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Interval training normalizes cardiomyocyte function, diastolic Ca^{2+} control and SR Ca^{2+} release synchronicity in a mouse model of diabetic cardiomyopathy

Short title: Exercise restore cardiac function in diabetic mice

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Abstract

In the present study we explored the mechanisms behind excitation-contraction (EC)-coupling defects in cardiomyocytes from mice with type-2 diabetes (db/db), and determined whether 13-weeks of aerobic interval training could restore cardiomyocyte Ca^{2+} cycling and EC-coupling. Reduced contractility in cardiomyocytes isolated from sedentary db/db was associated with increased diastolic sarcoplasmic reticulum (SR)-Ca^{2+} leak, reduced synchrony of Ca^{2+} release, reduced transverse (T)-tubule density, and lower peak systolic and diastolic Ca^{2+} and caffeine-induced Ca^{2+} release. Additionally, the rate of SR Ca^{2+} ATPase (SERCA2a)-mediated Ca^{2+} uptake during diastole was reduced, whereas a faster recovery from caffeine-induced Ca^{2+} release indicated increased Na^{+}/Ca^{2+}-exchanger (NCX) activity. The increased SR-Ca^{2+} leak was attributed to increased Ca^{2+}-calmodulin-dependent protein kinase (CaMKIIδ) phosphorylation, supported by the normalization of SR-Ca^{2+} leak upon inhibition of CaMKIIδ (AIP). Exercise training restored contractile function associated with restored SR Ca^{2+} release synchronicity, T-tubule density, twitch Ca^{2+} amplitude, SERCA2a and NCX activities, and SR-Ca^{2+} leak. The latter was associated with reduced phosphorylation of cytosolic CaMKIIδ. Despite normal contractile function and Ca^{2+} handling after the training period, phospholamban was hyperphosphorylated at Serine-16. Protein kinase A (PKA) inhibition (H-89) in cardiomyocytes from the exercised db/db group abolished the differences in SR-Ca^{2+} load when compared with the sedentary db/db mice. EC-coupling changes were observed without changes in serum insulin or glucose levels, suggesting that the exercise training-induced effects are not via normalization of the diabetic condition. These data demonstrate that aerobic interval training almost completely restored the contractile function of the diabetic cardiomyocyte to levels close to sedentary wild type (WT).

Keywords:

Diabetes mellitus, exercise training, Ca^{2+}-calmodulin-dependent protein kinase, ryanodine receptor and calcium handling

Non-standard Abbreviations and Acronyms:

excitation-contraction (EC), Ca^{2+}-calmodulin-dependent protein kinase (CaMK), sarcoplasmic reticulum (SR), transverse tubule (T-tubule), sarcoplasmic reticulum Ca^{2+} ATPase (SERCA), Na^{+}/Ca^{2+}-exchanger (NCX), Protein kinase A (PKA), wild type (WT), delayed afterdepolarisations (DADs), ryanodine receptor (RyR), maximal oxygen uptake (VO_{2max}), real-time polymerase chain reaction (RT-PCR), fatty acid (FA), Peroxisome proliferator activated receptor γ co-activator 1α (PGC-1α) and phospholamban (PLN)

Introduction

Diabetes mellitus (type 2 diabetes) is estimated to reach pandemic levels within the next two decades.[1] This has severe implications, because cardiovascular mortality is ~2-4 fold higher in diabetic, compared to non-diabetic patients,(e.g.[2]) and accounts for ~80% of the mortality in type 2 diabetes,[3] of which ~50% die of sudden cardiac death.[4] Furthermore, diabetics are 2.5 times more likely to develop congestive heart failure compared to non-diabetics.[5]

The db/db diabetic mouse model develops cardiomyopathy in a similar manner as type 2 diabetes in humans,[6] and presents with reduced whole-heart[7] and isolated cardiomyocyte[8] excitation contraction (EC) coupling function. This can partly be explained by a reduced L-type Ca^{2+} channel activity and increased Na^{2+}/Ca^{2+} exchanger (NCX) and depressed Ca^{2+} handling by the sarcoplasmic reticulum (SR).[8, 9] Furthermore, increased SR Ca^{2+} leak in db/db mice[8] can further reduce SR Ca^{2+} content. Recently, arrhythmias have been linked to increased diastolic Ca^{2+} leak via the SR release channels, the ryanodine receptor 2 (RyR2), causing delayed afterdepolarisations (DADs).[10] In failing hearts, phosphorylation of RyR2 by the Ca^{2+}-calmodulin-dependent protein kinase IIδ (CaMKIIδ) and/or the protein kinase A (PKA) is thought to sensitize RyR2 to Ca^{2+} and thus increase its open probability.[11, 12] In contrast, exercise training in healthy mice increases the level of phosphorylated cytosolic CaMKIIδ and in so doing increases CaMKIIδ activity. Under these circumstances, increased phosphorylation of CaMKIIδ was associated with an increased cardiac performance.[13]

Alongside increased SR Ca^{2+} leak, reduced transverse (T)-tubule structure leading to less synchronous SR Ca^{2+} release contributes further to the depressed EC coupling in models of cardiac dysfunction.[14] The mechanism for increased SR Ca^{2+} leak, and whether T-tubule structure in diabetic cardiomyopathy is conserved, has currently not been studied

In the present study, we explored the mechanisms behind the impaired cardiomyocyte function and increased SR Ca^{2+} leak in db/db cardiomyocytes, and then re-examined the same parameters in the db/db mice after an aerobic interval exercise training program. Since the activities of both CaMKIIδ and PKA are associated with both pathological and physiological remodeling, we also investigated the contributions of CaMKIIδ and PKA for the observed exercise training-induced changes.

Materials and Methods

For a detailed description, see online supplement.

Mouse model of diabetes and exercise training

The db/db mice model has been proven to be a suitable model to study the consequences of diabetes on the heart. Here we studied the male diabetic (BKS.Cg-m +/+ Lepdb/Bom Tac) (20 exercised and 20 sedentary mice) and sedentary (n=23) and exercise trained (n=6) non-diabetic healthy heterozygote (BKS.Cg-m +/+ Lepdb/+ lean); all age-matched (7 weeks at study start). To determine maximal oxygen uptake (VO_{2max}), mice ran until exhaustion on a customized treadmill in a metabolic chamber, and high intensity aerobic interval training was performed as uphill running, alternating between 4 min at 85%-90% of VO_{2max} and 2 min at 50% of VO_{2max} for 80 min/day, 5 days/week, for 13 weeks.[15] We and others have previously demonstrated the efficacy and relevance of this exercise regime by both clinical trials and experimental studies (e.g.[16]).

Cardiomyocyte isolation and Ca^{2+} measurements

Left ventricular myocytes were isolated as previously described.[15] The Norwegian council for Animal Research approved the study, which was in accordance with Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1996). Fura-2/AM-loaded cardiomyocytes were stimulated by bipolar electrical pulses for Ca^{2+} handling measurements including SR Ca^{2+} leak. CaMKII inhibitor and PKA inhibitor were used to determine the influence of the two kinases. Contractility was recorded by video-based sarcomere spacing.

Confocal imaging of Ca^{2+} waves, Ca^{2+} release synchrony and T-tubules

Cardiomyocytes loaded with Fluo-3/AM were used to count Ca^{2+} waves and determine Ca^{2+} release synchrony. Quiescent, non-perfused cardiomyocytes loaded with the membrane specific Di-8-ANEPPS were confocal Z-stack scanned. The relative density of T-tubules normalized to cell size was obtained from 5 images/cell captured from the middle of each cell

Western blot analyses and real-time quantitative RT-PCR

Western Blot and real-time quantitative RT-PCR analysis were performed using standardized protocols and normalized to housekeeping proteins and genes. For a detailed description see online supplement.

Statistics

Data are shown as mean±SD. One-way ANOVA with Bonferroni post-hoc test adjusted for multiple comparisons was used to identify the statistical differences between the groups and Mann-Whitney U was used when appropriate. $P<0.05$ was considered statistically significant.

Results

Aerobic capacity and echocardiography
Exercise training improved aerobic capacity, and exercised db/db mice had a 13% higher VO_{2max} than sedentary db/db and WT mice (Figure 1A). Improved aerobic fitness was also reflected by increased maximal running speed to a level above that of sedentary mice (0.23 vs. 0.12 $m \cdot s^{-1}$, respectively; $P<0.001$). Exercise training did not significantly change the body weight (Figure 1B). Left ventricular dysfunction in sedentary db/db mice was confirmed by reduced fractional shortening and stroke volume by high-resolution echocardiography, whereas endurance training improved both parameters to wild-type levels (Figure 1C & D).

Exercise training in WT animals
To compare the functional response to exercise training in diabetic mice to normal mice, we also exercise trained WT mice. Overall, results from exercised WT were superior to those observed in sedentary and exercised db/db and sedentary WT mice (Figures 1-6 and Table 1).

Free FAs, triglycerides, insulin, and blood glucose
Sedentary db/db mice had higher plasma levels of free FAs (648±90 vs. 263±70 mM, $P<0.05$) and similar triglycerides (0.74±0.06 vs. 0.68±0.07 mM, NS) when compared to sedentary WT mice. Exercise training reduced free FAs by ~16% (to 544±66 mM, $P<0.05$) and triglycerides by ~23% (to 0.56±0.05 mM, $P<0.05$). Plasma glucose was higher in db/db mice (20.1±0.9 vs. 10.3±0.8 mM, $P<0.01$) than in sedentary WT, and exercise training did not lower it significantly (17.0±1.2 mM, $P=0.19$). As expected, insulin was substantially higher in sedentary db/db mice (6.65±0.92 µg/L) vs. sedentary WT (0.98±0.01 µg/L), with no change observed after the training period (6.89±1.22 µg/L).

Cardiac PGC-1α
Cardiac mRNA levels of PGC-1α is a critical factor associated with the activation of metabolic genes required for substrate utilization and mitochondrial biogenesis. These levels were similar in sedentary db/db and sedentary WT (0.97±0.11 arbitrary units) and exercise training did not change PGC-1α (0.89±0.12).

Fractional shortening and Ca^{2+} cycling
Fractional shortening was impaired in sedentary db/db mice, but recovered to a level comparable to sedentary WT mice after the exercise program (Figure 2A, C). In line with this, we observed lower twitch Ca^{2+} release in sedentary, but not in exercised db/db mice compared to sedentary WT mice (Figure 2B, D). Sedentary db/db mice had lower diastolic and systolic Ca^{2+} levels, as well as reduced amplitude of the Ca^{2+} transient, compared to sedentary WT. After exercise training, no differences were observed between db/db and sedentary WT mice (Figure 2B, D). Lower SR Ca^{2+} load in sedentary db/db mice was confirmed by lower caffeine-induced Ca^{2+} release (Table 1). Exercise training increased SR Ca^{2+} load, demonstrated by increased caffeine-induced Ca^{2+} release; however, it did not reach sedentary WT levels (Table 1).

Diastolic function, measured as time to 50% re-lengthening, was impaired in sedentary db/db mice compared to sedentary WT mice, but was restored after exercise training (Figure 2E). The same pattern of change was observed for time to 50% Ca^{2+} transient decay (Figure 2F). SERCA2a is the main contributor to removal of cytosolic Ca^{2+} during diastole, and also indirectly affects NCX by virtue of controlling $[Ca^{2+}]_i$. Rate constants Ca^{2+} decay, indicated that sedentary db/db mice had a ~34% suppression of SERCA2a function and 59% increased NCX function compared to sedentary WT mice. Exercise training restored the functions of SERCA2a and NCX to levels comparable to sedentary WT mice (Figure 3B-D). This suggests that exercise training normalizes diastolic function in db/db mice by shifting the control of diastolic Ca^{2+} to SERCA2a and thus increasing the rate of Ca^{2+} removal. This also increased the SR Ca^{2+} load, which may have also contributed to increased systolic function.

SR Ca^{2+} leak

Measuring $[Ca^{2+}]_i$ in quiescent cardiomyocytes over a prolonged period of time (1 minute) with and without tetracaine provides a quantitative assessment of SR (RyR2) Ca^{2+} leak (Figure 4A). After normalizing for differences in SR Ca^{2+} content, we observed an increased SR Ca^{2+} leak in sedentary db/db mice compared to sedentary WT mice, whereas exercise training normalized SR Ca^{2+} leak in db/db mice to levels comparable to sedentary WT mice. 13% of the total SR Ca^{2+} content leaked during this period in sedentary db/db mice, versus 3-4% in both exercised db/db and sedentary WT mice (Figure 4B). This was also true when expressed as absolute Ca^{2+} leak (not normalized for differences in SR Ca^{2+} content, data not shown). In line with this, the frequency of non-stimulated Ca^{2+} waves was higher in sedentary db/db mice compared to exercised db/db and sedentary WT mice (Figure 5A, B). To further elucidate the mechanism behind increased SR Ca^{2+} leak, we inhibited CaMKIIδ with AIP and PKA with H-89. SR Ca^{2+} leak was unaffected by PKA inhibition, while CaMKIIδ inhibition reduced Ca^{2+} leak in sedentary db/db mice to levels comparable to exercised db/db and sedentary WT mice (Figure 4B, C).

Reduced synchrony of Ca^{2+} release, T-tubules and cardiomyocyte size

To further examine how diabetes may affect Ca^{2+} handling and contractility, we measured the synchrony of Ca^{2+} release during twitch stimulations. Compared to sedentary WT mice, Ca^{2+} release along the cardiomyocyte length was less synchronous in sedentary db/db mice, but exercise training reversed this to levels comparable to sedentary WT mice (Figure 6A, C). Synchrony of Ca^{2+} release is closely linked to the density and organization of T-tubules in the cardiomyocyte.[14] In line with this, we observed a reduced T-tubule density in sedentary db/db mice compared to sedentary WT mice, whereas exercise training restored the density (Figure 6B, D). Cardiomyocytes from sedentary db/db mice had an approximately 63% larger volume than cardiomyocytes from sedentary WT mice (P<0.01). Exercise training reduced cell volume to a level comparable to sedentary WT (Figure 6E).

Protein expression and phosphorylation status

SERCA2a expression was reduced in sedentary db/db mice compared to sedentary WT mice, but exercise training normalized this (Figure 7A). Total PLN expression levels did not differ between groups (Figure 7B). Phosphorylation of PLN at the CaMKIIδ site (Threonine-17) was increased in sedentary db/db mice compared to sedentary WT mice (Figure 7C), whilst PLN phosphorylation at the PKA site (Serine-16) was similar between sedentary db/db and sedentary WT mice (Figure 7D). Exercise training strongly increased PLN phosphorylation at Serine-16 (compared to sedentary db/db and sedentary WT mice), but reduced PLN phosphorylation at Threonine-17 (Figure 7C, D). Thus, exercise training normalized the phosphorylation status of PLN Threonine-17 but not Serine-16. Finally, expression levels of CaMKIIδ did not differ between the groups (Figure 7E), but phosphorylation of CaMKIIδ was strongly increased in sedentary db/db mice, whereas exercise training reduced this to sedentary WT levels (Figure 7F). Expression levels of RyR (1.17±0.06, 1.30±0.05 and 1.20±0.05 in sedentary db/db, exercised db/db and sedentary WT, respectively) and phosphorylation of RyR at the PKA and CaMKIIδ site Serine-2808 (Figure 7G) were similar between groups, whereas phosphorylation at the CaMKIIδ-specific site Serine-2814 was higher in sedentary and normalized in trained db/db, when compared to WT controls (Figure 7H).

Effects of CaMKII and PKA inhibitors

Overall, inhibition of PKA (with H89) had more profound effects than inhibition of CaMKIIδ (with AIP). Across all the groups, PKA inhibition induced the greatest reductions in fractional shortening, diastolic function, twitch Ca^{2+} amplitude and decay, and SR Ca^{2+} content (Table 1). Interestingly, inhibition of PKA in exercise trained db/db mice resulted in fractional shortening, diastolic function, twitch Ca^{2+} amplitude and decay, and SR Ca^{2+} content being similar to sedentary db/db (group differences ns, Table 1). Fractional Ca^{2+} release was enhanced in sedentary db/db mice compared to sedentary WT mice, but exercise training normalized this (P<0.05, Table 1). Adding AIP to the db/db sedentary cardiomyocytes reduced fractional Ca^{2+} release to levels comparable to exercised db/db and sedentary WT (Table 1).

Discussion

In the present study, we investigated subcellular mechanisms of dysfunction in diabetes-induced cardiomyopathic hearts, and the potential of regular exercise training for correcting the dysfunction. Dysfunction and cardiomyopathy in the present diabetes model was evidenced by reduced fractional shortening and stroke volume in-vivo, reduced cell contractility and Ca^{2+} handling, and induction of pathological hypertrophy, in concordance with previous findings.[8, 9] Moreover, two novel mechanisms of dysfunction in the diabetic cardiomyocyte were identified in the present study, (1) asynchronous EC-coupling that was associated with reduced density of T-tubules and (2) increase of diastolic SR Ca^{2+} leak that was associated with increased phosphorylation of cytosolic CaMKIIδ. Both are linked to reduced contractility and Ca^{2+} handling. High-intensity exercise training restored synchrony of EC-coupling and T-tubule density and reduced SR Ca^{2+} leak to levels comparable to sedentary WT mice. These effects also coincided with exercise-induced restoration of contractility and Ca^{2+} handling to normal levels. In contrast, exercise training did not alter serum glucose and insulin concentrations, in line with a previous study[17]. This suggests that normalized insulin and glucose transport cannot account for the restoration of the contractile function. Instead, other factors intrinsic to the myocardium explain the exercise training-induced effects. Previous studies suggest that aspects of the diabetic phenotype may be due to the disruption of a number of intracellular pathways, including those linked to glucose and insulin signaling. One possible candidate, namely mitochondrial biogenesis (PGC-1α), was ruled out by the current study. Furthermore, previous studies have shown that normalization of glucose and palmitate oxidation by high glucose and insulin treatment did not normalize cardiac function.[18] Other possible pathways including endogenous reactive oxygen species scavengers;[19] advanced glycation end products;[20] O-linked N-acetylglucosamine;[21] and Akt signaling;[22] have been suggested, clearly further studies are required to uncover the key pathways altered by exercise.

Increased diastolic SR Ca^{2+} leak and spontaneous waves in diabetes

Increased SR Ca^{2+} leak and increased frequency of spontaneous Ca^{2+} waves during diastole in quiescent cardiomyocytes were observed. These results are in line with previous reports of reduced RyR2 stability and subsequently increased SR Ca^{2+} leak in this model of diabetes,[8] as well as being consistent with increased RyR2 activity increasing the frequency of Ca^{2+} waves.[23] The increased diastolic SR Ca^{2+} leak appeared to be caused by increased CaMKIIδ activity, and not PKA activity. This observation was supported by differences in phosphorylation status of RyR at Serine-2814 (CaMKIIδ phosphorylation site on RyR), but similar phosphorylation at Serine-2808 (PKA and CaMKIIδ). Despite the increased diastolic Ca^{2+} leak, the diabetic cardiomyocytes had lower diastolic concentrations of Ca^{2+} compared to wild-type controls. This can be explained by the increased activity of NCX reported in this study, though the decreased SERCA2a function may result in diastolic Ca^{2+} not being reduced at higher stimulation frequencies (>3 Hz), because reduced SERCA2a tends to raise end-diastolic Ca^{2+}.

Asynchrony of intracellular Ca^{2+} release and reduced T-tubule density in diabetes

Cardiomyocytes isolated from sedentary db/db mice had a reduced synchrony of twitch-stimulated intracellular Ca^{2+} release. This was associated with a reduced density of the T-tubule network in the cell. Reduced synchrony of Ca^{2+} release and EC coupling has been observed regularly after reduced T-tubule density.[14] Thus, these reports suggest that the reduced T-tubule density contributes to reduce the synchrony of Ca^{2+} release and EC coupling. Further support for this hypothesis comes from the observation that the response time from stimulation to Ca^{2+} release is longer in cardiomyocytes from sedentary db/db mice compared to sedentary WT. The reduced T-tubule density suggests a disrupted spacing between L-type Ca^{2+} channels and RyR2 such that EC coupling becomes inefficient. To our knowledge, the current exercise training program is the first recorded intervention to restore T-tubule density and restore the synchrony of Ca^{2+} release and EC coupling. Whether restoration of T-tubule density after exercise training in db/db mice is a function of reduced cell size or increased amount of T-tubules *per se* is presently not fully understood. T-tubule density remained unchanged when cell size increased in exercise trained WT mice. This indicates that cell size and T-tubule density can vary independently.

Reduced cardiomyocyte inotropy in diabetes

The reduced inotropy in diabetic cardiomyocytes, observed as reduced amplitudes and decay rates of the fractional shortening and the Ca^{2+} transient, is most likely explained by several factors. The primary cause is reduced SR Ca^{2+} content as this reduces the amplitude of the Ca^{2+} transient.[23] The reduced SR Ca^{2+} loading may be caused by the increased NCX activity that competes with SERCA2a to remove intracellular Ca^{2+} during diastole. In addition, Ca^{2+} uptake rate via SERCA2a is also reduced in this model; the combination of the two would favor Ca^{2+} efflux via NCX rather than re-uptake into the SR. The increased diastolic SR Ca^{2+} leak is an additional factor contributing to a reduction in SR Ca^{2+} loading. Reduced synchrony of twitch-stimulated Ca^{2+} release would also impair EC coupling and reduce the inotropy. This is associated with, and may be caused by, reduced T-tubule density observed in this model. The relative contributions of increased NCX, increased SR Ca^{2+} leak, reduced SERCA2a Ca^{2+} uptake and reduced T-tubule density cannot easily be assessed, but all tend to reduce Ca^{2+} release during EC coupling and thereby impair cellular contraction.

The potential of exercise training to reverse mechanical cellular dysfunction

Here, we show for the first time that exercise training normalizes or reduces the dysfunction of both systolic and diastolic cellular parameters, such as T-tubule density, synchrony of SR Ca^{2+} release, diastolic SR Ca^{2+} leak, and the frequency of spontaneous Ca^{2+} waves, in cardiomyocytes from diabetic cardiomyopathy hearts. Also, SERCA2a uptake and NCX activity were all returned to normal levels. Finally, exercise training also induced reverse remodeling of diabetic cardiomyocytes, evidenced by reduced cellular dimensions as well as improved fractional shortening and stroke volume *in-vivo* in the exercise-trained group.

The observations in this study are consistent with exercise training improving cardiomyocyte Ca^{2+} handling and contractility in post-myocardial infarction heart failure,[24] as well as exercise training improving whole-heart function in diabetes.[25] Thus, despite the pathological remodeling and contractile dysfunction, cardiomyocytes maintain the ability to respond to exercise training.[13, 15]

Involvement of CaMKII and PKA

As discussed above, the cellular studies suggest that the increased SR Ca^{2+} leak in diabetic cardiomyocytes was caused by the increased CaMKIIδ activity. This is in line with the observation that CaMKIIδ was constitutively hyperphosphorylated in these cardiomyocytes. The reason for the hyperphosphorylation is unknown, but it appears despite reduced diastolic Ca^{2+} levels. Exercise training was able to fully reverse the SR Ca^{2+} leak and the constitutive hyperphosphorylation of CaMKIIδ. This suggests that exercise training-induced dephosphorylation of CaMKIIδ abolished the abnormally high SR Ca^{2+} leak in the diabetic model. This is consistent with the data from comparable heart failure models that link increased CaMKIIδ with higher SR Ca^{2+} leak, and that show inhibition of CaMKIIδ, but not PKA, abolishes the leak.[11, 26] db/db mice are more prone to arrhythmias[27] but the underlying mechanisms are unknown. Increased SR Ca^{2+} leak and increased NCX activity have been suggested as changes that would promote arrhythmias in whole hearts;[10] exercise training could therefore be an effective treatment for arrhythmias in diabetes.

In contrast to regulation of SR Ca^{2+} leak, the inotropy state of diabetic cardiomyocytes was sensitive to inhibition of both PKA and CaMKIIδ. This is also in contrast to previous studies of other forms of heart disease[11, 26] or exercise training in healthy normal mice,[13] which have suggested CaMKIIδ and not PKA as the main kinase that chronically mediates inotropy. The reason for this paradox is unknown, but it suggests that exercise training may correct diabetes-induced cardiac cell abnormalities by both CaMKIIδ- and PKA-mediated effects. However, PLN was chronically hyperphosphorylated at the PKA-targeted Serine-16 residue in the diabetic model, suggesting that the myocardium may have a limited response to β-adrenergic stimulation.

Conclusions

A program of exercise training reversed the contractile abnormalities associated with diabetic cardiomyopathy and restored the cardiomyocyte Ca^{2+} handling and contractility to levels comparable to sedentary WT, mainly by CaMKIIδ de-phosphorylation and compensatory PKA-dependent

phosphorylation. This was achieved without normalizing serum insulin or glucose levels. One explanation is that exercise training has stimulated a different trophic pathway from that activated by diabetes. The consequence of the change in expression of a range of proteins that alter the cellular phenotype and as a consequence CaMKIIδ activity is reduced. Future studies will examine the upstream signaling pathways responsible for exercise-induced changes to determine whether these pathways can be accessed as a therapeutic strategy.

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1. Effects of CaMKII and PKA inhibition upon contractile parameters.

	Diabetes		**Wild type**	
	SEDENTARY	**EXERCISE**	**SEDENTARY**	**EXERCISE**
CELL SHORTENING, %				
Vehicle situation	3.5 ± 1.6	7.8 ± 2.3*	8.4 ± 1.4*	12.2 ± 2.1$^{*\#}$
CaMKI-inhibition with AIP	2.8 ± 0.6	6.2 ± 1.3*	6.6 ± 1.2*	8.3 ± 1.4*
PKA-inhibition with H-89	2.0 ± 0.6	4.5 ± 1.0*	5.2 ± 1.3*	6.3 ± 1.6*
TIME TO 50% RELENGTHENING, ms				
Vehicle situation	219 ± 28	144 ± 32*	150 ± 22*	108 ± 30*
CaMKII-inhibition with AIP	249 ± 18	169 ± 27*	178 ± 16*	158 ± 16*
PKA-inhibition with H-89	279 ± 12	200 ± 20*	223 ±18*	203 ± 23*
CA^{2+} AMPLITUDE TWITCH, Fura-2 ratio				
Vehicle situation	0.07 ± 0.02	0.12 ± 0.03*	0.15 ± 0.02*	0.23 ± 0.02$^{*\#}$
CaMKII-inhibition with AIP	0.06 ± 0.02	0.09 ± 0.02*	0.13 ± 0.03*	0.19 ± 0.02$^{*\#}$
PKA-inhibition with H-89	0.04 ± 0.03	0.05 ± 0.02$^{*\#}$	0.08 ± 0.02*	0.16 ± 0.02$^{*\#}$
TIME TO 50% CA^{2+} DECAY, ms				
Vehicle situation	232 ± 14	177 ± 24*	157 ± 16*	123 ± 16$^{*\#}$
CaMKII-inhibition with AIP	248 ± 10	179 ± 18*	178 ± 11*	158 ± 11*
PKA-inhibition with H-89	260 ± 11	209 ± 12*	200 ± 15*	204 ± 17*
CA^{2+} AMPLITUDE CAFFEINE, Fura-2 ratio				
Vehicle situation	0.07 ± 0.03	0.14 ± 0.02$^{*\#}$	0.18 ± 0.02*	0.27 ± 0.02$^{*\#}$
CaMKII-inhibition with AIP	0.07 ± 0.02	0.11 ± 0.01$^{*\#}$	0.16 ± 0.02*	0.23 ± 0.02$^{*\#}$
PKA-inhibition with H-89	0.05 ± 0.02	0.06 ± 0.01$^{\#}$	0.10 ± 0.01*	0.13 ± 0.02*
FRACTIONAL RELEASE, Tw/caffeine				
Vehicle situation	0.95 ± 0.04	0.83 ± 0.03*	0.83 ± 0.04*	0.86 ± 0.04*
CaMKII-inhibition with AIP	0.86 ± 0.03	0.80 ± 0.02*	0.81 ± 0.03	0.84 ± 0.03
PKA-inhibition with H-89	0.88 ± 0.02	0.83 ± 0.02*	0.80 ± 0.03*	0.82 ± 0.04*

Data are mean ± SD. CaMKII, Ca2+/calmodulin-dependent kinase II; PKA, protein kinase A. *, different from diabetes sedentary, $P<0.01$. $^{\#}$, different from Wild type sedentary, $P<0.05$.

FIGURE LEGENDS:

Figure 1. A, Pre- and post tests of VO_{2max}. Data presented as means±SD. Exercise training improved VO_{2max} in the db/db and WT, *=P<0.01 exercise db/db and exercise WT vs. sedentary db/db and sedentary WT. **B**, Weight increased in a similar manner in both exercise db/db and sedentary db/db and was higher than sedentary WT and exercise WT throughout the intervention period . ***=P<0.03 different from pre (within groups), **§**=P<0.001 different from both db/db groups at pre and post. **C** and **D**, *In vivo* heart function measured by echocardiography revealed reduced fractional shortening (C) and stroke volume (D) in sedentary db/db mice, of which both increased to sedentary WT levels after exercise training.

Figure 2. A, Representative sample traces of cardiomyocyte fractional shortening from sedentary and exercised db/db and WT mice. **B**, Representative traces of Fura-2 ratio from sedentary and exercised db/db and WT mice. **C**, Fractional shortening was reduces in sedentary db/db mice, whereas exercise training restored normal fractional shortening. Exercised WT had higher fractional shortening than all other groups. **D**, Systolic and diastolic Ca^{2+} levels were lower in sedentary db/db mice, whereas exercise training increased both systolic and diastolic Ca^{2+} to WT levels. ***=P<0.01 different from exercised db/db, sedentary WT and exercised WT mice. $^{\#}$=P<0.01 different from all other groups. **E**, Time to 50% relenghtening in sedentary db/db mice was longer compared to sedentary WT, but exercised db/db mice were similar to sedentary WT. Exercised WT had a shorter time to 50% relenghtening than all other groups. **F**, Similarly, time to 50% Ca^{2+} decay was reduced in sedentary db/db mice, but this improved to sedentary WT levels after exercise training, whereas exercised WT mice had shorter time to 50% Ca^{2+} decay than all other groups.

Figure 3. A, Representative Ca^{2+} (Fura-2) decay after caffeine- and twitch-induced Ca^{2+} transients. **B**, Representative (normalized) Ca^{2+} transients and decays after sustained caffeine stimulation. This maneuver removes the SERCA2a component of cytosolic Ca^{2+} removal and indicates NCX function. **C**, Rate constant of Ca^{2+} removal after a normal twitch at 2 Hz indicating the global Ca^{2+} removal. Sedentary db/db mice had depressed global Ca^{2+} removal. Exercise training increased Ca^{2+} removal to sedentary WT levels. Exercised WT mice had a faster Ca^{2+} removal than all other groups. **D**, Sustained caffeine stimulation after a caffeine-induced transient indicating NCX function. Ca^{2+}ATPase functions were similar among all groups (data not shown). Sedentary db/db mice had increased NCX function. Exercise training reduced NCX Ca^{2+} removal to sedentary WT levels. Exercise in WT mice did not change NCX function.

Figure 4. A, Illustration of the SR Ca^{2+} leak protocol. **B**, SR Ca^{2+} leak normalized to SR Ca^{2+} content. Sedentary db/db mice had a larger leak compared to sedentary WT mice. Exercise training normalized SR Ca^{2+} leak to sedentary WT levels. Exercise in WT mice did not change SR Ca^{2+} leak. **C**, SR Ca^{2+} leak normalized to SR content in Sedentary db/db mice with PKA inhibitor (H-89) did not change Ca^{2+} leak whereas CaMKII inhibitor (AIP) reduced Ca^{2+} leak to levels indistinguishable from WT mice, as observed in panel B (P=ns).

Figure 5. A, Examples of non-stimulated Ca^{2+} waves measured by confocal microscopy in linescan mode in sedentary and exercised db/db mice. **B**, Ca^{2+} wave frequency was higher in sedentary db/db mice compared to WT levels. Exercise training reduced Ca^{2+} wave frequency to sedentary WT level. Exercise in WT mice did not change wave frequency.

Figure 6. A, Examples of linscan-recorded Ca^{2+} release along the longitudinal axis of the cardiomyocyte after twitch stimulations in sedentary and exercised db/db and sedentary and exercised WT mice. **B**, Examples of a z-stack scans with Di-8-ANEPPS visualized T-tubule structure in sedentary and exercised db/db and WT mice. **C**, We used the standard deviation (SD) of time to 50% Ca^{2+} release between different regions along the cell to determine synchrony of Ca^{2+} release. Sedentary db/db mice had significant higher SD of time to 50% Ca^{2+} release compared to sedentary WT mice, whereas exercise training reduced Ca^{2+} release variation to sedentary WT levels. Exercise in WT did not change Ca^{2+} release synchrony. **D**, T-tubule density normalized to cell size. Sedentary db/db mice had decreased T-tubule density compared to sedentary WT levels, whereas exercise training normalized T-tubule density to

sedentary WT levels. Exercise in WT did not change T-tubule density. **E**, Calculated left ventricular cardiomyocyte volume in sedentary db/db mice was larger than sedentary WT cardiomyocytes, while exercised db/db had reduced cell volume that were similar to sedentary WT levels. Exercised WT mice had larger cell volume than sedentary WT (indicating physiological hypertrophy), similar to exercised db/db and smaller than sedentary db/db.

Figure 7. Western blots from left ventricular tissue of **A,** SR Ca^{2+}-ATPase (SERCA2a), **B,** Total phospholamban (PLN), **C**, Phosphorylation levels of PLB at Threonine-17, **D**, Phosphorylation levels of PLB at Serine-16, **E**, Protein levels of total Ca^{2+}-calmodulin-dependent protein kinase (CaMKIIδ), **F**, Phosphorylated levels of CaMKIIδ. **G,** Phosphorylation of RyR2 at Serine-2808 (PKA and CaMKIIδ site), **H,** Phosphorylation of RyR2 at Serine-2814 (CaMKIIδ site).

Figure 1

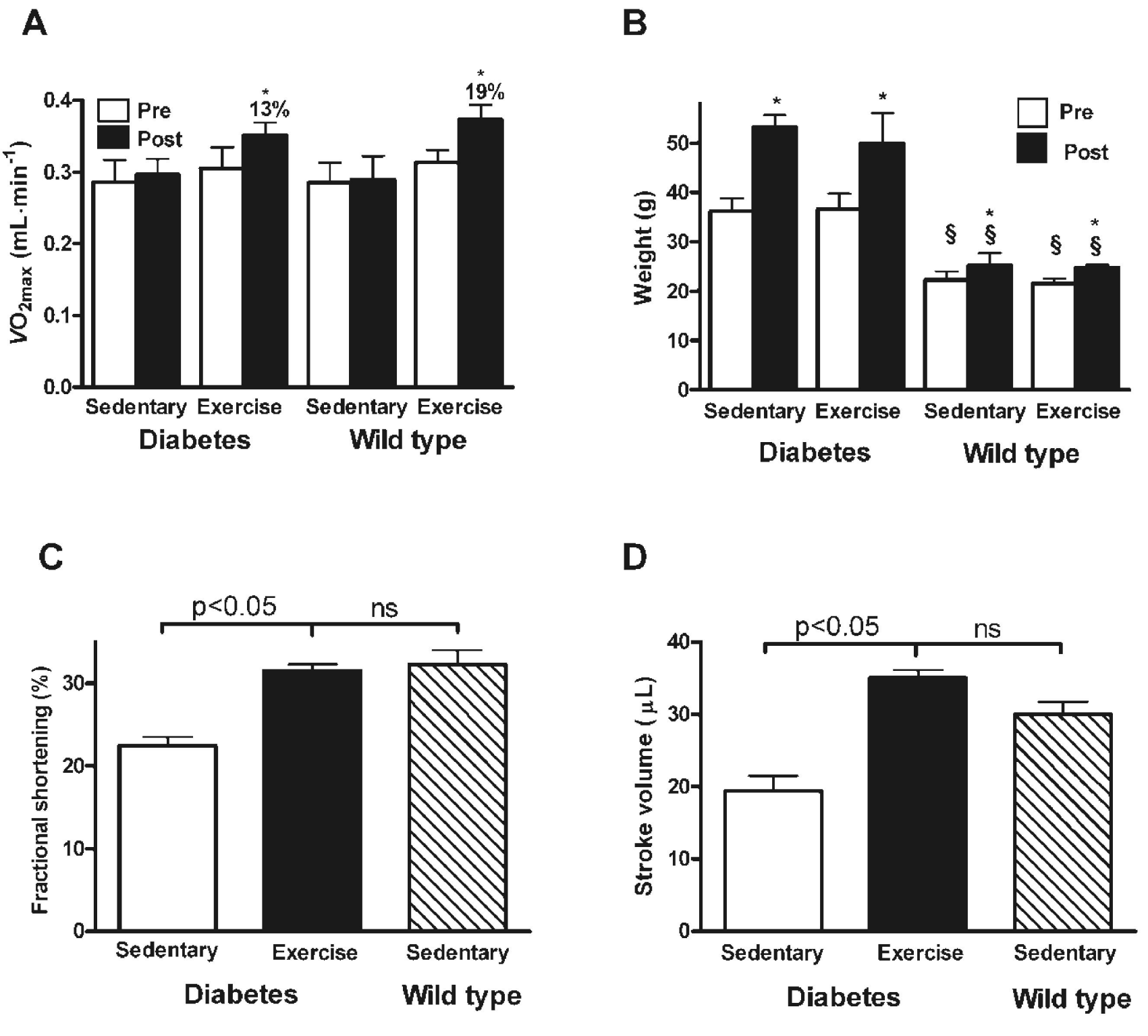

A
Pre
Post
*
13%
*
19%
0.4
0.3
0.2
0.1
0.0
VO2max (mL·min-1)
Sedentary
Exercise
Sedentary
Exercise
Diabetes
Wild type
B
Pre
Post
*
*
§
§
*
§
§
*
50
40
30
20
10
0
Weight (g)
Sedentary
Exercise
Sedentary
Exercise
Diabetes
Wild type
C
p<0.05
ns
30
20
10
0
Fractional shortening (%)
Sedentary
Exercise
Sedentary
Diabetes
Wild type
D
p<0.05
ns
40
30
20
10
0
Stroke volume (µL)
Sedentary
Exercise
Sedentary
Diabetes
Wild type

Figure 2

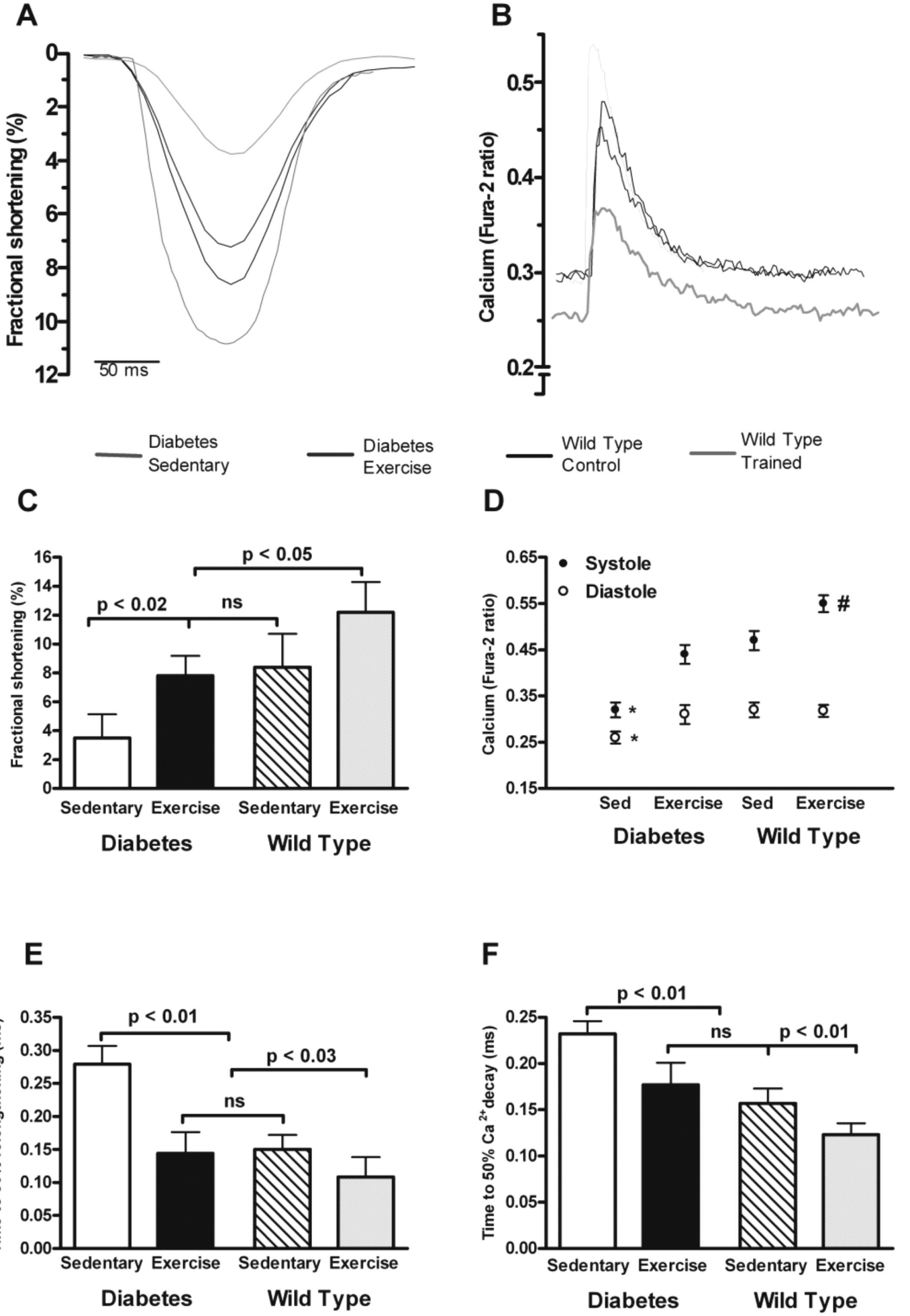
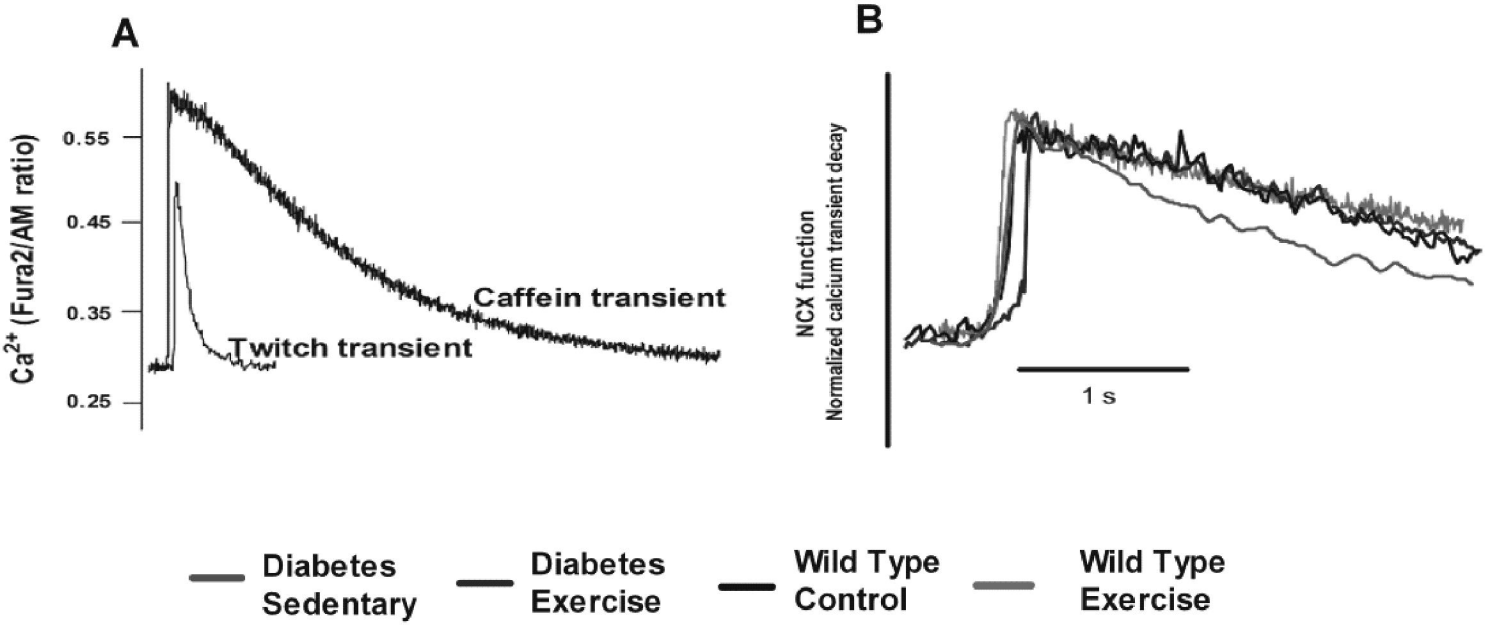
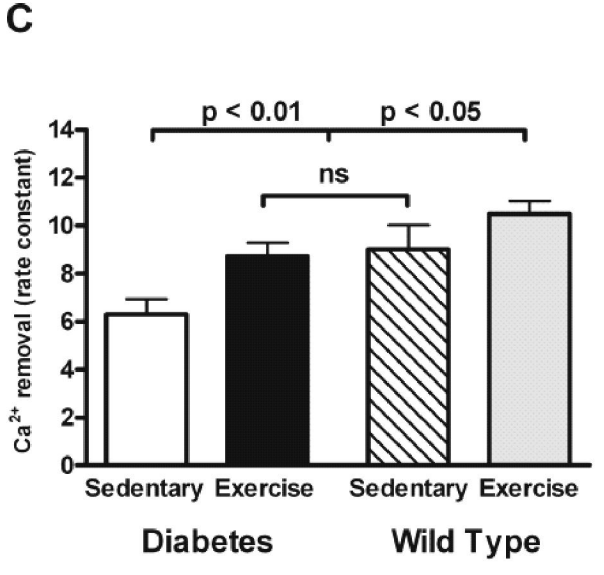

A
Fractional shortening (%)
0
2
4
6
8
10
12
50 ms
Diabetes Sedentary
Diabetes Exercise
B
Calcium (Fura-2 ratio)
0.5
0.4
0.3
0.2
Wild Type Control
Wild Type Trained
C
Fractional shortening (%)
p < 0.05
p < 0.02
ns
Sedentary Exercise Sedentary Exercise
Diabetes
Wild Type
D
Calcium (Fura-2 ratio)
Systole
Diastole
#
*
*
Sed Exercise Sed Exercise
Diabetes
Wild Type
E
Time to 50% relengthening (ms)
p < 0.01
p < 0.03
ns
Sedentary Exercise Sedentary Exercise
Diabetes
Wild Type
F
Time to 50% Ca 2+ decay (ms)
p < 0.01
ns
p < 0.01
Sedentary Exercise Sedentary Exercise
Diabetes
Wild Type

Figure 3


A
B
Ca^{2+} (Fura2/AM ratio)
0.55
0.45
0.35
0.25
Caffein transient
Twitch transient
NCX function
Normalized calcium transient decay
1 s
Diabetes Sedentary
Diabetes Exercise
Wild Type Control
Wild Type Exercise


C
Ca^{2+} removal (rate constant)
p < 0.01
p < 0.05
ns
14
12
10
8
6
4
2
0
Sedentary
Exercise
Sedentary
Exercise
Diabetes
Wild Type

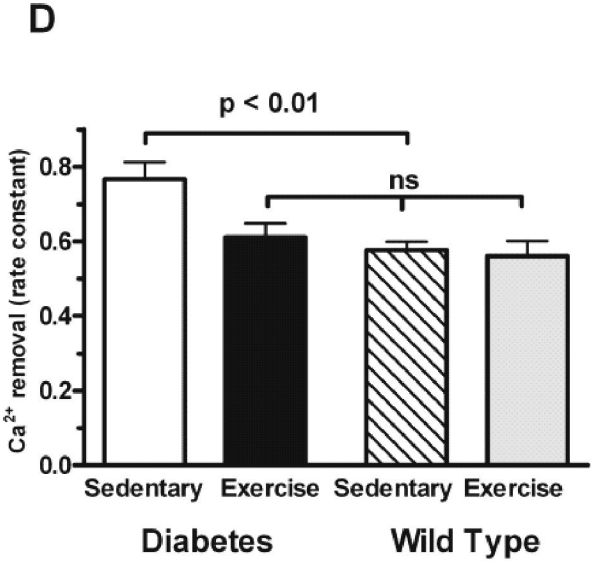

D
Ca^{2+} removal (rate constant)
p < 0.01
ns
0.8
0.6
0.4
0.2
0.0
Sedentary
Exercise
Sedentary
Exercise
Diabetes
Wild Type

Figure 4

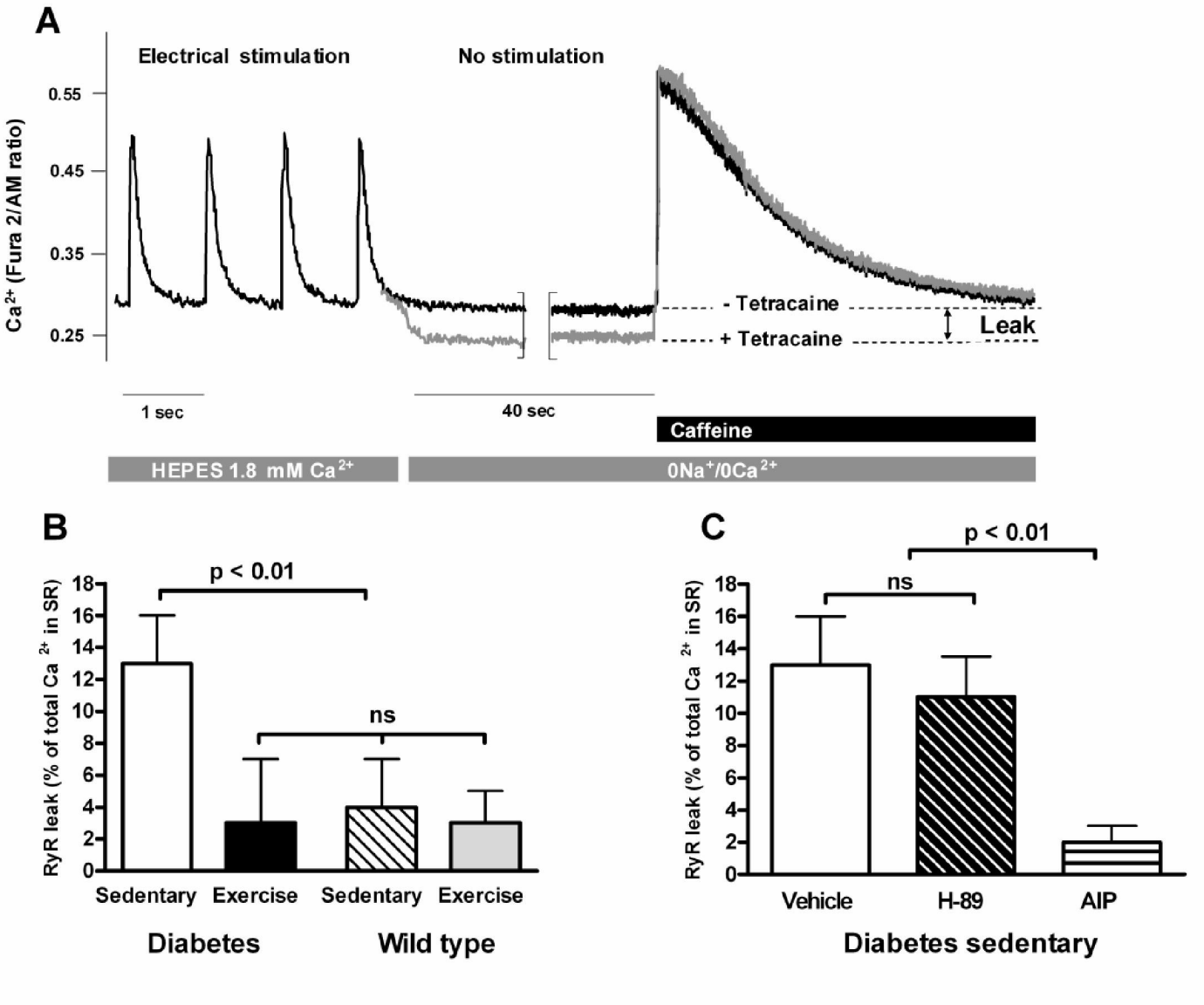

A
Electrical stimulation
No stimulation
0.55
0.45
0.35
0.25
Ca2+ (Fura 2/AM ratio)
- Tetracaine
+ Tetracaine
Leak
1 sec
40 sec
Caffeine
HEPES 1.8 mM Ca2+
0Na+/0Ca2+
B
p < 0.01
ns
RyR leak (% of total Ca 2+ in SR)
18
16
14
12
10
8
6
4
2
0
Sedentary
Exercise
Sedentary
Exercise
Diabetes
Wild type
C
p < 0.01
ns
RyR leak (% of total Ca 2+ in SR)
Vehicle
H-89
AIP
Diabetes sedentary

Figure 5

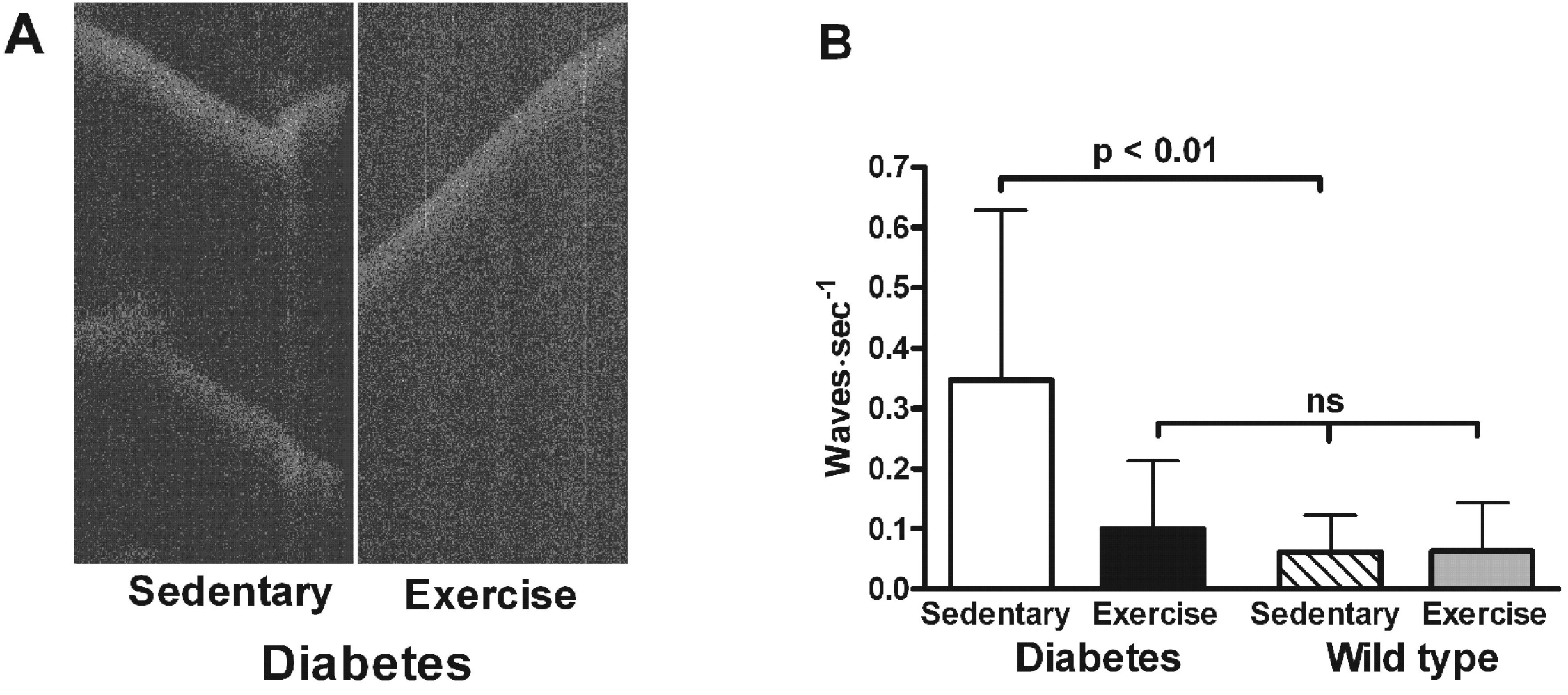
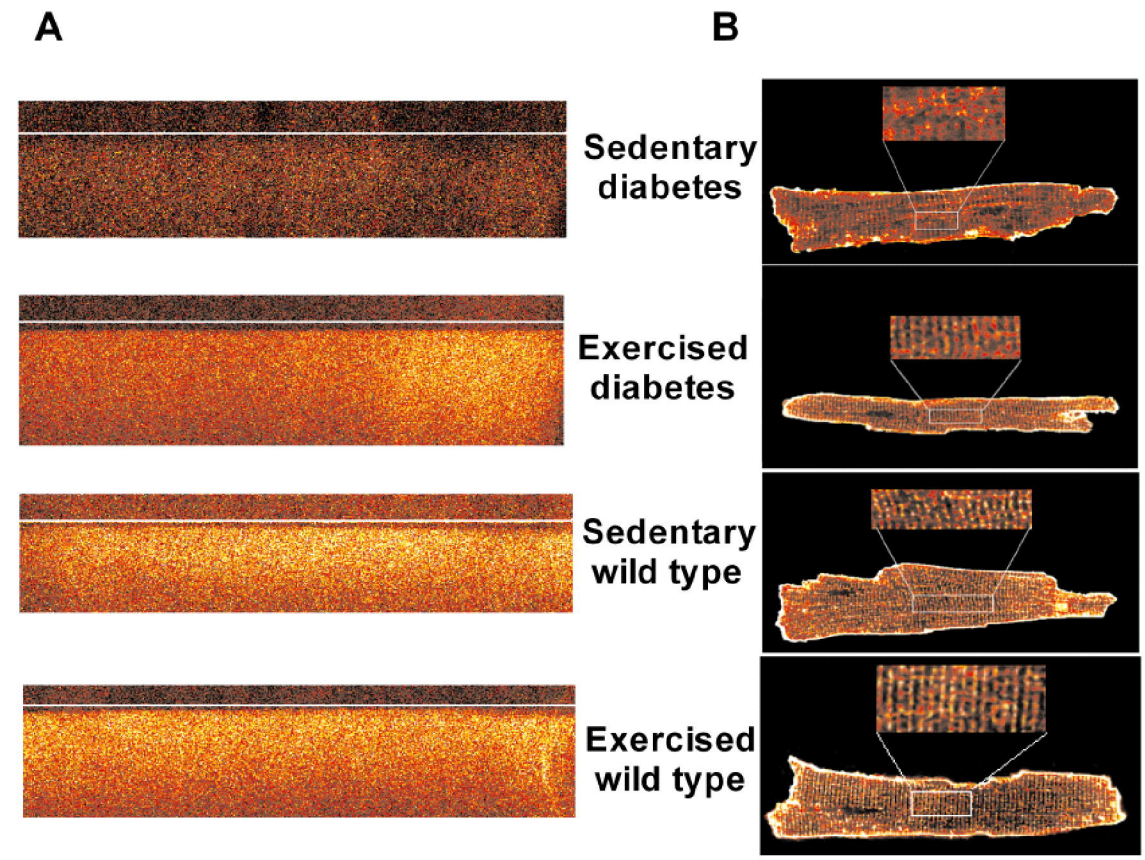
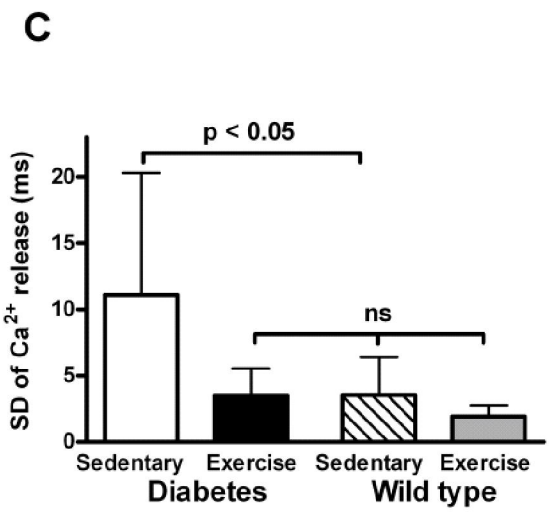

A
Sedentary
Exercise
Diabetes
B
p < 0.01
ns
Waves·sec-1
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Sedentary
Exercise
Sedentary
Exercise
Diabetes
Wild type

Figure 6


A
B
Sedentary diabetes
Exercised diabetes
Sedentary wild type
Exercised wild type


C
p < 0.05
ns
SD of Ca2+ release (ms)
0
5
10
15
20
Sedentary
Exercise
Sedentary
Exercise
Diabetes
Wild type


D
p < 0.01
ns
T-tubule density (%)
0
5
10
15
20
25
Sedentary
Exercise
Sedentary
Exercise
Diabetes
Wild type

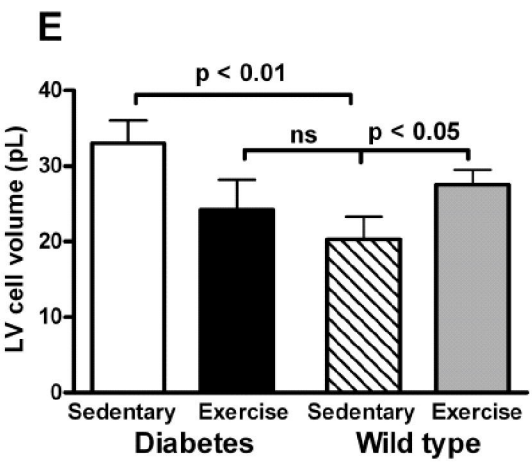

E
p < 0.01
ns
p < 0.05
LV cell volume (pL)
0
10
20
30
40
Sedentary
Exercise
Sedentary
Exercise
Diabetes
Wild type

Figure 7