

Nitric oxide and CaMKII: Critical steps in the cardiac contractile response To IGF-1 and swim training



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ABSTRACT

Cardiac adaptation to endurance training includes improved contractility by a non-yet clarified mechanism. Since IGF-1 is the main mediator of the physiological response to exercise, we explored its effect on cardiac contractility and the putative involvement of nitric oxide (NO) and CaMKII in control and swim-trained mice.

IGF-1 increased cardiomyocyte shortening ($128.1 \pm 4.6\%$ vs. basal; $p < 0.05$) and accelerated relaxation (time to 50% relengthening: $49.2 \pm 2.0\%$ vs. basal; $p < 0.05$), effects abrogated by inhibition of: AKT with MK-2206, NO production with the NO synthase (NOS) inhibitor L-NAME and the specific NOS1 inhibitor nitroguanidine (NG), and CaMKII with KN-93. In agreement, an increase in NO in response to IGF-1 ($133.8 \pm 2.2\%$) was detected and prevented by both L-NAME and NG but not KN-93, suggesting that CaMKII activation was downstream NO. In addition, we determined CaMKII activity (P-CaMKII) and phosphorylation of its target, Thr17-PLN. IGF-1, by a NO-dependent mechanism, significantly increased both ($227.2 \pm 29.4\%$ and $145.3 \pm 5.4\%$, respectively) while no changes in the CaMKII phosphorylation site of ryanodine receptor were evident. The improvement in contractility induced by IGF-1 was associated with increased Ca^{2+} transient amplitude, rate of decay and SR content. Interestingly, this response was absent in cardiomyocytes from transgenic mice that express a CaMKII inhibitory peptide (AC3-I strain). Moreover, AC3-I mice subjected to swim training did develop physiological cardiac hypertrophy but not the contractile adaptation.

Therefore, we conclude that NO-dependent CaMKII activation plays a critical role in the improvement in contractility induced by IGF-1 and exercise training. Interestingly, this pathway would not contribute to the adaptive hypertrophy.

1. Introduction

Exercise training is a consolidated approach to reduce cardiovascular risk factors that in the last years has emerged as a promising strategy for the treatment of cardiovascular disease. Several reports have demonstrated its ability not only to positively shift pathologic cardiac remodeling but also to recover abnormal Ca^{2+} handling and improve contractility [1,2]. The first large prospective randomized study to actually provide evidence for a training-induced improvement of ejection fraction and reduction of LV end-diastolic diameter in a mixed population of subjects with ischemic and dilated cardiomyopathy came from Hambrecht et al. [3]. Later, these findings were corroborated by the Exercise in Left Ventricular Dysfunction and Chronic Heart Failure study [4].

Exercise training raises oxygen consumption, and as a consequence

cardiac output and oxygen transport increase to match the metabolic demand. Moreover, there is a cardiac adaptation to aerobic exercise training that includes cardiomyocyte hypertrophy and improved contractility, as the most important events. This response is mediated by the production and release of several growth factors and other humoral mediators among which, the insulin-like growth factor 1 (IGF-1) seems to be the most relevant. IGF-1 increases in response to exercise training both in experimental animal models and in athletes compared with healthy sedentary controls [5,6]. Furthermore, IGF-1 has been demonstrated to be produced in cardiovascular tissue, where it likely exerts autocrine/paracrine effects. Indeed, IGF-1 binding to its specific receptor (IGF-1R) stimulates, at least, two canonical pathways in cardiomyocytes: the PI3K/AKT and the ERK cascade [7].

The key role played by IGF-1 in the exercise-induced hypertrophic response has been convincingly demonstrated by several research

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groups using different experimental models. Transgenic mice with cardiomyocyte-specific knockout of the IGF-1 receptor (IGF-1R) failed to develop cardiac hypertrophy in response to 5 weeks of swim training [8]. Moreover, inhibition or genetic ablation of PI3K or AKT1 prevents physiological cardiac hypertrophy, supporting a key role for this signaling pathway in stimulating cardiomyocyte growth in response to physical exercise [9–12].

With respect to the cardiac positive inotropic response to exercise training, the underlying subcellular mechanism has not been completely clarified yet. Importantly, there is still contradictory data regarding the inotropic consequences of IGF-1 in experimental animal models of cardiomyopathies [13–15]. The PI3K/AKT [16] pathway, different isoforms of the nitric oxide synthases (NOS) [16–18] and/or the activation or inhibition of the Ca^{2+} /calmodulin-dependent kinase II (CaMKII) [19,20] are among the most relevant signaling cascades proposed to be responsible for the improvement in Ca^{2+} handling that leads to the enhanced contractility associated with exercise training. In addition, Kemi et al. [19,21] reported that aerobic interval training increases the expression of the sarcoplasmic reticulum Ca^{2+} pump (SERCA2a), significantly improving myocardial Ca^{2+} handling. Similar results were also reported in mouse and dog models of heart failure subjected to exercise training [22,23]. Moreover, Kim et al. proposed that chronic treatment with IGF-1 enhances myocyte contraction and relaxation functions due to an AKT-dependent up-regulation of SERCA2a in isolated rat cardiomyocytes [24]. On the other hand, there is strong evidence supporting a key role played by NO in the positive inotropic response to exercise [17]. The PI3K/AKT pathway has, as one of its downstream molecular targets, the NO synthases (NOS) that once phosphorylated increase NO production [25–27]. In this regard, Ziolo's group showed in isolated cardiomyocytes from wild type mice that exercise training increases contraction and improves relaxation in an NOS1 dependent manner and that the exercise-induced increase in cardiac function was prevented in trained mice with cardiomyocyte specific deletion of NOS1 (NOS1KO) [17,18]. They proposed as the molecular mechanism responsible for the enhanced Ca^{2+} cycling a NOS1-mediated increase in the phosphorylation of phospholamban (PLN) at the residue serine 16 (Ser16) probably due to inhibition of a phosphatase, leading to an increase in sarcoplasmic reticulum (SR) Ca^{2+} content that results in an enhancement in SR- Ca^{2+} release [17,18]. However, the connection between NO and the phosphatase is only speculative at present. At this point it, is interesting to remember that PLN is also phosphorylated by CaMKII, and this kinase can be activated not only by an increase in intracellular Ca^{2+} but also by NO [27,28]. Interestingly, CaMKII activation has been recently proposed to be involved in the cardiac contractile adaptation to exercise training [19].

The aim of the present work was to investigate in mice the cardiac contractile response to a swim routine and IGF-1 stimulation, specifically focusing on the role played by CaMKII and NO and the putative relationship between both. We hypothesize that a NO-dependent activation of CaMKII is involved in the contractile cardiac adaptation to exercise training.

2. Materials and methods

All procedures followed during this investigation conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health Eighth Edition (National Academies Press, 2011) and the experimental protocol was approved by the Animal Welfare Committee of La Plata School of Medicine.

Wild-type (WT) C57BL/6 mice (WT, Jackson Laboratories, Bar Harbor, ME, USA) (18 to 20 g) were used as controls. Transgenic mice, generated in the C57BL/6 background, with cardiomyocyte-delimited transgenic expression of either a CaMKII inhibitory peptide (AC3-I) or a scrambled control peptide (AC3-C) were used [29]. We chose the transgenic mice model instead of a shRNA knock down model because

we were interested in exploring the cardiac consequences of a chronic intervention (6 weeks swim routine) and the efficiency of cardiomyocyte transduction (i.e. shRNA) is variable and not homogeneous, usually requiring repeated injections for sustained long term expression [30,31]. Even though disparity exists between sexes in cardiovascular physiology and disease that importantly include differential activation and modification of myocardial CaMKII [32,33]; we conducted this study only in male mice in order to minimize the amount of variation in the results. Animals, studied at the age of 8–10 weeks, were kept at constant temperature (23 °C) in a standard light/dark cycle (12:12 h), fed ad libitum and had free access to water.

2.1. Cardiomyocyte isolation

Male C57BL/6 and transgenic mice were sacrificed by cervical dislocation and hearts were rapidly excised. Cardiac myocytes were isolated by collagenase-based enzymatic digestion according to a technique previously described [34]. After isolation cells were kept in a HEPES solution, containing (in mmol/L): 146.2 NaCl, 4.7 KCl, 1.8 CaCl_2 , 10.0 HEPES, 0.4 NaH_2PO_4 , 1.1 MgCl_2 , 10 glucose (pH adjusted to 7.4 with NaOH), at room temperature (20–24 °C) until use.

2.2. Calcium transient and cell shortening measurements

Ca^{2+} transient and cardiomyocyte shortening measurements were performed at room temperature, as previously described [15]. Briefly, the cardiomyocytes were placed in a perfusion chamber on the stage of an inverted microscope (Nikon), continuously superfused with a HEPES solution and stimulated via 2-platinum electrodes on either side of the bath at 0.5 Hz. Resting cell length and cell shortening were measured using a video-based motion detector (Ionoptix) and stored by software for an off-line analysis. The average values of the inotropic response were calculated after 5, 10 or 15 min of incubation with IGF-1 or no drug (control). The time to 50% relengthening was measured to evaluate the effect of IGF-1 or chronic training on relaxation.

For intracellular Ca^{2+} measurements isolated cardiomyocytes were loaded with 10 $\mu\text{mol/L}$ Fura-2 AM (Molecular probes) during 12 min at room temperature. The ratio of the Fura-2 fluorescence (510 nm) obtained after exciting the dye at 340 and 380 nm was taken as an index of free cytosolic Ca^{2+} . Time to 50% relaxation was used as a parameter of the rate of Ca^{2+} decay. For SR Ca^{2+} content measurement, a solution containing 10 mmol/L caffeine was rapidly applied onto cells. The amplitude of the caffeine-induced Ca^{2+} transient was used to estimate sarcoplasmic-reticulum Ca^{2+} content. The pharmacologic inhibitors, when corresponding, were preincubated 10 min before IGF-1.

2.3. Measurement of intracellular nitric oxide production in cardiac myocytes

Cardiac myocytes were loaded with 5 $\mu\text{mol/L}$ DAF-FM diacetate (Molecular probes) for 30 min at room temperature, and imaged by epifluorescence on a Zeiss 410 inverted confocal microscope (LSM Tech, Pennsylvania, USA). Excitation at 488 nm was provided by an argon laser and emission was collected in a range of 510–530 nm as previously described [35]. Photographs were taken every minute, during 18 min. in the absence of any drug (control) or stimulated with IGF-1 10 nmol/L. The inhibitors, when indicated, were preincubated 10 min before IGF-1. Image J software was used for the analysis. Results were expressed as a percentage of time zero.

2.4. Western blot analysis

Mouse hearts were perfused for 15 min on a Langendorff apparatus in the presence or absence of 10 nmol/L IGF-1 with/without different pharmacologic inhibitors and subsequently homogenized. Equal amounts of proteins were seeded in a 10% SDS polyacrylamide gel and

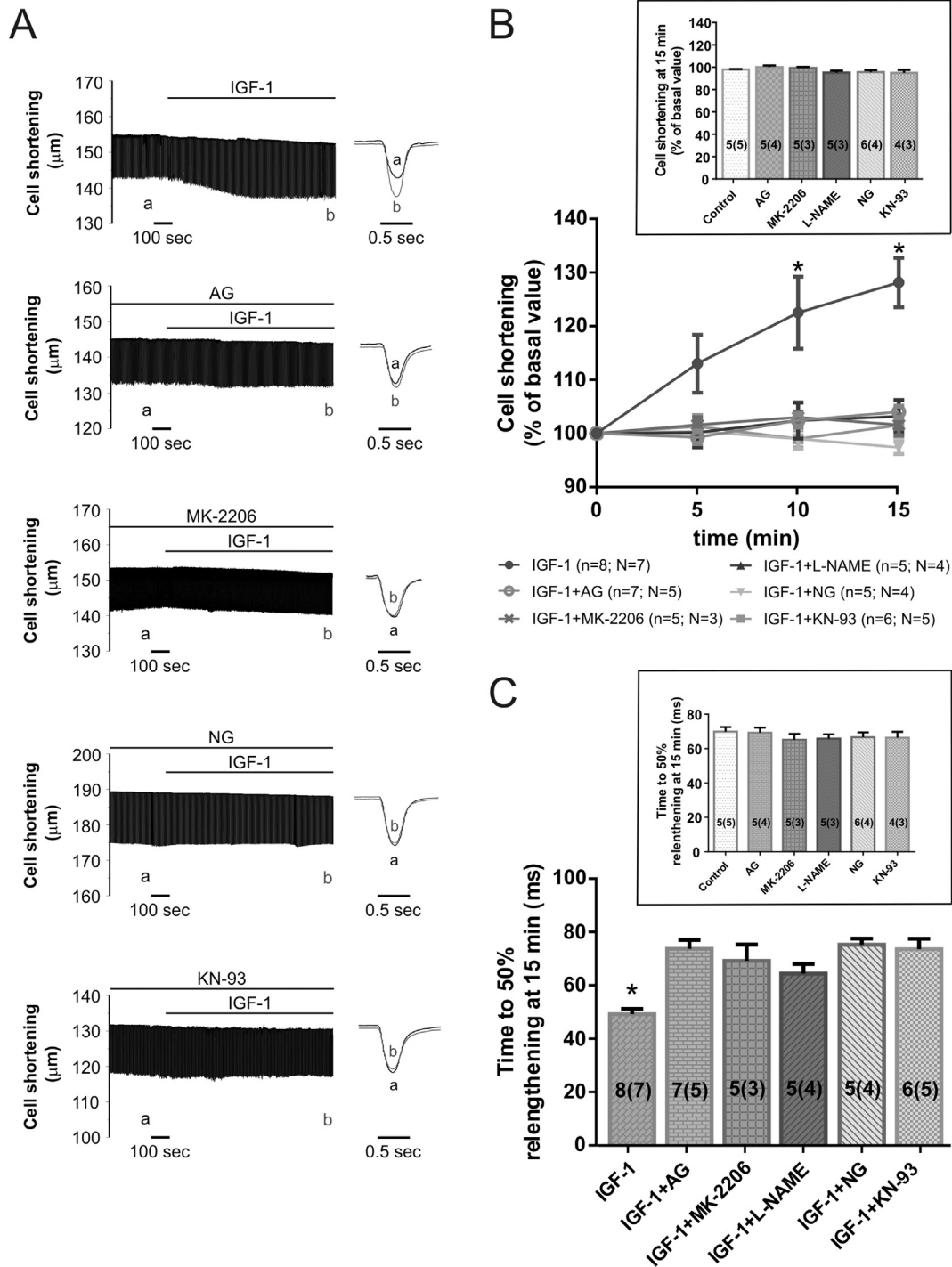


Fig. 1. IGF-1 produces a positive inotropic (PIE) and lusitropic effect (PLE) that depends on NOS1 and CaMKII activation. A. Representative original tracings of cardiomyocyte shortening showing the effect of IGF-1 (10 nmol/L) in the absence and presence of the IGF-1 receptor inhibitor, AG1024 (AG); the AKT inhibitor, MK-2206; the NOS1 inhibitor, nitroguanidine (NG); and the CaMKII inhibitor, KN-93. On the right, individual twitch recordings before (a) and after (b) IGF-1 are shown superimposed. B. Average results of cell shortening for all the experimental groups exposed to IGF-1. In the inset, the effect of the pharmacologic inhibitors, in the absence of IGF-1, on basal cell shortening is shown. C. IGF-1 significantly reduces the time to 50% relengthening after 15 min of incubation, effect abrogated by the inhibition of IGF-1 receptor, AKT, NO production and CaMKII. The inhibitors alone did not exert any effect on this parameter (inset). The number of cells assayed in each group (“n”) is depicted inside the bars while the number of hearts from where they came from (“N”) is specified between parentheses. * indicates $p < 0.05$ vs. control, Kruskal Wallis 1-way ANOVA and Dunn’s Method.

transferred to polyvinylidene difluoride membranes. Blots were probed overnight with the antibodies raised against phospho-Ser473 protein kinase B (P-AKT, 1:1000; Cell Signaling), neuronal NO synthase (NOS1, 1:1000; Santa Cruz Biotechnology) phospho-Ser1417-NOS1 (P-NOS1, 1:2000; Abcam), phospho-Thr286-CaMKII (P-CaMKII, 1:1000; Abcam), phospho-Thr17-PLN (2:500; Badrilla), phospho-Ser16-PLN (2:500;

Millipore) and phospho-Ser2814-RyR2 (1:5000; Badrilla). GAPDH detection (1:10,000; Santa Cruz Biotechnology) was used as loading control. Immunoreactivity was visualized by a peroxidase-based chemiluminescence detection kit (Immobilon Western Millipore) using a Chemidoc Imaging System. The signal intensity of the bands in the immunoblots was quantified by densitometry using Image J software

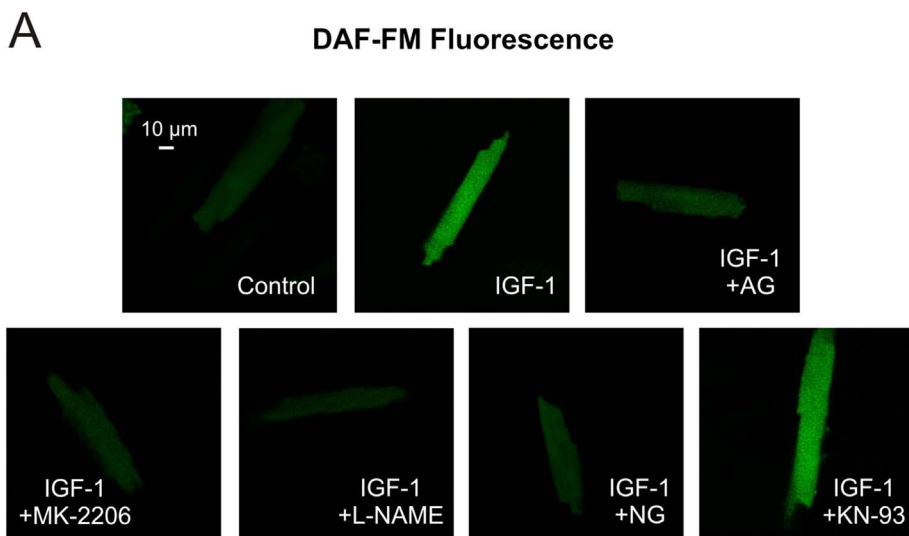
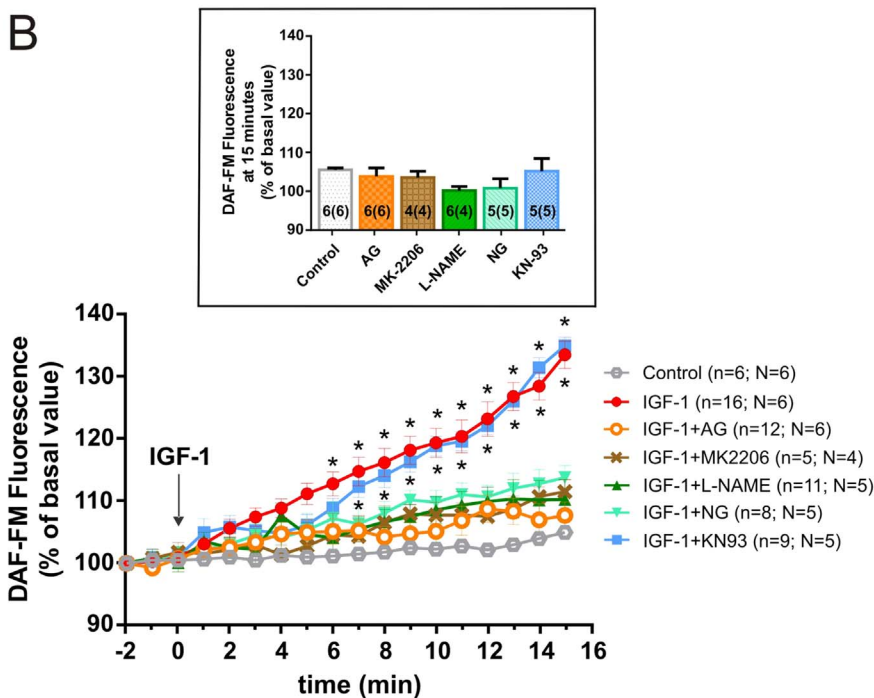


Fig. 2. IGF-1 stimulates cardiomyocyte NOS1-dependent NO production. **A.** Representative fluorescent images of cardiomyocytes loaded with the NO sensitive fluorescent dye DAF-FM and exposed to IGF-1 in the absence or presence of different inhibitors. **B.** Average results of DAF-FM fluorescence during 15 min of incubation with IGF-1 for the different experimental groups. The inhibition of CaMKII with KN-93 did not alter IGF-1-induced NO production. None of the pharmacologic inhibitors used exert an effect on cardiomyocyte NO production in the absence of IGF-1 (inset). The number of cells assayed in each group (“n”) is depicted inside the bars while the number of hearts from where they came from (“N”) is specified between parentheses. * indicates $p < 0.05$ vs. control at the same time points, Kruskal Wallis 1-way ANOVA and Dunn’s Method.



(NIH, USA).

2.5. Murine exercise protocol

Mice were randomly assigned to a sedentary (Sed) or swim-trained (Swim) group and divided in six groups: (1) C57 Sed, (2) C57 Swim (3) AC3-C Sed, (4) AC3-C Swim, (5) AC3-I Sed and (6) AC3-I Swim. The swim training protocol was modified from a previously published procedure [36]. Briefly, adult mice in the Swim group were trained, free-of-loading, 5 days/week, twice a day for 6 weeks in warm water (30–32 °C). The first week consisted of a training adaptation period; starting with 20 min/day of swimming and increased daily by 20 min up to 90 min. Mice in the Sed group were placed in the swimming tank for 10 min twice a week to mimic the water stress associated with the experimental protocol. After the final swim session mice were overnight fasted, then weighed and sacrificed. Hearts were washed with PBS, drained by gentle squeeze using cotton gauze and weighed. Right tibias were gently excised and all muscle was removed in order to expose the

whole bone for precise measurements with caliper rule. Heart weight (HW)/tibia length (TL) was calculated.

2.6. Echocardiographic evaluation

Echocardiography was performed with the Vevo 770 coupled to a 30 MHz transducer (Visual Sonics). Images were acquired in M-mode on the left ventricular muscle thickness and analyzed by a blinded investigator.

2.7. Histological analysis studies

Ventricular tissue was fixed in buffered 10% formaldehyde and paraffin-embedded. LV coronal sections (4 μm thick) at the equator were stained with hematoxylin-eosin or picrosirius red (Direct Red 80 Aldrich) for blinded determination of cardiomyocyte cross-sectional area (CSA) and quantitation of fibrosis (% of interstitial collagen). CSA was calculated in a minimum of 50 cardiomyocytes per experimental

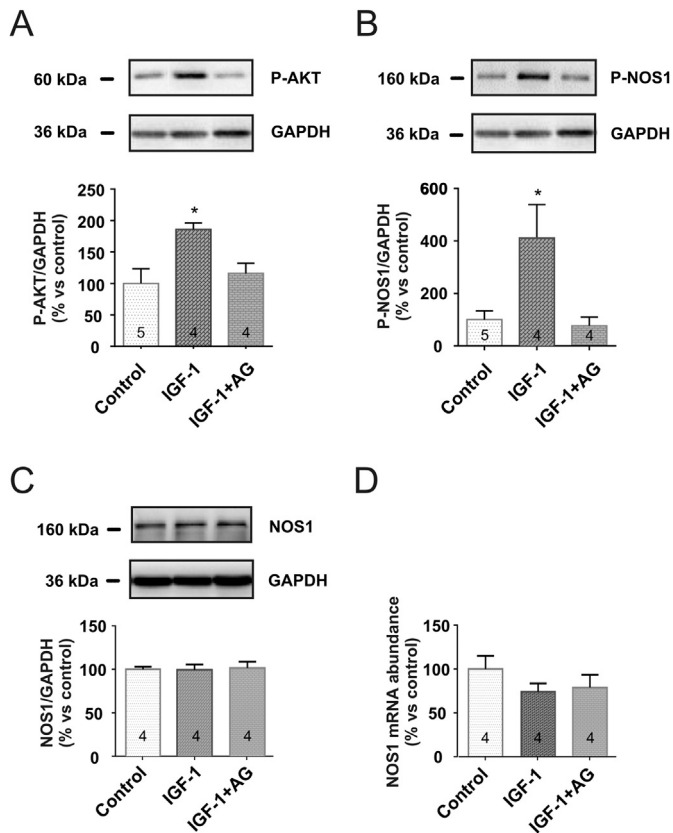


Fig. 3. IGF-1 increases AKT and NOS1 activity. Typical western blots and average data showing that IGF-1 induces both AKT (A) and NOS1 (B) phosphorylation while not increasing NOS1 protein expression (C) through an IGF-1 receptor-dependent mechanism. D. NOS1 mRNA abundance was quantified by real time RT-PCR demonstrating that IGF-1 does not up-regulate this enzyme under acute exposure. The number of experiments (n) in each group is depicted inside the bars. * indicates $p < 0.05$ vs. all other groups, Kruskal Wallis 1-way ANOVA and Dunn's Method.

condition from at least 4 different mice after digitalization at $200\times$ magnification in an image analysis system (Image-Pro Plus software). The investigator was unaware of the treatment groups and codes were disclosed after statistical analyses.

2.8. Real time RT-PCR

Brain natriuretic peptide (BNP) and NOS1 mRNA expression were assessed by real-time RT-PCR and normalized to GAPDH following the procedure described previously [1]. Briefly, total RNA was isolated from cardiac tissue using Trizol™ Reagent (Invitrogen). RNA (0.8 μ g) was reverse transcribed using the MMLV (Invitrogen). A dilution of the resulting cDNA was used to quantify the relative content of mRNA by real-time PCR (iCycler iQ real-time PCR detection system, Bio-Rad) using appropriate primers and SYBR Green as the fluorescent probe. The following primers, designed using Primer3 software, were used: 5'-GCTGTAACGCACTGAAGTTGT-3' (forward) and 5'-TCAAAGGTGGTCCAGAGCT-3' (reverse) for BNP; 5'-CAACAGCGTCTCCATT-3' (forward) and 5'-CGAAGACTGAGAACCTCACATT-3' (reverse) for NOS-1; 5'-CATGGCCTTCGTGTTCTTA-3' (forward) and 5'-TGCTTACCACC-TTCTTGATG-3' (reverse) for GAPDH.

2.9. Chemicals

All drugs used in the present study were analytical reagents. IGF-1: recombinant mouse insulin like growth factor -1 (10 nmol/L, Cat # PMG0075 from Gibco by Life Technologies); AG-1024 (100 nmol/L Cat # sc-205,907 from Santa Cruz Biotechnology); AKT inhibitor MK-2206

(500 nmol/mL Cat # S1078 from Selleck Chemicals); KN-93 (2.5 μ mol/L Cat 422,708 from Calbiochem); nitroguanidine (NG 240 nmol/L, Cat 490,070 from Calbiochem), nitro-l-arginine methyl ester (L-NAME 2.5 mmol/L, Cat N5751 from Sigma). KN-93, MK-2206 and AG-1024 were diluted in DMSO (final concentration $\leq 0.01\%$).

2.10. Statistical analysis

Results are expressed as mean \pm SEM. The non-parametric Wilcoxon rank-sum test and Kruskal-Wallis 1-way ANOVA, Dunn's Method were used for statistical comparisons when appropriate. Differences were considered significant at $p < 0.05$. All analyses were made using GraphPad Prism 5.0 (GraphPad Software, USA).

3. Results

3.1. IGF-1 improves cardiomyocyte contractility

Cardiac contractility was explored by measuring cell shortening in isolated cardiomyocytes. IGF-1 (10 nmol/L) acutely increased cardiomyocyte shortening, reaching its peak at ~ 15 min of incubation as it can be appreciated in the typical recordings and overall results shown in Fig. 1A–B. This positive inotropic effect was associated with an acceleration of cardiomyocyte relaxation as revealed by the significant reduction in the time to 50% relengthening (Fig. 1C). As expected, both effects were abrogated by the inhibition of the IGF-1 receptor (IGF-1R) with AG-1024 (100 nmol/L) and its downstream kinase AKT with MK-2206 (500 nmol/L). To get further insight into the subcellular mechanism involved, we examined cell shortening after inhibition of the NOS that are well recognized targets of AKT. We used L-NAME (2.5 mmol/L), a non-isoform specific NOS inhibitor, as well as nitroguanidine (NG, 240 nmol/L) a specific inhibitor of the neuronal isoform of NOS (NOS1). Under these experimental conditions, the positive inotropic and lusitropic response to IGF-1 were significantly attenuated. Similar results were obtained when CaMKII, a putative target of NO, was inhibited using KN-93 (2.5 μ mol/L). None of these inhibitors exerted an effect on contractility or the time to 50% relengthening when added in the absence of IGF-1 (insets of panels B and C, respectively). Control cells not exposed to IGF-1, showed no significant changes in contractility during the same experimental period as those exposed to IGF-1.

3.2. IGF-1 stimulates cardiomyocyte NO production

To confirm the participation of NO in the IGF-1-induced inotropic response we evaluated its production by epifluorescence in isolated cardiomyocytes exposed to IGF-1. IGF-1 increased the NO-dependent fluorescent signal, effect that was significantly attenuated by the blockade of IGF-1R, AKT and NOS1 (Fig. 2). Interestingly, the inhibition of CaMKII did not alter IGF-1 induced NO production, suggesting that, if they were both components of the same signaling pathway, CaMKII should be downstream of NO. This data was additionally supported by immunoblot determinations which confirmed that IGF-1 induced both AKT and NOS1 phosphorylation through an IGF-1R dependent mechanism (Fig. 3A–B). We excluded the possibility that an increase transcription of NOS1 was collaborating in the augmented production of NO by quantifying NOS1 protein and mRNA abundance (Fig. 3C–D).

3.3. IGF-1 and intracellular Ca^{2+} handling

In order to gain further understanding of the signaling cascade underlying the positive inotropic/lusitropic effect of IGF-1, we evaluated CaMKII activation by determining its autophosphorylation and the phosphorylation of its downstream targets, residue threonine 17 (Thr17) of PLN and serine 2814 of the ryanodine receptor (Ser2814-RyR2). IGF-1 significantly increased the phosphorylation of CaMKII and

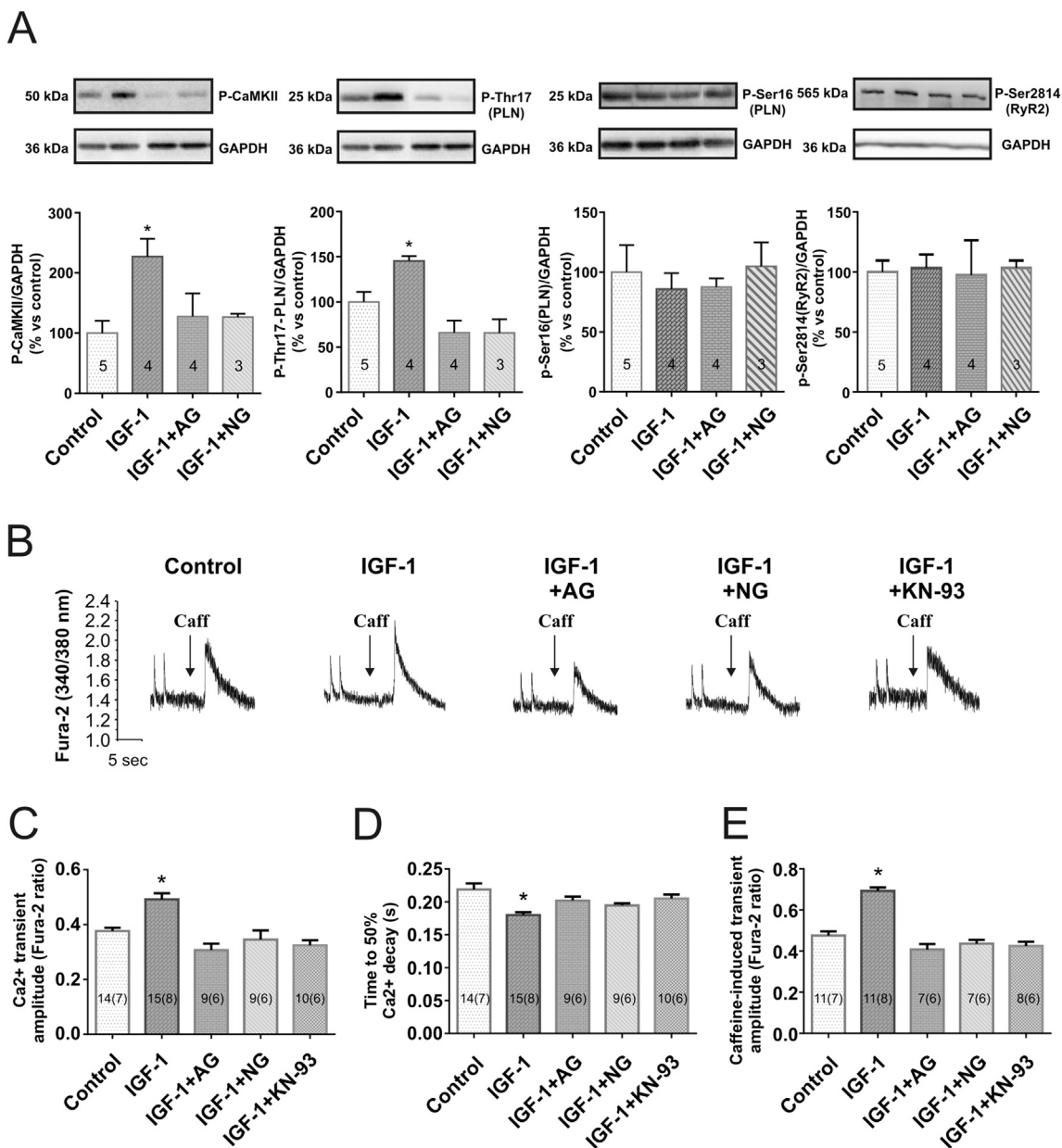


Fig. 4. IGF-1 activates CaMKII in a NOS1-dependent manner and improves SR Ca²⁺ handling. **A.** Representative Western blots and average results showing the effect of IGF-1 on the phosphorylation of CaMKII; threonine 17 (Thr17-PLN, target of CaMKII) and serine 16 of phospholamban (Ser16-PLN, target of PKA) and serine 2814 of the ryanodine receptor (Ser2814-RyR2, target of CaMKII); in the absence and presence of AG1024 (AG) and nitroguanidine (NG). **B.** Typical tracings showing the effect of IGF-1 on the amplitude and decay of the Ca²⁺ transient and on the amplitude of the caffeine-induced Ca²⁺ transient. **C–D.** Overall results for the amplitude and time to 50% decay of the Ca²⁺ transient for each experimental group, respectively. **E.** Effect of IGF-1 on SR Ca²⁺ content as indicated by the amplitude of the caffeine-induced Ca²⁺ transient. The number of cells assayed in each group is depicted inside the bars while the number of hearts from where they came from is specified between parentheses. * Indicates *p* < 0.05 vs. all other groups, Kruskal Wallis ANOVA and Dunn's Method.

Thr17-PLN, effects that were cancelled not only by the blockade of IGF-1R with AG but also by the inhibition of NOS1. On the contrary, no significant differences were detected in the phosphorylation of either the CaMKII site on RyR2 (Ser2814) or the PKA site on PLN (Ser16) (Fig. 4A). These results are in line with those of Fig. 2, suggesting that NO production and CaMKII activation are steps of the same intracellular signaling cascade, being CaMKII downstream of the increase in NO. Since PLN phosphorylation is expected to increase the activity of the SR Ca²⁺ pump (SERCA2a) augmenting SR Ca²⁺ content, we analyzed the amplitude and decay of Ca²⁺ transients in isolated cardiomyocytes exposed to IGF-1. We found that IGF-1 induced a significant increase in the amplitude of the Ca²⁺ transient while reducing its time to 50% decay (Fig. 4B–D). Moreover, IGF-1 significantly increased the SR Ca²⁺ content as revealed by the greater amplitude of the caffeine-

induced Ca²⁺ transient (Fig. 4E).

3.4. IGF-1 on cardiomyocyte contractility in a transgenic mice model

To confirm the participation of CaMKII in the inotropic/lusitropic effects of IGF-1 we performed another set of experiments in transgenic mice that express an inhibitory peptide of the kinase (AC3-I strain, and their corresponding control AC3-C). As shown in Fig. 5, IGF-1 failed to increase cell shortening and accelerate relaxation in isolated cardiomyocytes from AC3-I mice, while this effect was preserved in AC3-C mice.

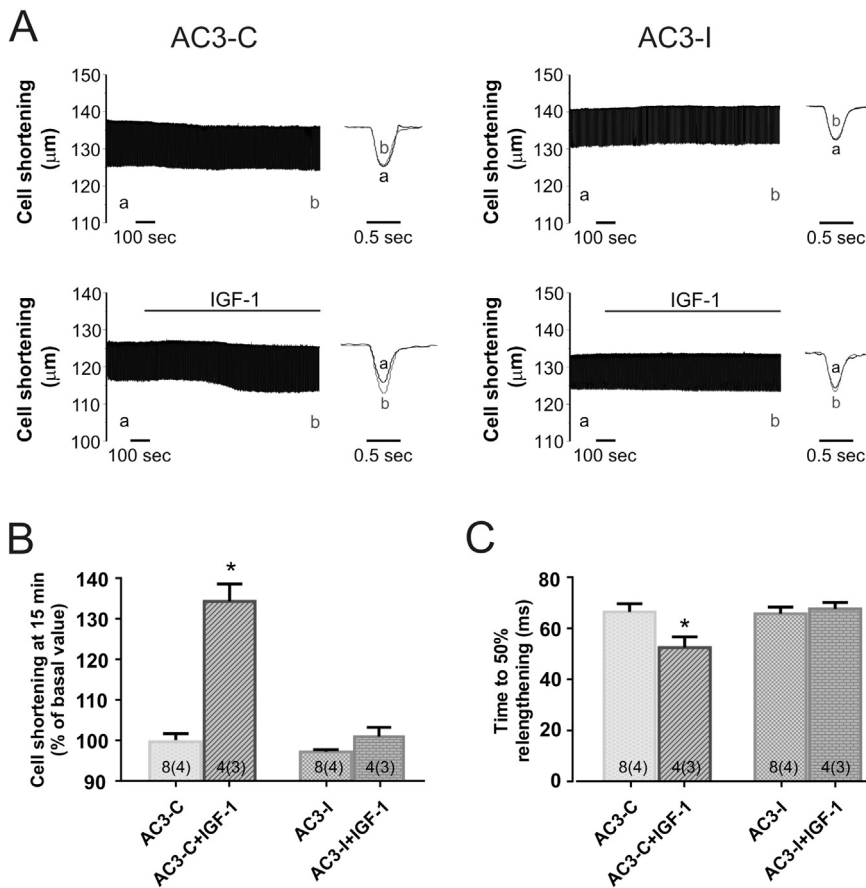


Fig. 5. IGF-1 fails to affect cardiomyocyte contractility in transgenic AC3-I mice. Typical tracings (A) and overall results (B) showing that the positive inotropic effect of IGF-1 was present in isolated cardiomyocytes from control transgenic AC3-C mice and absent in myocytes that express the CaMKII inhibitory peptide (AC3-I strain). The number of cells assayed in each group is depicted inside the bars while the number of hearts from where they came from is specified between parentheses. * Indicates $p < 0.05$ vs. all other groups, Kruskal Wallis ANOVA and Dunn's Method.

3.5. Cardiac adaptation to swim training in wild type and transgenic mice

Having demonstrated the key role played by CaMKII in the positive inotropic/lusitropic effect of IGF-1 in cardiac myocytes we further explored its role in the setting of hearts with physiological cardiac hypertrophy. In pursuing this aim, we subjected mice to 6 weeks of exercise training by swimming. At the end of this period, physiological cardiac hypertrophy was evidenced by the increase in cardiac mass and cross sectional area of cardiomyocytes without the presence of fibrosis. The physiological nature of hypertrophy was reinforced by the absence of up-regulation of brain natriuretic peptide, a molecular marker of pathological hypertrophy. Physiological cardiac hypertrophy was accompanied by an increase in fractional shortening detected by echocardiography (Fig. 6A–F). In homogenates from these hearts we determined the phosphorylation state of AKT, CaMKII, Thr17-PLN and Ser2814-RyR2. In the hypertrophied hearts, we found an increased phosphorylation in AKT, CaMKII and Thr17-PLN but not in Ser2814-RyR2, in agreement with the results obtained with IGF-1 stimulation (Fig. 6G).

Transgenic AC3-I and AC3-C mice, were subjected to the same swim protocol, and even though AC3-I myocytes had not shown the positive inotropic response to IGF-1 (see Fig. 5), they did develop physiological cardiac hypertrophy (Fig. 7A–D). As expected, only in the AC3-C mice the swim routine induced CaMKII activation increasing P-Thr17-PLN. Again, no significant changes in the phosphorylation status of Ser16-PLN or Ser2814-RyR2 were detected (Fig. 7E). In agreement with these biochemical modifications an increase in cardiac contractility and acceleration of relaxation were detected exclusively in the AC3-C trained mice (Fig. 7F–H). As a whole, these results support that CaMKII is not involved in the hypertrophic response although it is critical for the adaptive contractile response to exercise training.

4. Discussion

The results presented herein provide strong evidence that CaMKII plays an essential role in the positive inotropic/lusitropic response to IGF-1 and in the adaptive improvement in contractility associated to exercise training. Taken together our findings allow us to propose that exercise training, through the release of IGF-1, induces the phosphorylation of AKT which in turn activates NOS1 increasing myocardial NO production. The increase in NO would be responsible for CaMKII activation improving SR Ca^{2+} handling and therefore contractility. The underlying mechanism would involve the phosphorylation of Thr17-PLN increasing SERCA2a activity. Interestingly, the experiments performed in AC3-I/AC3-C transgenic mice also revealed that CaMKII, although critically involved in the inotropic/lusitropic response to IGF-1 and swim training, is not required for the development of physiological cardiac hypertrophy.

In this work, we specifically investigated the role played by the NO-CaMKII axis in the inotropic response to IGF-1, the major mediator of the cardiac adaptation to exercise training. NO is a well-established regulator of cardiac contractility. Both NOS1 and NOS3 -the endothelial isoform- are constitutively expressed in cardiomyocytes; however, while NOS1 is localized to the SR, NOS3 is localized to the caveolae, mediating independent, and in some cases opposite, effects on cardiac function [37]. It is known that NOS1 and CaMKII are spatially coupled in the SR, with a facilitative effect on contractility [37,38]. Interestingly, NO has been recently demonstrated to be responsible for a novel activation scheme for CaMKII in cardiomyocytes, being the increase in NO the consequence of AKT-dependent NOS1 stimulation [27,28,39]. In this pathway, activation of CaMKII occurs likely by nitrosylation of residues within the regulatory domain of the kinase, thus allowing for its increased activity [40]. The increase in NO bioavailability would be the result not only of an AKT-dependent phosphorylation of NOS1

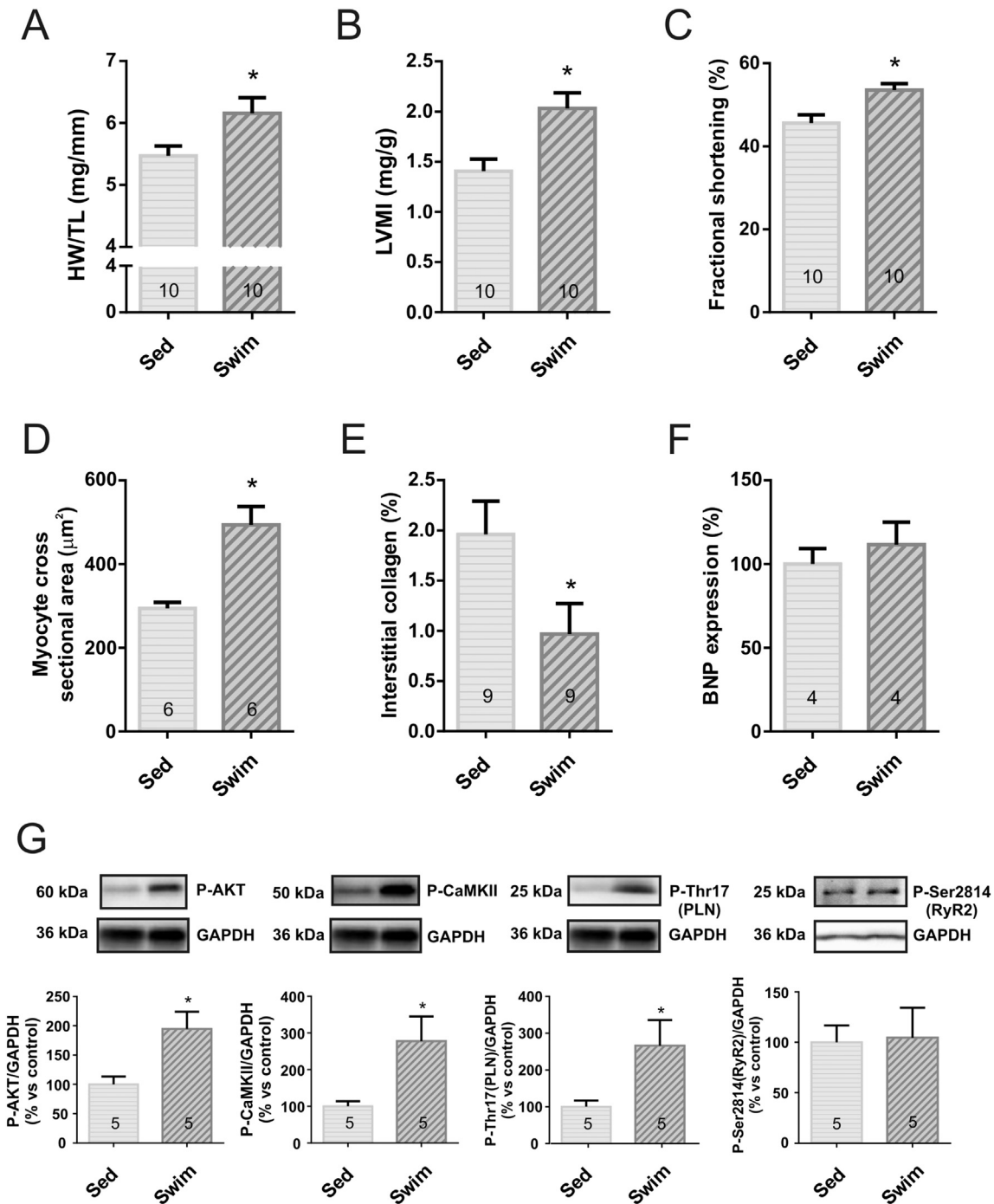


Fig. 6. Swim training induced physiological cardiac hypertrophy and contractile adaptation in mice. Chronic swimming (6 weeks) induced cardiac hypertrophy in mice as revealed by a significant increase in heart weight/tibia length ratio (HW/TL; A) and the left ventricular mass index (LVMI) calculated by echocardiography (B). The echocardiographic studies also revealed a significant increase in fractional shortening in trained mice (C). In the histological analysis we detected an increase in the cross sectional area of cardiomyocytes (D). The physiological nature of this increase in cardiac mass was confirmed by the absence of fibrosis (E) and no change in the myocardial expression of BNP, a molecular marker of pathological cardiac hypertrophy (F). G. Representative Western blots and average results showing the effect of exercise training on the phosphorylation of AKT, CaMKII, Thr17-PLN and Ser2814-RyR2. The number of experiments (n) in each group is depicted inside the bars. * means $p < 0.05$, Wilcoxon rank-sum test.

(present results) but also due to a greater expression induced by chronic training [17]. Regarding cardiomyocyte NOS3, it has been recently demonstrated that it is not stimulated by exercise training, and thus, does not contribute to cardioprotection [41]. Our results exploring the effect of IGF-1 on cardiomyocyte cell shortening in the presence and absence of NOS1 inhibition, as well as those revealing the stimulatory effect of IGF-1 upon NO production and the prevention of IGF-1 induced CaMKII activation by the NOS1 inhibitor NG, support that this pathway is critically involved in the positive inotropic response to IGF-

1. Hence, NO-induced CaMKII activation would be responsible for the increase in contractility detected in the presence of IGF-1. Interestingly, these results are in agreement with the recent demonstration of Erickson et al. [40] that NO-induced S-nitrosylation of the Cys-290 site results in persistent increase in autonomous CaMKII activity. Exploring the effect of IGF-1 upon cardiac contractility in isolated cardiomyocytes simplified the interpretation of the results by avoiding the consequences of IGF-1 on other tissues, such as the reduction in afterload due to the endothelium-dependent vasodilation.

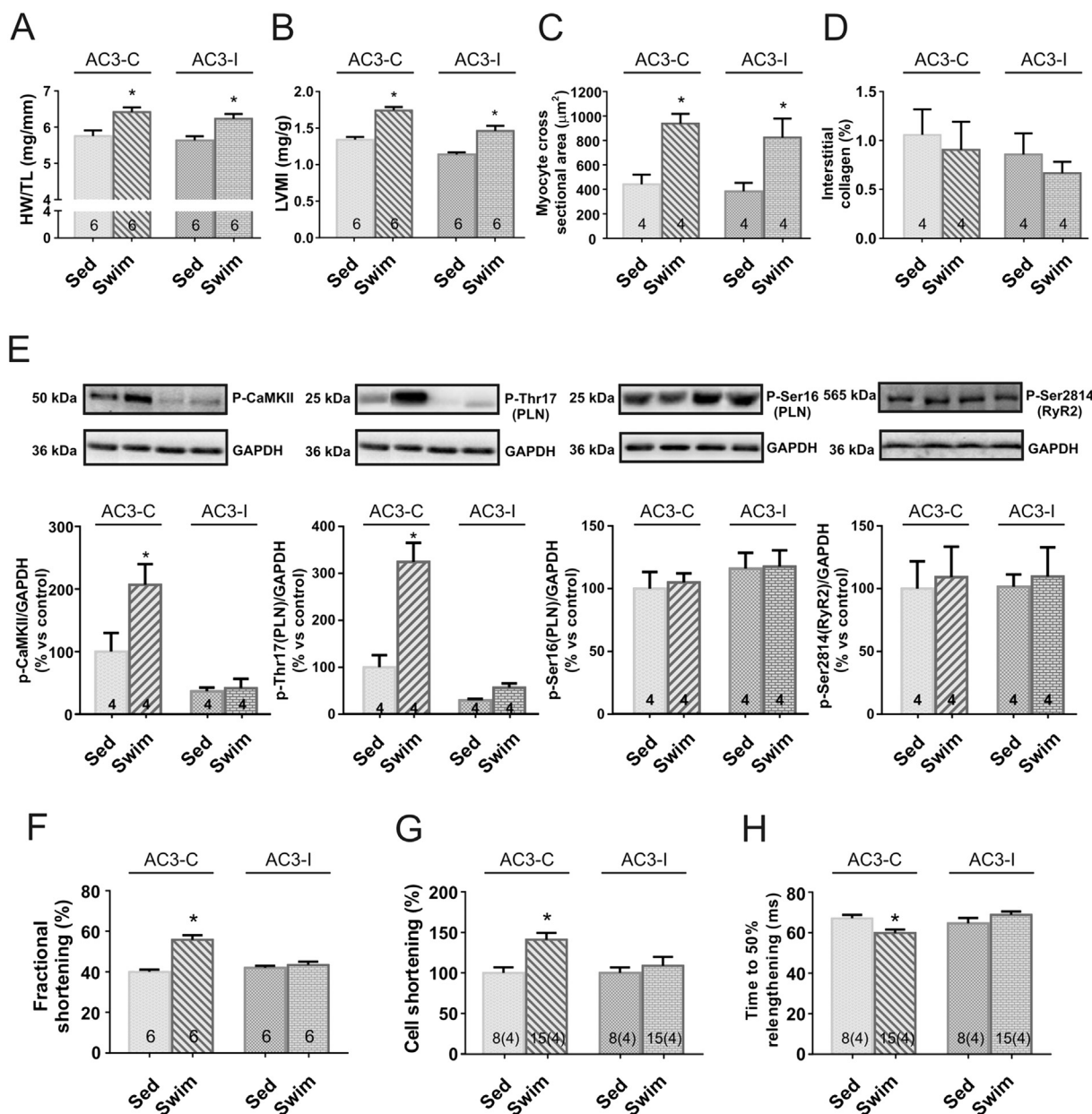


Fig. 7. Role of CaMKII in the cardiac adaptive response to swim training. Transgenic mice that express a CaMKII inhibitory peptide (AC3-I strain) developed physiological cardiac hypertrophy when subjected to swim training similarly to their corresponding controls, AC3-C mice (expressing a scrambled control peptide), as indicated by the increase in HW/TL (A), LVMI (B) and cross sectional area of cardiomyocytes (C) without an increase in interstitial collagen (D). Representative Western blots and average results showing the effect of swim training on the phosphorylation of CaMKII; Thr17-PLN, Ser16-PLN, and Ser2814-RyR2 (E). Exclusively in AC3-C mice chronic training induced a positive inotropic and lusitropic effect evidenced by the increase in fractional shortening (F) and cell shortening (G), and the reduction in the time to 50% relengthening (H). The contractile adaptation to training was absent in mice with cardiac specific inhibition of CaMKII (AC3-I mice). The number of cells assayed in each group is depicted inside the bars while the number of hearts from where they came from is specified between parentheses. * Indicates $p < 0.05$ vs. sedentary mice for each strain, Wilcoxon rank-sum test.

The fact that CaMKII activation was mandatory for the contractile response to IGF-1, as demonstrated by the absence of the positive inotropic effect not only in the presence of the specific inhibitor of CaMKII, KN-93, but also in AC3-I transgenic mice; in combination with normal development of physiological cardiac hypertrophy induced by the swim routine in AC3-I mice, unmasked a key point of divergence in the signaling pathway that leads to either cardiac hypertrophy or to the contractile adaptation to exercise training. CaMKII activation while being critical for the inotropic response would not be required for the increase in cardiac mass in response to exercise training/IGF-1 release. PLN, a constitutive regulator of SERCA2a function, is a well-known substrate for CaMKII. Once CaMKII is activated in the SR microdomain, in this case by the NOS1-dependent increase in NO, it phosphorylates

the residue Thr17 of PLN allowing a higher activity of SERCA2a and therefore improving Ca^{2+} handling and contractility. Our results showing that IGF-1 increases Ca^{2+} transient amplitude and SR Ca^{2+} content -as revealed by the greater amplitude of the caffeine-induced Ca^{2+} transient in the presence of IGF-1- support this hypothesis and are in agreement with those of Kemi et al. [19]. These authors described an increase in CaMKII and Thr17-PLN phosphorylation together with enhanced myocardial contractility in mice subjected to endurance training during 6 weeks. The relationship between NOS1 and PLN phosphorylation is also supported by the slower rate of intracellular Ca^{2+} decline and relengthening in NOS1 knockout mice (NOS1^{-/-}) [42–44]. Interestingly, the work of Roof et al. [17,18] also support NOS1-dependent CaMKII activation during exercise training. However,

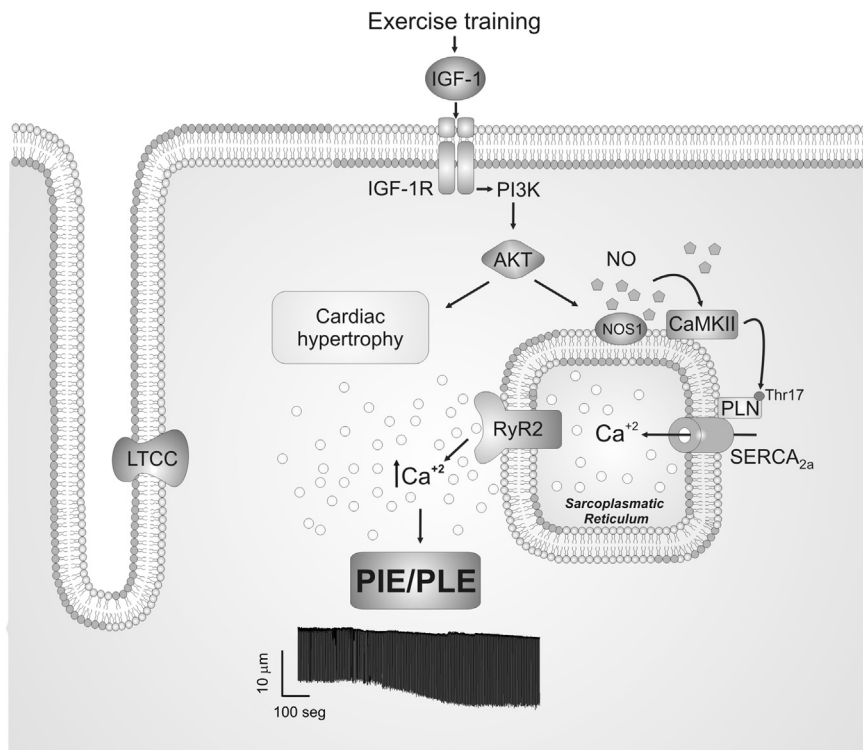


Fig. 8. Schematic summary of the molecular events proposed to underlie the adaptive cardiac contractile response to exercise training/IGF-1 release. Briefly, exercise training, through the release of IGF-1, induces the phosphorylation of AKT which in turn activates NOS1 increasing myocardial NO production. This increase in NO would be responsible for CaMKII activation that phosphorylates residue Thr17-PLN increasing SERCA2a activity and SR Ca²⁺ content, being the functional consequences a positive inotropic and lusitropic effect. LTCC: L-type Ca²⁺ channel; RyR2: ryanodine receptor 2; PIE: positive inotropic effect; PLE: positive lusitropic effect.

these authors attributed the improvement in cardiomyocyte Ca²⁺ handling to an increase in Ser16-PLN phosphorylation with no detectable changes in Thr17-PLN phosphorylation. Indeed, they ruled out an enhancement in PKA activity, the kinase responsible for Ser16-PLN phosphorylation, and proposed a CaMKII-dependent inhibition of phosphatases as the mechanism underlying the positive inotropic effect [17,18]. We do not have an explanation for this discrepancy at present.

PLN is not the only target of CaMKII that can be involved in the positive inotropic effect of IGF-1. Among the other possibilities, an increase in Ca²⁺ current (I_{CaL}) through CaMKII-dependent phosphorylation of the L-type Ca²⁺ channel, may also enhance Ca²⁺ transients and contractility. Regarding this mechanism, Sun et al. [45] demonstrated that IGF-1 enhances cardiac L-type Ca²⁺ current through a PI3K/AKT dependent mechanism. Moreover, in the setting of chronic exercise training it was recently proposed that AKT directly controls L-type Ca²⁺ channel complex protein density and I_{CaL} by preventing degradation of the pore-forming subunit of the L-type Ca²⁺ channel complex [46]. Our results showing that pharmacological or genetic inhibition of CaMKII are able to completely abolish the IGF-1-induced positive inotropic effect suggest that if L-type Ca²⁺ current contributes to the acute IGF-1-induced positive inotropic effect it is through a CaMKII-dependent mechanism. Whether direct, AKT-dependent enhancement of I_{CaL} coexists with Thr17 of PLN in the chronic response to exercise training warrants further investigation.

Interestingly, the NOS-CaMKII pathway described herein has been shown to contribute significantly not only to the inotropic effect but also to the arrhythmogenic potential of β -AR stimulation. The latter seems to be the consequence of CaMKII-dependent phosphorylation of RyR2 favoring SR Ca²⁺ leak and spontaneous Ca²⁺ waves [27,28]. Since the cardiac physiologic adaptation to moderate intensity exercise training does not involve a greater susceptibility to arrhythmias [47–51] and we did not detect an increase in the phosphorylation of Ser2814-RyR2 either in IGF-1 stimulated hearts or trained mice, it is tempting to speculate that beneficial or detrimental consequence on Ca²⁺ handling could depend on spatial/temporal compartmentalization of NO-dependent activation of CaMKII. Thus, under physiologic conditions, i.e. exercise training; CaMKII nitrosylation improves

SERCA2a activity through the phosphorylation of Thr17-PLN. However, under pathologic circumstances, i.e. pressure overload, CaMKII would also phosphorylate RyR2 inducing SR Ca²⁺ leak and providing an arrhythmogenic substrate.

In summary, based on the presented data we propose that CaMKII plays an essential role in the adaptive improvement in contractility associated to exercise training, but not in the expected physiological increase in cardiac mass. Exercise training, through the release of IGF-1, would induce the sequential activation of IGF-1 receptor, AKT and NOS1. The increase in myocardial NO would activate CaMKII leading to the phosphorylation of Thr17-PLN increasing SERCA2a activity and therefore contractility. Fig. 8 schematically summarizes this proposal.

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Disclosures

None to declare.

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