (repolarization) and RV effective refracter period along with decreased gene expression of potassium channels. We could not induce any ventricular arrhythmia by programmed stimulation.
Conclusions Regular swim training induced physiological RV hypertrophy that was associated with functional improvement and hypophosphorilation of troponinI. A balanced exercise program without excessive exercise sessions might not be associated with induction of pathological alterations.

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Long-term intense exercise training is associated with benign myocardial remodeling in the right ventricle in an experimental model of athlete's heart

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Running title: Exercise induces physiological RV remodeling

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26 **Abstract**

27 **Purpose** Research projects have focused on exercise-induced alterations of the right ventricle (RV) of
28 the heart, because the exercise-associated disproportionate load on the RV might lead to pathological
29 consequences, such as interstital fibrosis, chamber dilation or pro-arrhytmic remodelling. We aimed at
30 providing a complex characterization of RV alterations induced by regular exercise training in a rat
31 model of athlete's heart.

32 **Methods** Young, adult rats were divided into control (Co) and exercised (Ex) groups. Exercised rats
33 swam 200min/day for 12 weeks. In vivo cardiac electrophysiological study and in vitro force
34 measurements on isolated permeabilized RV cardiomyocytes were performed to investigate electrical
35 and functional alterations, respectively. Molecular biological and histological investigations were
36 carried out.

37 **Results** Exercise training was associated with mild increased RV hypertrophy (cardiomyocyte diameter:
38 12.5±0.1µm Co vs. 13.7±0.2µm Ex, $p<0.05$) and corresponding hyperphosphorylation of protein kinase
39 B (Akt). Absence of pathological remodelling was revealed by unchanged pro-fibrotic and pro-apoptotic
40 markers. We found increased maximal force development (12.1±1.0kN/m^2 Co vs. 16.7±1.1 kN/m^2 Ex,
41 $p<0.05$) and improved calcium sensitivity in the cardiomyocytes of exercised animals. Sarcomere
42 protein investigations revealed marked overall and site-specific (Ser22/23, Ser43 and Thr143)
43 hypophosphorylation of troponinI. We found prolonged QT interval (repolarization) and RV effective
44 refracter period along with decreased gene expression of potassium channels. We could not induce any
45 ventricular arrhythmia by programmed stimulation.

46 **Conclusions** Regular swim training induced physiological RV hypertrophy that was associated with
47 functional improvement and hypophosphorilation of troponinI. A balanced exercise program without
48 excessive exercise sessions might not be associated with induction of pathological alterations.

49

50 Key words: right ventricle, athlete’s heart, exercise training, in vivo electrophysiology, contractility,
51 physiological hypertrophy

52

53 **Introduction**

54 Regular exercise is associated with unequivocal health benefits and results in many structural
55 and functional changes of the myocardium those enhance performance and prevent
56 cardiovascular diseases (1,2). The accurate characterization of exercise induced cardiovascular
57 alterations have already been started more than a century ago and for a long time athlete's heart
58 had been described as a purely benign phenomena (3, 4). Similar to left ventricle, right ventricle
59 (RV) is also associated with increased internal diameter and free-wall thickness in highly
60 trained athletes and the amount of heart remodeling has been traditionally considered balanced
61 between the left and the right heart chambers (5,6). However, in contrast to the LV, the
62 functional consequences of RV hypertrophy have remained controversial (5,7). Although
63 resting RV global systolic function has shown to be decreased in endurance athletes, the novel
64 non-invasive imaging techniques suggest unaltered or even enhanced systolic performance (8).

65 On the other hand, the unexplained sudden cardiac death cases and recent research findings
66 about excessive exercise raised a question about the physiological nature of intense exercise
67 related to elite sport (9). Accordingly, the number of research projects about exercise-induced
68 right ventricular alterations have extensively been increased, looking for pathophysiological
69 characteristics (10, 11). Indeed, right ventricular wall stress is disproportionate during exercise
70 due to the relatively thin wall of the RV and a substantial increase of pulmonary pressure during
71 physical exertion (12). As a consequence, intense prolonged exercise bouts are associated with
72 a transient measurable reduction in right ventricular function, even when left side of the heart
73 is relatively unaffected (13). Chronic high-intensity exercise can not only aggravate structural
74 diseases, such as arrhythmogenic right ventricular cardiomyopathy (ARVC), but itself might
75 cause a pathological remodelling associated with dilation and fibrosis, a possible pro-
76 arrhythmic remodeling (14, 15).

77 The purpose of our study is to investigate the extent and nature of exercise-induced
78 alterations in the RV in a small animal model, where a marked left ventricular hypertrophy was
79 induced by regular long-lasting swim training without excessive exercise bouts (16). Our
80 animal model corresponds to relevant athlete's heart model with robust LV hypertrophy and
81 contractility improvement. We aimed to characterize both the beneficial and potentially harmful
82 changes in RV structure and function that result from sustained physical activity. Therefore a
83 detailed characterization of regular aerobic exercise training-induced right ventricular
84 alterations has been provided in a rat model of athlete's heart.

85

86 ## Materials and Methods

87 ### Ethical approval, animals

88 All animals received humane care in compliance with the Principles of Laboratory Animal
89 Care formulated by the National Society for Medical Research and the Guide for the Care and
90 Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and
91 published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996). Our
92 research conform to Directive 2010/63/EU. All procedures and handling of the animals during
93 the study were reviewed and approved by the Ethical Committee of Hungary for Animal
94 Experimentation.

95 Young adult (12 wk old, m=250-300 g) Wistar rats (n=24) (Toxi-Coop, Dunakeszi,
96 Hungary) were housed in a room with constant temperature of 22±2 ^{o}C with a 12/12h light-
97 dark cycle and fed a standard laboratory rat diet ad libitum and free access to water.

98 ### Experimental groups

99 After acclimation, rats of the exercise group (Ex, n=12) underwent a 12-week swim training
100 program, untrained animals served as controls (Co, n=12). Body weight was measured three
101 times a week during the whole study period. Invasive electrophysiological experiments were
102 performed after completion of the training program in six-six animals and after the protocol
103 hearts were removed and heart weight was measured immediately after sacrifice. Six-six
104 animals of each groups were sacrificed without in vivo experiments, their myocardial samples
105 were used for molecular analysis. Heart was removed under anesthesia.

106 ### Swim training protocol - rat model of exercise-induced cardiac hypertrophy

107 Based on the results of own preliminary pilot studies we provided a training plan to establish
108 a rat model for inducing robust cardiac hypertrophy (16, 17). Shortly, exercised rats swam for
109 a total period of 12 weeks, 200min session/day and 5 days a week. For adequate adaptation, the
110 duration of swim training was limited to 15min on the first day and increased by 15min every
111 second training session until the maximal swim duration (200min) was reached. Untrained
112 control rats were placed into the water for 5min each day during the 12-week training program.

113 ### In vivo right ventricular electrophysiology

114 The procedure was performed under general anaesthesia with 2%-isoflurane in six control
115 and six exercised animals. Body temperature was strictly maintained between 36.7ºC and
116 37.3ºC. An incision was made in the right supraclavicular region, and a 1.6F octapolar electrode
117 catheter (Millar EPR-802; Millar Instruments, Houston, US) was placed in the right internal
118 jugular vein. The catheter was advanced through the right atrium to the right ventricle using
119 electrogram guidance and pacing capture to verify intracardiac position. A computer-based data
120 acquisition system (PowerLab 16/30; ADInstruments, Colorado Spring, US) was used to record
121 a 1-lead body surface ECG (lead II) and up to 4 intracardiac bipolar electrograms (LabChart
122 Pro software v7; AD Instruments). Bipolar pacing through the distal electrodes was carried out
123 with an impulse generator (STG3008-FA, Multi Channel Systems, Reutlingen, Germany)
124 triggered by a special software (MC Stimulus II, Multi Channel Systems). Pacing capture
125 intensity threshold was explored and double value of threshold intensity were used during
126 pacing protocols.

127 In order to determine right ventricular refractory period (RVERP) the measurements were
128 continued after 10 minute stationary period, paying special attention to the body temperature.
129 RVERP was tested through programmed right-atrial stimulation with a 10-beat train (S1, CL
130 150 ms) followed by an extrastimulus (S2) that was decreased 2 ms per step until refractoriness.
131 Atrial effective refractory period was defined as the longest coupling interval failing to produce
132 a propagated ventricular response.

133 Ventricular arrhythmia inducibility was tested with double extrastimulation (DES) and burst
134 pacing. DES was performed following a 10-beat atrial-pacing train at a CL of 150 ms, followed
135 by one extrastimulus (S2) 10 ms longer than RAERP, while third extrastimulus (S3) was
136 decreased by 2 ms until refractoriness was reached. Ventricular burst pacing trains at 60 and 40
137 ms CL were applied for 15 and 30 seconds. Ventricular tachycardia (VT) was defined by >3
138 ventricular beats. VT was considered non-sustained (nsVT) when it lasted between 1 and 30
139 seconds.

140 **In vitro isometric force measurements in permeabilized cardiomyocytes**

141 Deep-frozen RV tissue samples were mechanically disrupted in isolating solution (ISO, (1
142 mM $MgCl_2$, 100 mM KCl, 2 mM EGTA, 4 mM ATP, 10 mM imidazole; pH 7.0, 0.5 mM
143 phenylmethylsulfonyl fluoride, 40 µM leupeptin and 10 µM E-64, all from Sigma-Aldrich, St.
144 Louis, MO, USA) and thereafter permeabilization was performed with 0.5% Triton X-100
145 detergent for 5 min, as described elsewhere (18). Briefly, single permeabilized cardiomyocytes

146 were mounted with silicone adhesive (DAP 100% all-purpose silicone sealant; Baltimore, MD,
147 USA) to two stainless steel insect needles, which were connected to a sensitive force transducer
148 (SensoNor, Horten, Norway) and to an electromagnetic high-speed length controller (Aurora
149 Scientific Inc., Aurora, Canada) in ISO at 15°C. The average sarcomere length was adjusted to
150 2.3 µm. The contractile machinery was activated by transferring the cardiomyocyte from a
151 relaxing to an activating solution. The Ca^{2+} concentrations ([Ca^{2+}]) expressed in pCa units refer
152 to -log [Ca^{2+}]. The pCa of the activating and relaxing solutions was 4.75 and 9.0, respectively.
153 When a steady force level had been reached, a rapid release-restretch maneuver (30 ms) was
154 applied to determine the baseline of the force generation and hence the Ca^{2+}-activated total
155 force (F_{total}). Fitting of the force re-development phase to a single exponential following the
156 release-restretch maneuver allowed the characterization of the maximal turnover rate of actin–
157 myosin cross-bridges (rate constant of force redevelopment in the presence of saturating
158 [Ca^{2+}]). About 6 s after the onset of force redevelopment, the Ca^{2+}-independent passive tension
159 ($F_{passive}$) was measured by shortening to 80% of the original preparation length at pCa 9.0 for 8
160 s. The active force (F_{active}) was calculated as a difference of the F_{total} and $F_{passive}$. Maximal
161 activation at pCa 4.75 was used to determine the maximal Ca^{2+}-activated isometric force (F_{max}),
162 while activations with intermediate [Ca^{2+}] (pCa 5.4– 7.0) yielded the pCa–isometric force
163 relationship. Isometric forces at submaximal [Ca^{2+}] normalized to F_{max} were plotted and then
164 fitted to a modified Hill-equation (Origin 6.0, Microcal Software, Northampton, MA., USA)
165 and to determine the Ca^{2+}-sensitivity of force production (pCa50). Original forces of every
166 individual cell were normalized to cardiomyocyte cross sectional-area, calculated from the
167 width and height of the cardiomyocytes. Force values were expressed in kN/m2 units.

168 **Quantitative Real-Time Polymerase Chain Reaction**

169 Right ventricular tissue samples were homogenized in Buffer RLT (Qiagen, Venlo, The
170 Netherlands) using Bertin Precellys 24 Tissue Homogenizer with Bertin Cryolys cooling
171 system (Bertin Technologies, Montigny-le-Bretonneux, France) to ensure adequate and
172 constant cooling (~0°C) of samples throughout the procedure. Then, total RNA was isolated
173 using the RNeasy Fibrous Tissue Kit (Qiagen) as per the manufacturer's protocol. RNA
174 concentration was measured photometrically at 260 nm, while RNA purity was ensured by
175 obtaining 260/280 nm and 260/230 nm optical density ratio of ~ 2.0, respectively.

176 Reverse transcription of RNA to cDNA was conducted with QuantiTect Reverse
177 Transcription Kit (Qiagen) by using 1µg RNA of each sample and random primers, as per

protocol. Then, quantitative real-time polymerase chain reaction (qRT-PCR) was performed on StepOnePlus RT PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using TaqMan Universal PCR MasterMix and TaqMan Gene Expression Assays (Thermo Fisher Scientific) for the following targets: α-MHC (assay ID: Rn00691721_g1); β-MHC (assay ID: Rn00568328_m1); atrial natriuretic factor (ANF, assay ID: Rn00561661_m1); Bcl-2 associated X protein (Bax, assay ID: Rn02532082_g1); Bcl-2 (assay ID: Rn99999125_m1); connexin (Cx) 40 (Gja5, assay ID: Rn00570632_m1); Cx43 (Gja1, assay ID: Rn01433957_m1); potassium channels: Kcna5, assay ID: Rn00564245_s1; Kcnd2, assay ID: Rn00581941_m1; Kcnd3, assay ID: Rn04339183_m1; Kcnj2, assay ID: Rn00568808_s1; Kcnj3, assay ID: Rn00434617_m1 and transforming growth factor β1 (TGFβ, assay ID: Rn00572010_m1). Every sample was quantified in duplicates or triplicates in a volume of 10μl in each well containing 1μl cDNA. Data were normalized to the housekeeping GAPDH, then to a positive calibrator (a pool of cDNA from all samples of the Co group) in each case. Accordingly, gene expression levels were calculated using the comparative method ($2^{-\Delta\Delta CT}$).

Protein expression - Western blot

Western blot experiments were performed as described earlier. Snap-frozen RV samples from 6 animals of each group were homogenized with RIPA buffer (Sigma Aldrich, Budapest, Hungary) containing Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland). Protein concentration was measured by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). Protein concentration was assessed with BCA kit (Thermo Fisher Scientific). Protein samples were resolved on precast 4–20% Criterion TGX gels (Bio-Rad, Hercules, CA, USA) and transferred to Immun-Blot PVDF membranes (Bio-Rad). Equal protein loading was verified with Ponceau staining. Membranes were blocked with bovine serum albumin (BSA; Santa Cruz Biotechnology, Dallas, TX, USA) in Tris-buffered saline with 0.05% Tween 20 (TBS-T) for 2 h. Membranes were incubated with primary antibodies in BSA in TBS-T against phospho-Akt [Ser473] (p-Akt, #4060, Cell Signaling), Akt (#9272, Cell Signaling), connective tissue growth factor (CTGF, sc-14939, Santa Cruz) and connexion-43 (ab11370, Abcam). After three washes with TBS-T, horseradish peroxidase-conjugated secondary antibody was added for 1 h at room temperature (in BSA in TBS-T; Cell Signaling). Signals were detected with an enhanced chemiluminescence kit (Bio-Rad) by Chemidoc XRS+ (Bio-Rad) and quantitated in Image Lab 4.1 software (Bio-Rad). Antibodies bound to phospho-epitopes were removed with Pierce Stripping Buffer (Thermo Fisher Scientific) before

211 incubation with antibodies detecting the total protein. We included all intact samples in the
212 analysis.

213 **Histology**

214 A whole transversal section of myocardium were cut at the height of base and left
215 ventricular papillary muscles. Ventricular myocardial tissue samples of animals were removed
216 for histological processing and then they were fixed in neutral buffered formalin (4%) and
217 embedded in paraffin. Slices of the whole heart at the level of LV papillary muscles were
218 sectioned and processed conventionally for histological examination.

219 After staining these myocardial tissue sections with hematoxylin and eosin (H&E) the
220 tissue structure was examined by light microscopy. The transverse, transnuclear diameter of
221 100 randomly selected RV cardiomyocytes per animal in longitudinal orientation of HE stained
222 sections were measured using the ImageJ software (National Institutes of Health, Bethesda,
223 MD, USA), and then averaged per animal.

224 RV wall thickness was measured on scanned H&E slices at the height of LV papillary
225 muscles. RV free wall thickness was measured three times, perpendicularly to RV endocardial
226 surface and these values were averaged.

227 To investigate RV collagen content, picrosirius red positive area was calculated using
228 ImageJ image analysis software. Three randomly selected RV fields (magnification 200x) were
229 investigated from each-each animal. The fractional area (picrosirius red positive area to total
230 area ratio) was determined on each images and the mean value of the images represents each
231 animal.

232 Apoptosis in cardiomyocytes was determined with terminal deoxynucleotidyl transferase-
233 mediated dUTP nick-end labeling (TUNEL) technique. TUNEL staining was performed using
234 DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI, USA) according to the
235 manufacturers instruction. Three visual fields of right ventricular sections were randomly
236 selected in each animal, and TUNEL-positive cells were counted.

237 **Proteomic analysis**

238 Cryopreserved LV and RV myocardial samples were homogenized in 0.1% Rapigest and
239 100 mM HEPES (pH 7.5) solution. Reductive alkylation was performed using 5 mM tris(2-
240 carboxyethyl)phosphine-hydrochloride (TCEP, Sigma-Aldrich) and 20 mM iodoacetamide
241 (Sigma-Aldrich). Proteins were digested overnight by Trypsin (Worthington, Lakewood, NJ,

242 sequencing grade), and the resulting peptides were desalted on PreOmics columns (PreOmics,
243 Bavaria, Germany). Further steps were performed as described previously. Briefly, peptides
244 were labeled with TMT11plex isobaric label reagents (Thermo Fisher Scientific). Reverse-
245 phase prefractionation (pH = 10) was performed on an XBridge C18 column, 150 mm × 1 mm
246 column containing 3.5 µm particles (Waters) inserted into an Agilent 1100 high performance
247 liquid chromatography system (HPLC). Fractions were analyzed on a Q-Exactive Plus (Thermo
248 Scientific, Bremen, Germany) operating in a data-dependent acquisition (DDA) mode. Mass
249 spectra were analyzed using MaxQuant version 1.6.17.0 with the Uniprot rat database
250 downloaded in November 2020. Quantified peptide intensities were then summarized by
251 MSstatsTMT (R package).

252 **Statistics**

253 Graphs were created and statistical analysis was performed in GraphPad Prism 6.0 software.
254 Differences between groups were calculated by Student's test after confirmation of normal
255 distribution of the parameters. Group descriptions were based on the mean ± SEM values.
256 Statistical significance was accepted at $p < 0.05$.

257

258 **Results**

259

260 **Right ventricular hypertrophy**

261 Exercise training was associated with significant, marked myocardial hypertrophy, indexed
262 by the heart weight values normalized to body weight (Figure 1.A). We experienced RV
263 hypertrophy in trained animals at the macroscopic and microscopic levels. RV free wall was
264 mildly thickened in exercised rats compared to control ones. Accordingly, RV cardiomyocyte
265 width was also increased after the completion of swim training protocol (Figure 1.A). The
266 phosphorylation level of protein kinase B (Akt), a key regulator of physiological hypertrophy,
267 was also increased in exercised rodents, while the total Akt level did not differ between groups
268 (data not shown).

269 We investigated the nature of the observed RV hypertrophy. Therefore, gene expression
270 level of common markers of pathological hypertrophy were measured. Neither ANF, nor the
271 ratio of α- and β-MHC did differ between exercised and control animals (Figure 1.B). The
272 histological analysis of picrosirius-stained RV sections allowed us to estimate the collagen
273 content of the myocardium and according to our results, exercise training was not related to
274 myocardial fibrosis (Figure 1.B). Accordingly, the gene expression level of TGF-β1 and the
275 protein expression of CTGF did not reveal the induction of profibrotic processes. Moreover,
276 TUNEL staining did not suggest any apoptotic activity of the cells (lack of stained cell nuclei)
277 and the gene expression ratio between proapoptotic Bax and anti-apoptotic Bcl-2 remained
278 unchanged after exercise training. These results suggest physiological nature of the observed
279 RV hypertrophy.

280 Proteomic analysis suggested less pronounced exercise-induced alterations in the RV
281 compared to the changes in LV (Figure 2.). While we detected 293 altered protein levels in the
282 LV, this number was only 38 in case of the RV. There was also a significant overlap in the RV
283 proteomic pattern of control and exercised animals, while our proteomic method could not
284 identify the presence of distinct clusters.

285

286 **Myocardial sarcomerdynamics and phosphorylation pattern of myofilaments**

287 Ca^{2+}-activated force (F_{active}) development was followed in permeabilized cardiomyocytes
288 isolated from RV tissue samples in vitro at different Ca^{2+} concentrations (pCa: from 4.75 to
289 7.0) (Figure 3A). Mean values of F_{active} (incl. F_{max}) were significantly higher following exercise
290 training (Ex group) than those of controls (Co group) between pCa 6.2 and 4.75. Ca^{2+}-sensitivity
291 of force production (pCa50) was significantly higher in the Ex group than in the Co group.

292 Mean pCa50 values did not differ after detraining (Figure 3B). Passive tension ($F_{passive}$) of
293 cardiomyocytes and the the rate of tension development ($k_{tr,max}$) were similar in the Co and Ex
294 groups (data not shown).

295 Following exercise training, overall phosphorylation level of cTnI decreased markedly in
296 RV cardiomyocytes. Overall phosphorylation levels of cMyBP-C and titin were similar in
297 permeabilized RV cardiomyocytes in both experimental groups (Figure 4A). To elucidate the
298 molecular background of increased F_{max} and pCa50 values of exercised animals, site-specific
299 phosphorylation assays were included for cTnI. Hypophosphorylation both at the PKA-specific
300 Ser22/23 and at the PKC-specific Ser43 and Thr143 sites of cTnI were observed in the Ex
301 groups (Figure 4B). These sites might be associated with the observed improved contractility
302 in exercised animals.
303

304 **Electrophysiological alterations**

305 We performed in vivo invasive electrophysiology to obtain electrical properties of the RV
306 myocardium. Refractoriness of RV myocardium was measured by standard protocol and we
307 found a significant prolongation of RVERP in exercised rats (Figure 5A). Right ventricular
308 arrhythmia inducibility was tested by burst pacing and DES stimulation protocols (Figure 5B).
309 No sustained ventricular arrhythmia was triggered by these programmed stimulations. We
310 could detect one induced VES in the case of one control and one exercised animal (Figure 5C).
311 These data suggest that our training protocol did not result in increased risk of arrhythmia in
312 exercised animals. We found decreased RV gene expression of potassium channels (Kcnd2,
313 Kcnd3 and Kcnj3) in exercised animals compared to controls. The gene and protein expression
314 of connexions did not differ between groups (Figure 5D).

315 **Discussion**

316 The knowledge of exercise-induced cardiovascular alterations have been substantially
317 widened in the recent years. However, the role of right ventricle in cardiovascular adverse
318 events observed in elite athletes is still dubious. According to our knowledge, this is the first
319 comprehensive experimental study to characterize long-lasting, intense training associated right
320 ventricular alterations. We investigated different aspects of right ventricular remodelling in a
321 rat model, where relevant, significant hypertrophy and contractility improvement was described
322 in the left ventricle (16, 19).

323

324 **Right ventricular remodelling**

325 Indeed, 12-week long swim training program has induced ~20-25% myocardial hypertrophy
326 in our exercised animals compared to the control ones, that is comparable to highly-trained
327 athletes, other relevant animal models and our previous results (17, 20, 21) (Figure 1A). We
328 also detected hypertrophy of the right ventricular cardiomyocytes and increased RV free wall
329 thickness values (unloaded conditions), that should be the consequence of increased pulmonary
330 pressure and the general volume overload during swim training sessions. Accordingly,
331 endurance elite and master athletes were both characterized by significantly increased RV mass
332 in MRI studies, where usually sport was associated RV hypertrophy and dilation (22, 23). We
333 also detected increased protein kinase B (Akt) phosphorylation in RV cardiomyocytes that
334 suggest the activation of the main pathway that is associated with physiological hypertrophy
335 (Figure 1A, 24).

336 To further confirm the physiological nature of the detected hypertrophy, we examined
337 markers of pathological remodelling at the cellular level and in the myocardium. These markers
338 can clearly differentiate between physiological and pathological myocardial processes (24, 25).
339 We could not detect any induction of genes as ANF or the ratio of MHC isoforms (Figure 1B).
340 Myocardial pathological processes and excessive oxidative stress might lead to increased
341 number of programmed cell death in the myocardium (24, 25). Nor TUNEL staining, neither
342 the ratio of pro-apoptotic Bax and anti-apoptotic Bcl-2 have suggested increased apoptotic
343 activity in the RV of trained animals (Figure 1B).

344 Perhaps, the most dubious question about exercise-induced RV alterations is about the long-
345 term remodelling and fibrosis that was raised mostly by the persistence of RV dilation in veteran

346 athletes. Human cardiac MRI studies have found delayed gadolinium enhancement (focal
347 pattern, especially at the interventricular septum and the site of RV attachment) in
348 approximately 10-40% of active and veteran endurance athletes (10, 26, 27). Besides that, there
349 are several small animal studies, where a marked right ventricular fibrosis and induction of pro-
350 fibrotic processes were observed after intense exercise training program (28, 29). However,
351 most of these small animal studies used treadmill training, where shock grids have been used
352 to motivate the animals, and the role of stress and electrical impulses were not deeply evaluated.
353 Our data indicated lack of fibrosis in swim-trained rats, despite the significant hypertrophy
354 (Figure 1B). We could not detect the induction of profibrotic key regulator molecules (such as
355 TGF-β1 and CTGF), that is in line with our histological analysis. There are also several human
356 studies that are in line with our findings and show absence of myocardial fibrosis (30, 31). The
357 results of our study suggest that long-term balanced exercise alone may not induce RV fibrotic
358 processes. Indeed, there are considerations about the pathological precipitating role of recurrent
359 excessive exercise sessions (such as races) and related extreme pulmonary hypertension (15).

360 Proteomic analysis revealed no relevant changes in the myocardial protein pattern, only the
361 expression of few proteins were upregulated in the RV compared to the LV (Figure 2.). This is
362 in contrast with pathological conditions where a huge number of proteins might be altered. We
363 could also not identify specific molecular groups associated with significant alterations.

364

365 **Function: sarcomerdynamics and sarcomeric alterations**

366 The hemodynamic load on the right ventricle is considered more pronounced during exercise
367 than that on the left ventricle. The thin wall of RV with a disproportionately increased
368 pulmonary pressure can cause a suboptimal coupling throughout high-intensity exercise. These
369 alterations can lead to exercise-induced transient RV fatigue that has been widely described:
370 not only the systolic function but also the relaxation can be impaired following prolonged
371 exercise (32, 33).

372 Regarding RV function, the long-term consequence of regular training remained
373 controversial. Most of the studies showed that echocardiographic parameters of global RV
374 systolic function (ejection fraction, fractional area change) were slightly reduced or comparable
375 at rest compared with the non-athletic controls (34, 35). Indeed, a mild reduction in
376 conventionally measured RV function at rest has been considered a physiological phenomen
377 associated with RV dilation and comparable contractile reserve was shown during exercise (5,

378 10). However, these parameters are largely dependent on chamber sizes and loading conditions
379 of the RV. According to our knowledge, our experimental study is the first one to show that
380 RV cardiomyocytes are associated with improved contractile force, Ca^{2+}-sensitivity and
381 contractile capacity (Figure 3). We should also add, that the increment in maximal force
382 (~+40%) is relatively smaller compared to LV (almost doubled) according to our previous
383 results (18).

384 The mechanism in the background of improved RV contractile reserve is still unknown.
385 While there were slight proteomic alterations, we investigated the phosphorylation pattern of
386 myofilaments (Figure 4). While the total phosphorylation of cMyBP-C did not show difference,
387 we have found marked hypophosphorylation of cTnI. Indeed cTnI holds a central role in the
388 regulation of contraction and relaxation in the heart muscle (36). Furthermore, we investigated
389 phosphorylation sites those are associated with alteration of contractile status and Ca^{2+}-
390 sensitivity (Figure 4, 15769444). While Ser22/23 is dependent by other proteins as protein
391 kinase A or G, all of the investigated sites (Ser22/23, Ser43, Thr143) are regulated by protein
392 kinase C (PKC). PKC-dependent phosphorylation of cTnI has been suggested to contribute to
393 the reduction in maximum Ca^{2+}-activated force in failing human myocardium. Increased
394 phosphorylation of Ser43 was related to decreased Ca^{2+}-activated force and phosphorylation of
395 Ser22/23 was associated with decreased Ca^{2+}-sensitivity (37). Although the interpretation of
396 myofilament phosphorylation is complex, here we found quite similar phosphorylation pattern
397 as in the LV that can be unique in exercise-induced hypertrophy (18). Further studies are needed
398 to understand the regulation of troponin I in athletes.

399 Moreover, in this study we found no difference regarding $F_{passive}$ (indicating passive diastolic
400 function) and no alteration in titin phosphorylation was observed (Figure 3 and 4.). This is in
401 line with the absence of fibrotic remodelling and shows similar result to our previous study
402 about LV (18).

403

404 **Electrical alterations**

405 In those minority of athletes who develop ventricular arrhythmias, the origin of arrhythmias
406 is most likely to be in the RV and is frequently associated with more pronounced dilation (15).
407 Although small animal models hold considerable limitations we examined the electrical
408 properties of RV.

409 Exercise-induced RV hypertrophy was associated with increased refractory period (RVERP,
410 Figure 5A). This is in line with this alteration, where our research groups and others have found
411 prolonged repolarization (QT length) after completion of exercise training (38, 39). This is also
412 in accordance with a study, where longer VERP was detected on isolated heart of trained rabbits
413 (40). Longer refractory period, without excessive prolongation, might hold rather a protective
414 role against re-entrant arrhythmias (41).

415 We could not induce ventricular arrhythmias by forceful programmed stimulation (Figure
416 5B). Our finding is in contrast with previous studies that showed inducible ventricular
417 arrhythmia in treadmill-trained small and large animals (28, 39). However, in these
418 investigations, the increased arrhythmia inducibility was associated with significant RV fibrotic
419 remodelling. Our data is in line with the previously described benign nature of exercise-induced
420 hypertrophy in a healthy myocardium and underline the beneficial effects of regular intense
421 training (30, 31, 38).

422 Decreased RV expression of certain potassium channels was observed in the RV and similar
423 alterations have been described in the atrium of exercised animals (38). This alteration might
424 explain the prolonged repolarization and refractoriness of the myocardium, although the
425 markedly distinct repolarization in rodents makes it difficult to extrapolate this result to athletes.
426 However, this data is in line with a previous observation that suggested a reduction in density
427 of one component of repolarizing K^+-currents (42).

428

429 **Conclusions**

430 Regular prolonged endurance training resulted in a physiological, beneficial remodelling of
431 the right ventricle without induction of pathological processes. We have found a significant
432 right ventricular functional improvement by increased maximal activated force and calcium
433 sensitivity of isolated cardiomyocytes. In the background significant hypophosphorylation of
434 troponin I was observed, quite similar pattern to left ventricle. In vivo electrophysiology has
435 not revealed increased myocardial vulnerability towards ventricular arrhythmias. According to
436 our results the previously observed pathological concerns about right ventricle in athletes might
437 be the consequence of excessive exercise sessions (prolonged competitions) or other triggers
438 (persistent hypertension) or might be based on other right ventricular pathologies.

439 The unexplained sudden cardiac death cases and recent pathological findings about
440 excessive exercise raised a question about the physiological nature of intense exercise related
441 to elite sport. Especially the role of right ventricular remodelling has been suspected in the
442 background, because of the disproportionate load during exercise sessions and sporadic
443 findings of right ventricular fibrosis. We investigated right ventricular alterations in our small
444 animal model that is associated with training-induced robust cardiac hypertrophy and functional
445 improvement. A significant right ventricular hypertrophy was observed with the absence of
446 pathological myocardial processes. Contractile reserve and calcium sensitivity improvement
447 was shown on right ventricular isolated cardiomyocytes. A marked hypophosphorylation of
448 troponin I was observed, both total and contractility-associated sites were involved. In vivo
449 electrophysiology could not show any sign of increased arrhythmia vulnerability. Our results
450 strengthen the hypothesis that regular intense exercise does not lead to pathological right
451 ventricular remodelling in a healthy myocardium. The previously observed sporadic cases
452 might be the consequence of non-severe structural heart disease or might be related to excessive
453 prolonged exercise sessions or other trigger factors.

454

Data availability statement The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflict of interest None to declare.

Ethics approval statement 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). All procedures and handling of the animals during the study were reviewed and approved by the Ethical Committee of Hungary for Animal Experimentation.

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592

593 **Figure 1. Exercise-induced tissue remodelling in the right ventricle (RV).** Panel A: markers of
594 myocardial and RV hypertrophy: heart weight index (normalized to body weight), RV free wall
595 thickness and RV cardiomyocyte diameter suggested mild RV hypertrophy in exercised (Ex) animals
596 compared to control (Co) ones. This was associated with hyperphosphorylation of protein kinase B
597 (Akt). Panel B: markers of pathological remodelling (ANF, ratio of α-MHC and β-MHC), markers of
598 fibrotic processes (collagen content, TGF- β1, CTGF) and apoptosis (TUNEL positive nuclei, Bax/Bcl-
599 2 ratio) did not differ between groups.

600 Akt: protein kinase B; ANF: atrial natriuretic factor; Bax: Bcl-2 associated protein X; CTGF: connective
601 tissue growth factor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; H&E: MHC: myosin heavy
602 chain; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labelling. Scale bar on picrosirius
603 staining: 100 μm. Data: mean±SEM. *$p<0.05$ vs. Co.

604 **Figure 2. Proteomic analysis**. Panel A: Volcano-plot about the proteomic alterations in the left
605 ventricle (LV) and the right ventricle (RV), when the proteomic expression of exercised (Ex) animals
606 was related to control (Co) ones. Significantly altered proteins has been shown as red dots, while
607 unchanged proteins are marked with blue dots. Panel B: Principal component analysis in the RV
608 showed a marked overlap. Panel C: The numbers of significantly changed proteins in case of the LV
609 and RV. Note, that while proteomic analysis revealed 293 altered proteins in LV, this number was
610 only 38 in case of RV.

611 **Figure 3. Sarcomerdynamics in isolated cardiomyocytes of right ventricle (RV).** Panel A:
612 Calcium concentration (pCa)-developed force (F_{active}) relationships from one-one animal of each group
613 (between pCa 7 and 4.75). The maximal Ca^{2+}-activated force (F_{max}) was improved in the RV
614 cardiomyocytes of exercised (Ex) animals compared to control (Co) ones. Panel B: normalized pCa-
615 force relationships from one-one animal of each group (between pCa 7.0 and 4.75). The leftward shift
616 of the curve and consecutive increased pCa50 values in exercised animals revealed improved Ca2+-
617 sensitivity induced by exercise training.

618 Co: control group, Ex: exercised group. Data: mean±SEM. *$p<0.05$ vs. Co.

619 **Figure 4. Phosphorylation levels of sarcomeric proteins of right ventricular RV cardiomyocytes**
620 **following exercise training.** Upper panel: Pro-Q Diamond staining was used to detect total
621 phosphorylation level of cardiac troponin I (cTnI), cardiac myosin binding protein-C (cMyBP-C) and
622 titin in RV. Exercise training was associated with decreased TnI phosphorylation level, while there was
623 no difference regarding phosphorylation of cMyBP-C and titin. Total protein amounts were assessed
624 by Coomassie-blue staining. Lower panel: Site-specific phosphorylation levels of cTnI. cTnI
625 phosphorylation levels of the Ser-22/23, Ser-43 and Thr-143 residues were determined by Western
626 immunoblotting in RV cardiomyocytes. We found marked hypophosphorylation on these sites.

627 The upper bands reflect the phosphorylation status of proteins and the lower bands indicate total protein
628 amounts. Co: control group, Ex: exercised group. Data: mean±SEM. *$p<0.05$ vs. Co.

629 **Figure 5. Electrophysiological properties of the right ventricle (RV) by invasive**
630 **electrophysiology and molecular measurements.** Panel A: Right ventricular refractery period
631 (RVERP) representative ECG (red), IAEG (yellow) and stimulation (green) curves from an exercised
632 (Ex) and a control (Co) animal. Panel B: Ventricular arrhythmia inducibility by using burst pacing or
633 double extrastimulation (DES) protocol and representative ECG (red), IAEG (yellow) and Stim curves
634 from an exercised and a control animal. Panel C: RVERP values were increased in exercised animals.
635 We could not induce non-sutained ventricular tachycardia (nsVT) or ventricular tachycardia (VT) in
636 our animals, while in case of one-one animal, we could induce ventricular extrasystole (VES). Panel
637 D: RV protein expression of connexion-43 (Cx43) did not differ between groups. RV gene expression
638 of different voltage-gated potassium channels (Kv1.5, Kv4.2 and Kv4.3), inward rectifier potassium
639 channels (Kir2.1 and Kir3.1) and connexins (Cx40 and Cx43) in control and exercised animals.

Figure 1

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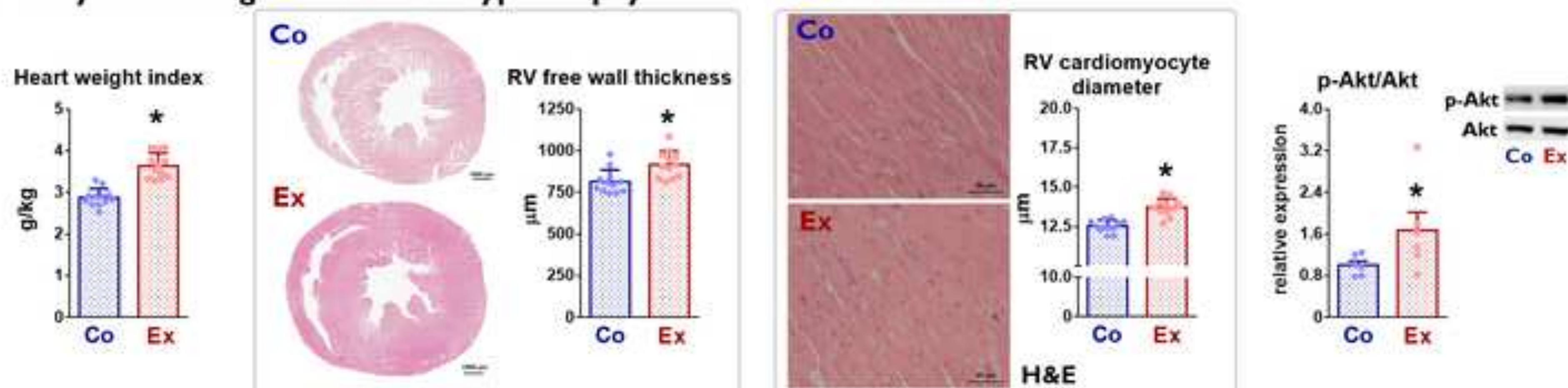

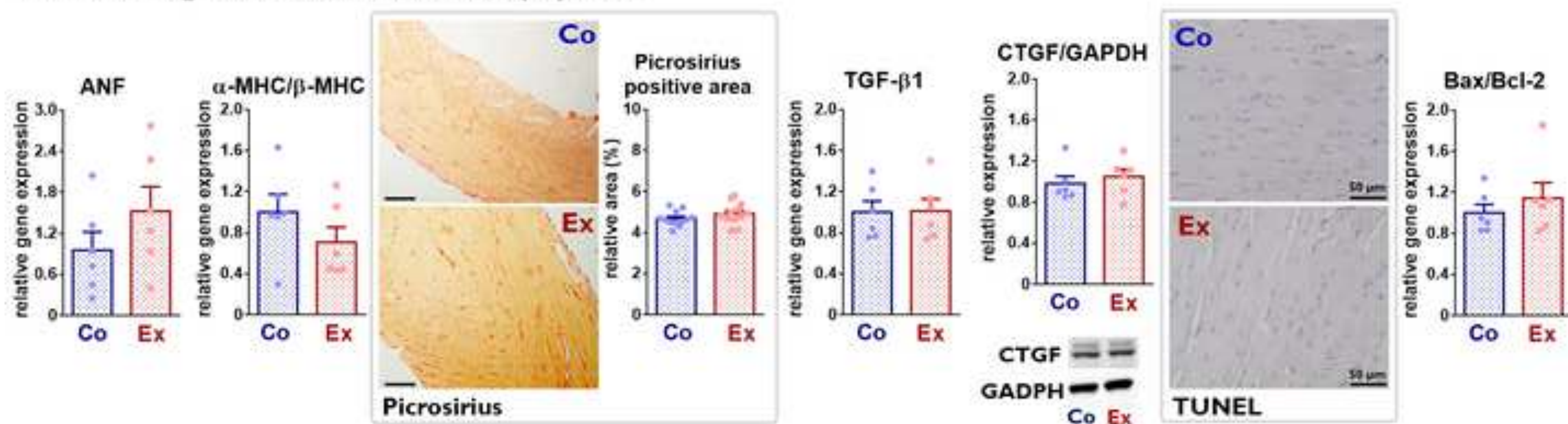

Figure 2

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A

Differential expression analysis

Ex vs Co (LV) | Ex vs Co (RV)

LV: Ex vs. Co

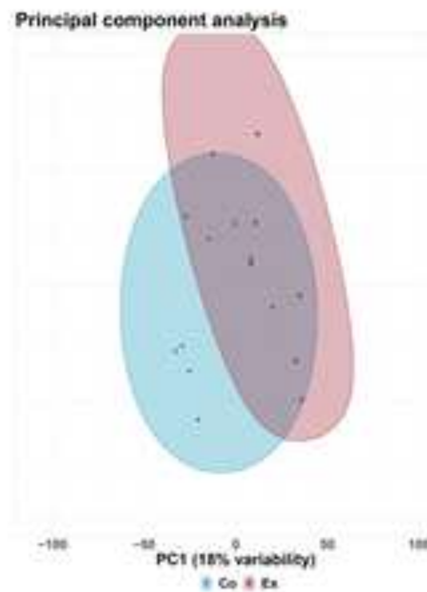
RV: Ex vs. Co

-log10(adj.P.Val)

log2 fold change

• significant change • not significant

B



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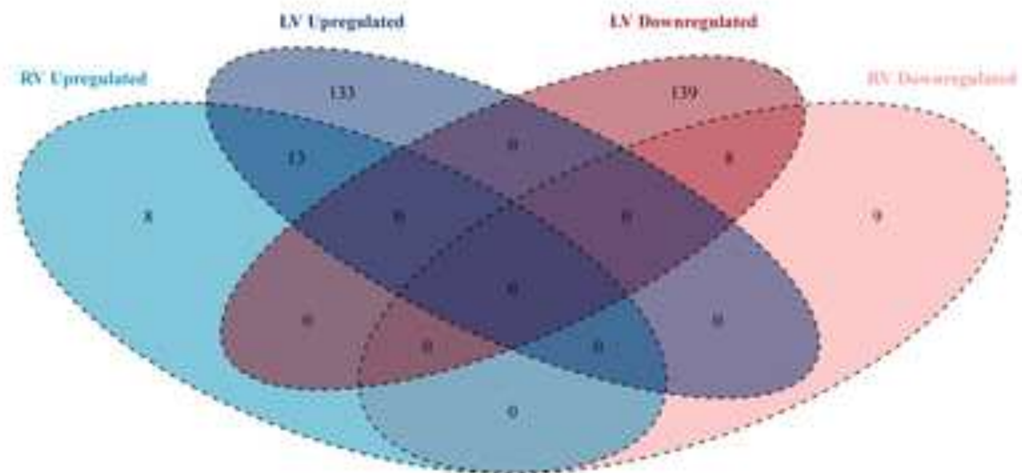

Figure 3

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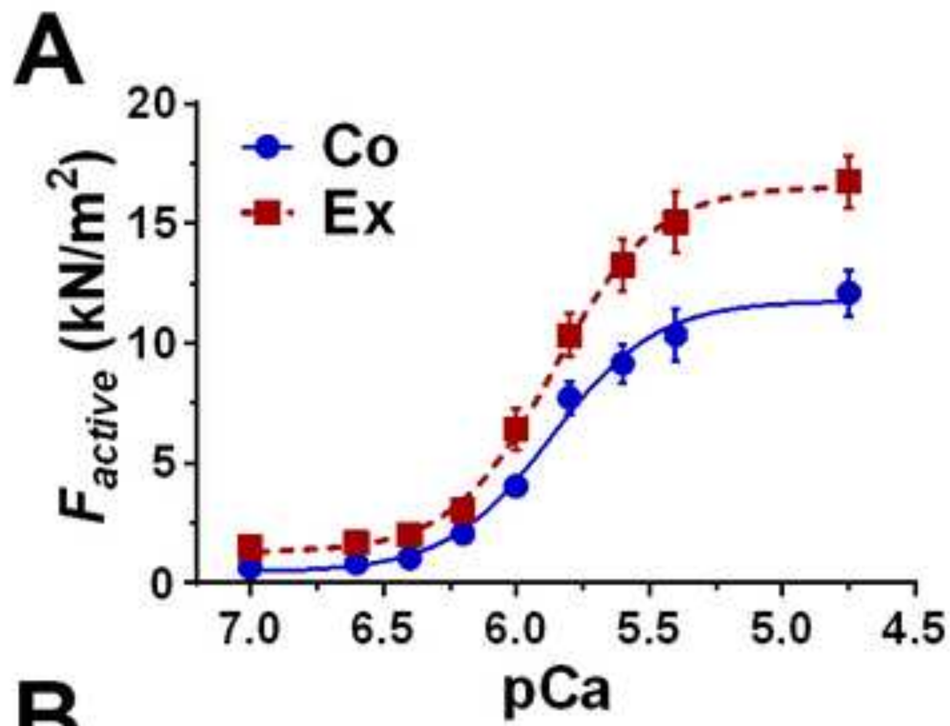

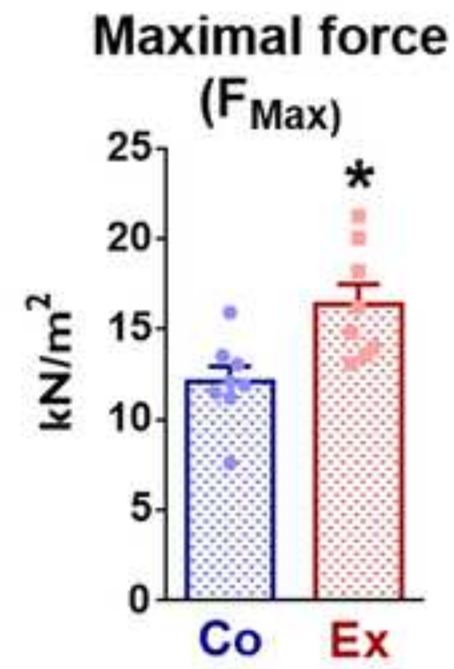

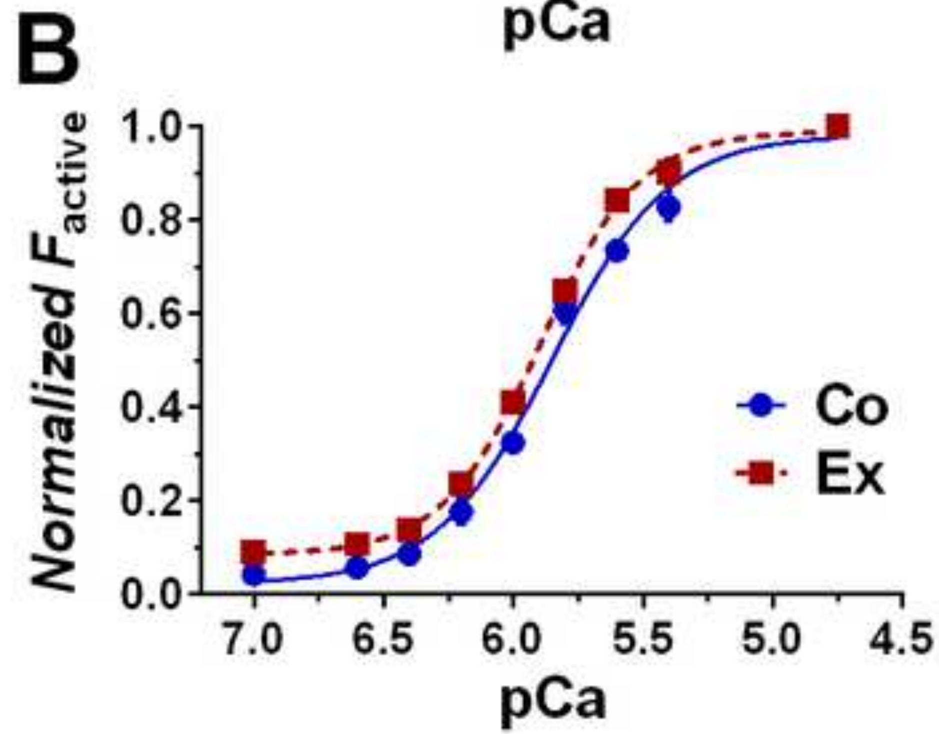

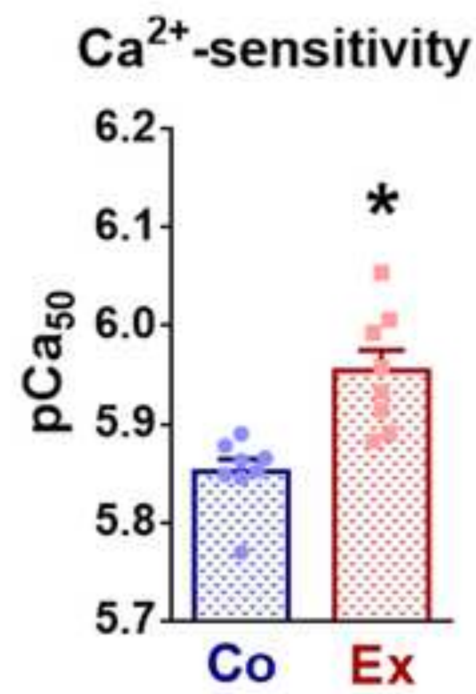

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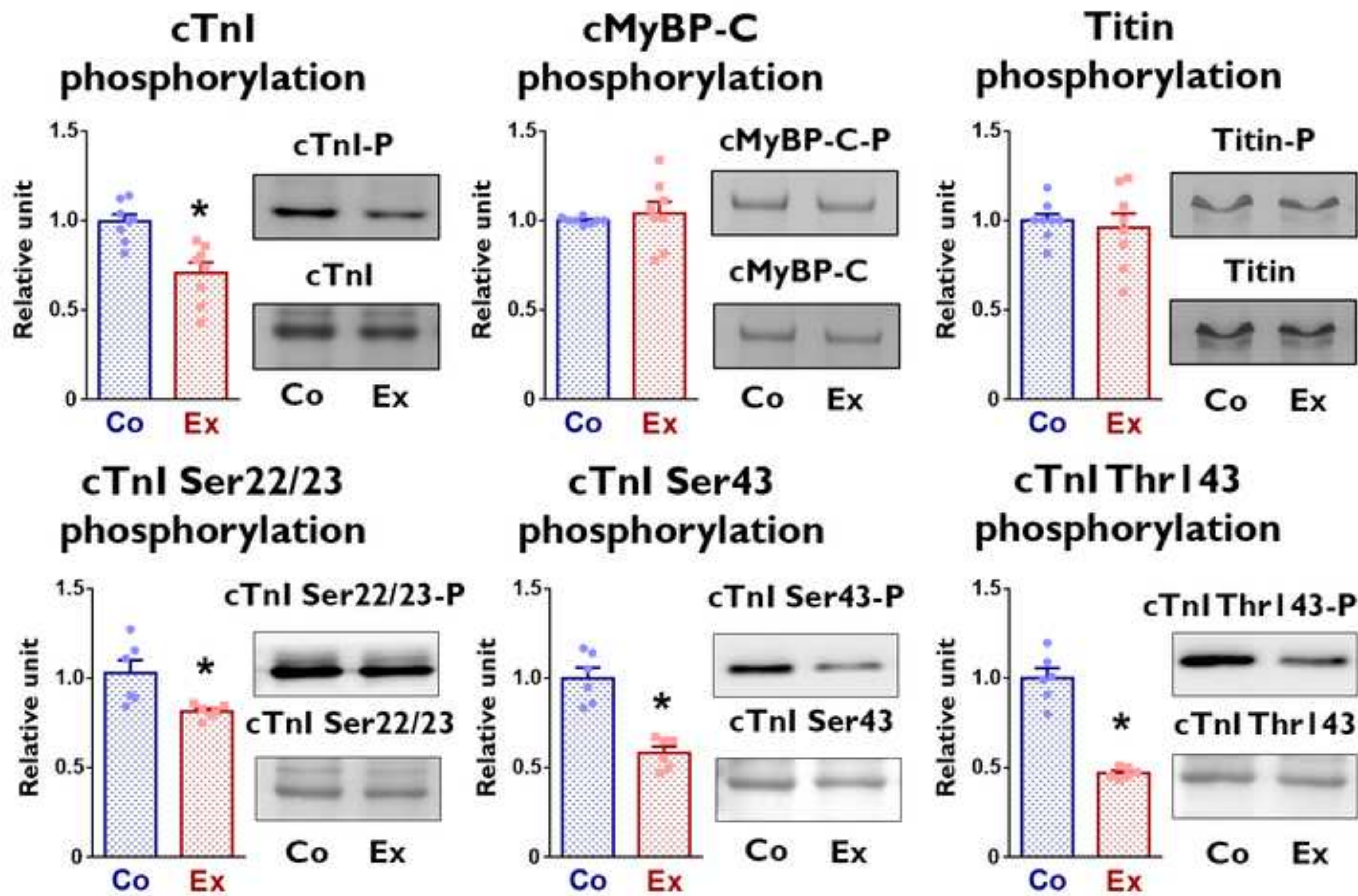

Figure 5

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