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Exercise training impacts exercise tolerance and bioenergetics in gastrocnemius muscle of non-obese type-2 diabetic Goto-Kakizaki rat in vivo

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Abbreviations:

³¹P-MRS, 31-phosphorus magnetic resonance spectroscopy; AMPK, AMP-activated protein kinase; ATP, adenosine triphosphate; CK, creatine kinase; GK, Goto-Kakizaki; GLUT-4, Glucose transporter type 4; Intracellular pH (pH_i); MR, magnetic resonance; NEFA, non-esterified fatty acids; PCr, phosphocreatine; P_i, inorganic phosphate; T2DM, type 2 diabetes mellitus.

Abstract

The functional and bioenergetics impact of regular physical activity upon type-2 diabetic skeletal muscle independently of confounding factors of overweight remains undocumented. Here, gastrocnemius muscle energy fluxes, mitochondrial capacity and mechanical performance were assessed noninvasively and longitudinally in non-obese diabetic Goto-Kakizaki rats using magnetic resonance (MR) imaging and dynamic ^{31}P -MRS throughout a 6-min fatiguing bout of exercise performed before, in the middle (4-week) and at the end of an 8-week training protocol consisting in 60-min daily run on a treadmill. The training protocol reduced plasmatic insulin level (-61%) whereas blood glucose and non-esterified fatty acids levels remained unaffected, thereby indicating an improvement of insulin sensitivity. It also increased muscle mitochondrial citrate synthase activity (+45%) but this increase did not enhance oxidative ATP synthesis capacity in working muscle in vivo while glycolytic ATP production was increased (+33%). On the other hand, the training protocol impaired maximal force-generating capacity (-9%), total amount of force produced (-12%) and increased ATP cost of contraction (+32%) during the fatiguing exercise. Importantly, these deleterious effects were transiently worsened in the middle of the 8-week period, in association with reduced oxidative capacity and increased basal $[\text{P}_i]/[\text{PCr}]$ ratio (an in vivo biomarker of muscle damage). These data demonstrate that the beneficial effect of regular training on insulin sensitivity in non-obese diabetic rat occurs separately from any improvement in muscle mitochondrial function and might be linked to an increased capacity for metabolizing glucose through anaerobic process in exercising muscle.

Keywords:

Regular physical activity; Skeletal muscle function; Diabetes; Mitochondrial capacity; Nuclear magnetic resonance.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a major public health problem growing all over the world, in part because of low physical activity and excessive consumption of fat and sugar. This pathology is initially caused by peripheral insulin resistance syndrome, i.e., the inability of insulin to stimulate glucose absorption in peripheral tissue, together with the progressive failure of the pancreatic cells to supply sufficient amount of insulin [1]. Skeletal muscle plays a crucial role in the development of insulin resistance because it is one of the major organs participating in the assimilation, storage and utilization of glucose provided by food [2]. The development of insulin resistance in muscle is strongly associated to microvascular complication and reduced mitochondrial capacity for glucose and lipid oxidation [3-5]. These defects lead to disturbances in muscle energy metabolism and reduce exercise tolerance and mechanical performances in diabetic subjects [6-8].

Lifestyle modification including regular physical activity, reduction of calorie intake and weight loss is recommended for the non-pharmacological treatment of T2DM. Hence, it is well established that these approaches improve insulin sensitivity and glycemia control as the result of an increased muscle mitochondrial capacity for oxidizing fatty acids and glucose [1, 9]. However, it remains unclear whether the effect of training on muscle mitochondrial function is beneficial per se or because it is associated with intervention for reduced caloric restriction and loss weight [10]. This issue warrants to be clarified given that a significant proportion of T2DM patients is lean [11]. Yet, the effect of training on mitochondrial capacity and more generally on muscle function remains to date poorly documented in these subjects. The very few studies having investigated the impact of long-term moderate regular physical activity on skeletal muscle independently of confounding factors of obesity have been performed in Goto-Kakizaki (GK) rat. This model is indeed highly interesting because it is insulin-resistant and moderate fasting hyperglycemic from early life, and spontaneously develops T2DM without obesity [12, 13]. Moreover, it is recognized as a powerful tool for investigating exercise tolerance to diabetes [14, 15]. It has been shown that long-term

moderate regular physical activity in GK rat improves insulin sensitivity [2, 13, 16, 17] in association at the muscle level with reduction of oxidative stress [13] and microvascular complication throughout angiogenesis capillary diameter increase [16, 17], upregulation of glucose and lactate metabolic proteins [18], increase of mitochondrial DNA content [13] and activity of both citrate synthase [18] and succinate deshydrogenase [16, 17]. Overall, these results suggest that regular physical activity impacts energy metabolism in GK muscle. However, given that these results were obtained only on the basis of in vitro experiments, the corresponding impacts of these changes upon muscle mechanical performance in relation to energy production remain undetermined under physiological condition. This gap warrants to be rapidly filled for a better understanding of the impact of regular physical activity upon diabetic muscle physiology.

The aim of this study was to characterize the effect of long-term moderate regular physical activity upon GK muscle exercise tolerance and bioenergetics in vivo. Especially, we have tested whether regular training improves muscle mitochondrial function in this non-obese diabetic animal model. For this purpose, gastrocnemius muscle force-generating capacity, metabolic fluxes and anatomy were investigated noninvasively throughout a longitudinal approach using magnetic resonance (MR) imaging and dynamic ³¹P-phosphorus MR spectroscopy (³¹P-MRS) during a fatiguing bout of exercise performed before, in the middle (4-week) and at the end of an 8-week physical activity protocol consisting in 1-hour daily run on a treadmill.

2. Methods

2.1. Animal care and feeding

Twenty Goto-Kakizaki [GK/Par subline] 7-month-old male rats were used for these experiments conducted in strict accordance with the guidelines of the European

Communities Council Directive 86/609/EEC for Care and Use of Laboratory Animals. All animal procedures were performed with the approval of the animal experiment committee of Aix-Marseille University and under the supervision of BG (license number 13.164 2008/11/25). Every attempt was made to minimize the number and the suffering of animals. Rats were socially housed as 2-3 per cage in an environmentally controlled facility (12-12 h light-dark cycle, 22°C) with free access to commercial standard food (diet 113; SAFE, Augy, France) and water.

2.2. Experimental design

Animals were randomly assigned to two groups. Animals of the first group (untrained; $n = 10$) remained sedentary, whereas those of the second group (trained; $n = 10$) performed an 8-week period of regular physical activity consisting in daily running sessions repeated 5 times per week on a dedicated motor-driven treadmill (Exer 3/6 animal, Columbus Instruments, Columbus, OH, USA) with 0% inclination. In the first week, sessions' duration and running speed were gradually increased until 60-min and 20 m/min and kept during the following 7 weeks. Each training session began with a 10-min warm-up during which the belt speed was progressively increased from zero to the training speed. Running animals were closely monitored to ensure their safety and training compliance.

2.3. In vivo MR investigation of gastrocnemius muscle function and bioenergetics

Investigations were performed longitudinally and noninvasively using MR imaging and dynamic ^{31}P -MR spectroscopy. For each animal of the training group, investigations were repeated three times, i.e., before (t_0), in the middle (4-week) and at the end of the 8-week period of regular physical activity. To prevent the acute effects of the last running session, MR investigations were delayed for 72 h after the last running session of weeks 4 and 8. In the middle of the training protocol, daily running session started again the day after the 4-week MR investigation.

Animal preparation. Rats were initially anesthetized in an induction chamber with 4% isoflurane (Forene; Abbott France, Rungis, France) mixed in air (4 L/min). Once the right hindlimb was shaved, electrode cream was applied at the knee and heel levels in order to optimize transcutaneous electrical stimulation. Anesthetized animal was placed supine in a home-built cradle, which has been specially designed for the totally noninvasive MR investigation of gastrocnemius muscle anatomy, mechanical performance and bioenergetics [19]. This muscle was chosen because it is easily accessible for MR measurements and preferentially activated using our experimental methodology. The cradle integrates an ergometer consisting in a foot pedal coupled to a force transducer. The animal's foot was positioned on the pedal and the hindlimb was centered inside a 30 mm-diameter ^1H -MR Helmholtz imaging coil while the belly of the gastrocnemius muscle located above an elliptic (10 x 16 mm) ^{31}P -MRS surface coil. Corneas were protected from drying by applying ophthalmic cream and animal's head was placed in a facemask continuously supplied with 2.5% isoflurane in 33% O_2 (0.2 L/min) and 66% N_2O (0.4 L/min) throughout the MR exploration. Animal body temperature was controlled and maintained within a physiological range during anesthesia, using a feedback loop including an electrical heating blanket (Prang+Partner AG, Pfungen, Switzerland), a temperature control unit (Ref. No. 507137, Harvard Apparatus, Les Ulis, France) and a rectal probe.

Muscle electrostimulation procedure and force output measurement. Muscle contractions were achieved by transcutaneous electrostimulation using two rod-shaped 1.5-mm-diameter electrodes integrated in the experimental cradle and connected to a constant-current stimulator (Type 215/T; Hugo Sachs Elektronik–Harvard Apparatus GmbH, March–Hugstetten, Germany). One electrode was placed at the heel level and the other one was located just above the knee joint. Electrical signal coming out from the ergometer was amplified with a home-built amplifier and converted to a digital signal, which was monitored and recorded on a personal computer using the WinATS software version 6.5 (SYSMA, Aix-en-Provence, France).

Preliminary adjustments. Before each experiment, gastrocnemius muscle was passively stretched at rest by adjusting the pedal position, thereby modifying the angle between the foot and the hindlimb in order to produce a maximum isometric twitch tension in response to supramaximal square wave pulses (1-ms duration). The individual maximal electrostimulation intensity (6-8 mA) was determined by progressively increasing the electrical stimulus intensity until there was no further peak twitch force increase.

Fatiguing exercise. The fatiguing bout of exercise was performed simultaneously to the dynamic ^{31}P -MRS acquisition. It consisted in 6 min of repeated maximal isometric contractions induced electrically with 1-ms duration square-wave pulses at a frequency of 3.3 Hz. Specific force was defined as the absolute force normalized by muscle volume calculated from MR images (see below). Force-generating capacity was calculated as percent of the maximal force reached during the exercise.

MR data acquisition and processing. Investigations were performed in the 4.7 Tesla horizontal magnet of a 47/30 Biospec Avance MR system (Bruker, Karlsruhe, Germany). Sixteen consecutive non-contiguous axial scout slices (1-mm thickness; 0.5-mm spaced), covering the region from the knee to the ankle, were selected across the lower hindlimb. Rapid acquisition relaxation-enhanced (RARE) images of these slices (8 echoes; 49.3-ms effective echo time; 16-ms actual echo time; 2000-ms repetition time; 30 x 30 mm² field of view; 256 x 192 matrix size) were recorded at rest. ^{31}P -MR spectra (16 accumulations; 1.8-s repetition time; 8-kHz spectral width, 512 data points; temporal resolution of ~60 s) from the gastrocnemius muscle region were continuously acquired before (rest period; 6-min duration), during and after (recovery period; 15-min duration) the 6-min fatiguing exercise. MR data acquisition was gated to muscle electrostimulation in order to reduce potential motion artifacts due to contraction. MR data were processed using a custom-written image analysis program developed with the IDL software (Interactive Data Language, Research System, Inc., Boulder, CO, USA). For each MR image, regions of interest were manually outlined so that the corresponding cross-sectional areas could be measured. Gastrocnemius muscle volume was then calculated as the sum of the volumes

included between the consecutive slices. Relative concentrations of PCr, inorganic phosphate (P_i) and β -ATP were obtained by a time-domain fitting routine using the AMARES-MRUI Fortran code and appropriate prior knowledge of the ATP multiplets. Absolute concentrations of phosphorylated compounds were expressed relative to a resting β -ATP concentration determined by bioluminescence in muscle extracts as detailed below. Intracellular pH (pH_i) was calculated from the chemical shift of the P_i relative to the PCr signal.

2.4. Metabolic fluxes calculation and ATP cost of contraction

ATP productions from creatine kinase reaction, oxidative phosphorylation and glycolysis were calculated *in vivo* during the fatiguing bout of exercise according to the quantitative interpretation of ^{31}P -MRS bioenergetics data [20] and detailed in Appendix. This approach is the only one offering the possibility to assess noninvasively and simultaneously metabolic fluxes for a given muscle. ATP cost of contraction referred to ATP production scaled to force output during the same period.

2.5. *In vitro* analytical procedures

Blood and gastrocnemius muscle were collected in non-fasting animals the day after the 8-week MR investigation. In order to exclude any bias related to physiological and behavioral circadian oscillations between animals, all sacrifices were performed the same day and within a 60-min time frame starting six hours after light. Transcardiac blood samples (0.25 ml) were obtained during an anesthetic epoch. Plasma was immediately collected after blood centrifugation (15 min at 4,000 rpm) in EDTA-treated tubes. Animals were euthanized by cervical dislocation following isoflurane anesthesia and gastrocnemius muscles were quickly removed, freeze-clamped with liquid nitrogen-chilled metal tongs and stored at -80°C .

Plasmatic concentrations of insulin, glucose and non-esterified fatty acids (NEFA) were measured using insulin (Mercodia, Uppsala, Sweden), glucose (Randox Laboratories,

Crumlin, Antrim, UK) and NEFA (Roche Diagnostics, Roche Applied Science, Mannheim, Germany) determination kits.

ATP content was determined in 40-60 mg of freeze-clamped muscle homogenized in 1.2 mL of ice-cold 0.6 M perchloric acid using a Polytron PT2100 (Kinematica AG, Luzern, Switzerland). After incubation (15 min at 4°C), the homogenate was centrifuged (15 min, 2000 x *g*, 4°C), and the supernatant was neutralized with K₂CO₃, placed for 30 min at 4°C and centrifuged (15 min, 2000 x *g*, 4°C) to remove precipitates. ATP concentration was determined using the Bioluminescence ATP Determination Kit (ref. A22066, Invitrogen, Eugene, Oregon, USA).

Citrate synthase activity was measured in another part (20-30 mg) of the freeze-clamped muscle, which was homogenized with a lysis reagent (ref. C3228, Sigma-Aldrich) and a protease inhibitor cocktail (P8340, Sigma-Aldrich). Citrate synthase activity was measured using the colorimetric Citrate Synthase Assay Kit (ref. CS0720, Sigma-Aldrich) according to the manufacturer's instructions, and was normalized by the protein content measured using the colorimetric Pierce BCA Protein Assay Kit (ref. 23225, Thermo Fisher Scientific, Waltham, MA, USA). All in vitro measurements were done on a microplate reader (Victor X3, PerkinElmer, Waltham, MA, USA).

2.6. Statistical analysis

All data are expressed as mean \pm SD. Statistical analyses were performed using the Statistica 10 software (StatSoft France, Maisons-Alfort, France) and the R environment with package PMCMR (www.r-project.org), and the level of significance was set to $P < 0.05$. Sample distribution was tested with the Shapiro-Wilk test. For comparisons between sedentary and trained animals, all samples were normally distributed for each variable and significant differences were determined using parametric two-tailed Student's *t*-tests. For comparisons within the trained group, samples were normally distributed for all variables except for the basal ADP concentration, the PCr resynthesis rate, both the absolute and

relative ATP production rates, and the ATP cost of contraction. For these five variables, differences between exploration times (i.e., before, in the middle and at the end of the 8-week period of regular physical activity) were tested using the non-parametric Friedman's test followed when justified by pairwise post-hoc Nemenyi's test to determine difference between the exploration times. Otherwise, the effect of training was tested using one-factor ANOVAs with repeated measures on time followed when justified by pairwise post-hoc LSD Fisher tests.

3. Results

3.1. Physiologic changes

Body weight (421 ± 13 g at t_0 ; 429 ± 16 g at 4-week; 437 ± 20 g at 8-week) and gastrocnemius muscle volume (1.46 ± 0.08 cm³ at t_0 ; 1.50 ± 0.07 cm³ at 4-week; 1.52 ± 0.06 cm³ at 8-week) were larger at 4 weeks (body weight: +2%, $P = 0.039$; muscle volume: +3%, $P < 0.001$) and 8 weeks (body weight: +4%, $P = 0.038$; muscle volume: +5%, $P < 0.001$) of regular physical activity when compared to t_0 . However, the ratio between body weight and gastrocnemius muscle volume (289 ± 16 at t_0 ; 287 ± 13 at 4-week; 287 ± 8 at 8-week) did not differ ($P = 0.82$) among the three exploration times. Besides, 8-week regular physical activity did not affect the plasmatic levels of glucose ($P = 0.90$) and NEFA ($P = 0.96$) (Table 1), but decreased plasma insulin level (-61%, $P < 0.001$; Table 1 and Fig. S1A) and increased muscle citrate synthase activity (+45%, $P = 0.020$; Table 1 and Fig. S1B).

3.2. Mechanical performance

Contractile force production during the 6-min fatiguing bout of exercise was scaled to gastrocnemius muscle volume for normalizing mechanical performance between animals. The time courses of force production and force-generating capacity are shown in Fig. 1A and

1B. For each animal, force transiently increased in the early stage of the exercise to reach a maximal value that was reduced at 4-week (-28%, $P < 0.001$) and 8-week (-9%, $P = 0.006$) when compared to t_0 (Fig. 1C). Afterwards, force progressively decreased until the end of the exercise as a sign of fatigue development (Fig. 1A). Importantly, force-generating capacity at the exercise end was lower at 4-week compared to t_0 (-43%, $P < 0.001$) and 8-week (-37%, $P = 0.002$) (Fig. 1D). Overall, the total amount of force produced during the whole 6-min exercise was lower at 4-week (-45%, $P < 0.001$) and 8-week (-12%, $P = 0.039$) when compared to t_0 (Fig. 1E).

3.3. Phosphorylated compounds, pH_i and oxidative capacity *in vivo*

In resting muscle, there were no differences for [PCr]/[ATP] ratio ($P = 0.18$), [ADP] ($P = 0.70$) and pH_i ($P = 0.72$) between the three exploration times (Table 2). On the contrary, [PCr] was lower at 4-week compared to t_0 (-11%, $P = 0.030$; Table 2) and $[P_i]/[PCr]$ ratio was larger at 4-week compared to t_0 (+17%, $P = 0.042$; Table 2 and Fig. S1C) and 8-week (+21%, $P = 0.015$; Table 2 and Fig. S1C). At the onset of the 6-min fatiguing bout of exercise, PCr was rapidly degraded at a similar rate between the three exploration times ($P = 0.10$) and reached a plateau that was maintained until the exercise end (Fig. 2A). This plateau indicates the establishment of a metabolic steady state since the pool of PCr is considered at this stage of muscle activity as a shuttle for the transport of high-energy phosphates between the sites of production and consumption of ATP. Noteworthy, the extent of PCr depletion measured at the end of the exercise was larger at 4-week (+20%, $P < 0.001$) and 8-week (+19%, $P < 0.001$) when compared to t_0 (Table 2). During the first 3 minutes of the exercise, pH_i fell rapidly (then indicating a glycolytic flux acceleration) to reach a steady state that was maintained until the end of the exercise (Fig. 2B); at this stage, the extent of acidosis reduction (relative to rest) was larger at 4-week (+15%, $P = 0.009$) and 8-week (+17%, $P = 0.004$) compared to t_0 (Table 2). ATP level slightly decreased throughout the fatiguing bout of exercise (Fig. 2C) and the extent of ATP depletion measured at the end of the exercise did

not differ ($P = 0.48$) between the three exploration times (Table 2), which indicates that the regular physical activity did not alter ATP homeostasis.

During the post-exercise recovery period, phosphorylated compounds and pH_i progressively reached their respective basal values (Fig. 2A, 2B and 2C). The time constant of PCr resynthesis was larger at 4-week compared to t_0 (+34%; $P < 0.001$) and 8-week (+28%; $P = 0.002$) but did not differ ($P = 0.56$) between t_0 and 8 weeks (Fig. 3A), whereas the initial rate of PCr resynthesis was lower at 4-week compared to t_0 (-32%; $P = 0.013$) but was similar ($P = 0.98$) between t_0 and 8 weeks (Fig. 3B). Furthermore, the maximal oxidative capacity was lower at 4-week compared to t_0 (-39%; $P < 0.001$) and 8-week (-32%; $P < 0.001$) but did not differ ($P = 0.29$) between t_0 and 8 weeks (Fig. 3C). Taken together, these results indicate that mitochondrial function was transiently reduced at 4-week of regular physical activity. Besides, proton efflux from the muscle did not differ ($P = 0.59$) between the three exploration times (Fig. 3D).

3.4. Metabolic fluxes and ATP cost of contraction

Four weeks of regular physical activity did not affect ATP production from CK reaction ($P = 0.14$; Fig. 4A) and glycolysis ($P = 0.06$; Fig. 4B) taken individually but increased (+17%; $P = 0.026$) the total anaerobic ATP production (Fig. 4C) calculated as the sum of glycolytic and CK reaction contributions, and reduced (-25%, $P = 0.013$) the oxidative ATP production (Fig. 4D); as a result, the relative contribution of oxidative pathway to total ATP production was reduced (-19%; $P = 0.003$) at 4 weeks (Fig. 4E). When compared to t_0 , 8 weeks of physical activity increased by around 32% the ATP productions from CK reaction ($P < 0.001$; Fig. 4A) and glycolysis ($P < 0.001$; Fig. 4B), and the total anaerobic ATP production ($P < 0.001$; Fig. 4C), but did not affect oxidative ATP production ($P = 0.99$; Fig. 4D) and oxidative contribution ($P = 0.25$; Fig. 4E). Overall, 8 weeks of physical activity increased ($P = 0.005$) total ATP production (calculated as the sum of anaerobic and oxidative processes) by 22% when compared to t_0 (Fig. 4F). Besides, the averaged ATP cost of contraction (calculated

across the whole 6-min fatiguing exercise as the total amount of ATP production scaled to the total force output) was larger after 4- and 8-week of regular physical activity (4-week: +87%, $P < 0.001$; 8-week: +32%, $P = 0.048$) when compared to t_0 (Fig. 4G).

4. Discussion

This study represents the first attempt at investigating the effect of regular physical activity upon exercise tolerance and bioenergetics in type-2 diabetic skeletal muscle independently of confounding factors of overweight. We mainly found that an 8-week protocol of moderate daily run on a treadmill (i) reduces plasmatic insulin level whereas blood glucose and NEFA levels remains unaffected; (ii) increases muscle citrate synthase activity but this increase does not enhance oxidative ATP synthesis production in working muscle while glycolytic ATP production is increased concomitantly; (iii) impairs force-generating capacity and ATP cost of contraction. Importantly, we found that these impairments were transiently worsened in the middle (4-week) of the protocol in association with reduced oxidative capacity and increased basal $[P_i]/[PCr]$ ratio, an *in vivo* biomarker of muscle damage [21, 22].

We found that the 8-week regular physical activity protocol decreased serum insulin content whereas blood glucose and NEFA levels remained unaffected. These findings, which are consistent with other studies in trained GK rat [2, 13, 16, 17], demonstrate that our training protocol improves insulin sensitivity. Such an improvement has been put down to an increase in muscle oxidation of both fatty acids and glucose [1, 9]. In accordance with these data and as previously reported in GK rat [18], we measured that the 8-week training protocol increased the muscle citrate synthase activity, hence suggesting an increase in oxidative capacity and/or mitochondrial density. Our data further corroborates other experiments in GK rats showing that regular training is beneficial for mitochondrial function through the increase of mitochondrial DNA content [13] and succinate deshydrogenase

activity [16, 17]. Nevertheless, the increased citrate synthase activity we measured did not affect muscle basal bioenergetics and especially did not enhance mitochondrial capacity and oxidative ATP synthesis assessed in vivo using dynamic ^{31}P -MR spectroscopy during a fatiguing bout of exercise. The cause of this discrepancy has to be explained. It must be kept in mind that the ability of mitochondria to produce ATP critically depends on oxygen supply. Yet, histological and microscopy analyses have shown that muscle capillary diameter, tortuosity and content are significantly lower in GK rat when compared to age-matched control Wistar rats [23], and that 3 weeks of low-intensity running on a treadmill reverses these microcirculatory complications throughout angiogenesis and increased luminal capillary diameter [16, 17]. Therefore, one can postulate that the beneficial effect of regular physical activity over capillary architecture potentially improves blood flow and oxygen availability in contracting muscle. This eventuality can however be ruled out herein since we found that the training protocol did not affect the proton efflux leaving the muscular cells, hence indicating in accordance with previous studies [24, 25] that blood flow during exercise was not improved in vivo. On the other hand, it is possible that rather affecting mitochondrial capacity, the increase in muscle citrate synthase activity promotes cytosolic lipogenesis throughout the citrate-shuttle pathway [26]; This phenomena would favor fat accumulation, explaining by the way the body weight gain in animals performing the training protocol.

Interestingly, we noted that regular physical activity increased muscle acidosis during the fatiguing bout of exercise thereby suggesting a higher anaerobic energy flux. According to the quantitative interpretation of bioenergetics data [20], we actually found that glycolytic ATP production was enhanced in exercising muscle following the 8-week training, which is in agreement with in vitro experiments showing that glucose and lactate metabolic proteins are upregulated in skeletal muscle of exercise-trained GK rats [18]. In addition, we found that PCr consumption to produce ATP throughout the anaerobic CK reaction was increased during the fatiguing exercise session in trained GK animals. PCr level reduction raises the cytoplasmic concentrations of creatine and AMP in muscle, which results in the activation of AMP-activated protein kinase (AMPK). Yet, activation of AMPK is the mechanism by which

exercise induces translocation of the GLUT-4 isoform of the glucose transporter into the sarcolemma and stimulates glucose transport [27-29]. Then, our data are in line with the view that the improved insulin sensitivity results from an increase in glucose transport and utilization in the muscle [30, 31]. Besides, it must be underlined that glucose metabolism enhancement occurred independently of any mitochondrial impairment, which is consistent with the assumption that DT2M-induced mitochondrial dysfunction cannot be considered as the direct cause of the impaired glucose metabolism [32, 33].

In addition, we found that 8 weeks of regular physical activity increased the total amount of ATP produced during the whole fatiguing bout of exercise. Nevertheless, it is striking that this increased ATP generation did not improve muscle function whereas it is well established that increased energy supply in working muscle increases mechanical performance [34, 35]. We found on the contrary that the training protocol reduced the exercise tolerance and caused an increased ATP cost of contraction. These results are unexpected because it is usually accepted that regular physical activity is beneficial for muscle function in healthy or diabetic subjects [30, 31]. However, despite the treadmill training protocol we used is comparable in terms of duration and intensity to that of previous studies in GK rat [2, 13, 18, 36], we cannot excluded it was in fact too intensive for this animal model. In that sense, we found that mechanical performance reduction and increased ATP cost measured at 8-week training were both intensified in the middle (4-week) of the protocol in association with reduction of both oxidative capacity and relative contribution of the oxidative process to total ATP production. Moreover, we found that the basal $[P_i]/[PCr]$ ratio was significantly higher after 4 weeks when compared to t_0 and 8-week of training, which indicates that the first half of the training causes muscle injury [21, 37]. Our data are consistent with studies showing that unaccustomed physical exercise in healthy sedentary subject results in temporary, repairable skeletal muscle damage including cellular and subcellular disturbances [38, 39]. These disturbances are associated with various physiological events, e.g., inflammatory processes and oxidative stress, that are known to be deleterious for both muscle and mitochondrial function [38, 39]. This deleterious state was

however transitory given that mitochondrial indexes and $[P_i]/[PCr]$ ratio were restored after 8 weeks of training. Also, it is noteworthy that the reduced mitochondrial function observed at 4 weeks of training was not linked to any impairment of anaerobic contribution. These data go certainly against studies suggesting that impaired glucose transport/phosphorylation directly relates to mitochondrial dysfunction [3, 4] but echoes with the growing number of studies proposing that mitochondrial impairment is not the causative factor of insulin resistance development [32, 33]. Another attractive possibility for explaining the unexpected deleterious impact of training upon oxidative capacity might lie in the use of isoflurane for maintaining the general anesthesia during MR investigations. This anesthetic agent is actually known to alter mitochondrial bioenergetics mainly through the inhibition of complex I of the electron transport chain [40, 41]. Therefore, repeated anesthesia could counteract the potential beneficial effect of training upon mitochondrial function.

5. Conclusion

These data demonstrate that the beneficial effect of long-term regular physical activity on insulin sensitivity in non-obese diabetic GK muscle occurs independently of any improvement in muscle mitochondrial function and might be linked to an increased capacity for metabolizing glucose through anaerobic process in working muscle. Noteworthy, we found that the 8-week training protocol reduced the exercise tolerance of diabetic muscle and this deleterious effect was further transiently worsened in the middle of the protocol in association with reduced oxidative capacity and increased muscle damage. These findings therefore suggest that, despite already used in GK rat, this training protocol might be too intensive for this animal model, which emphasizes the importance of adapting training to the physical capacity of patient in the perspective of prevention and treatment of T2DM.

6. Appendix: Metabolic fluxes calculation and ATP cost of contraction

ATP productions from creatine kinase reaction, oxidative phosphorylation and glycolysis were calculated in vivo during the fatiguing bout of exercise according to the quantitative interpretation of ^{31}P -MRS bioenergetics data [20].

ATP production rate from PCr degradation throughout the CK reaction (D) was directly calculated using the [PCr] time-course: $D = -d\text{PCr}/dt$.

Oxidative ATP production rate (Q) was calculated considering that oxidative ATP synthesis is stimulated by [ADP] through a hyperbolic relationship: $Q = Q_{\max}/(1 + K_m/[\text{ADP}])$, in which K_m (the ADP concentration at half-maximal oxidation rate) is 50 μM and Q_{\max} is the maximal oxidative capacity. [ADP] was calculated from [PCr], [ATP] and pH_i using the CK equilibrium constant ($K = 1.67 \cdot 10^9 \text{ M}^{-1}$). Q_{\max} was calculated using the rate of PCr resynthesis at the start of the post-exercise recovery period ($V\text{PCr}_{\text{rec}}$) and the concentration of free cytosolic ADP measured at the end of the exercise: $Q_{\max} = V\text{PCr}_{\text{rec}} (1 + K_m/[\text{ADP}]_{\text{end}})$. $V\text{PCr}_{\text{rec}}$ was the product of k (the pseudo-first-order rate-constant of PCr recovery) and $[\text{PCr}]_{\text{cons}}$ (the amount of PCr consumed at the end of the exercise). In order to determine k , the PCr time-course during the post-exercise recovery period was fitted to a first-order exponential curve with a least mean-squared algorithm: $[\text{PCr}]_t = [\text{PCr}]_{\text{rest}} - [\text{PCr}]_{\text{cons}} e^{-kt}$.

Glycolytic ATP production rate (L) was determined considering that it is related to glycolytic proton generation (H_{Gly}) with a stoichiometry of 1.5 moles ATP per mole of proton: $L = 1.5 H_{\text{Gly}}$. Indeed, the degradation of a mole of glycosyl unit generates 3 moles of ATP while the hydrolysis of 3 moles of ATP is coupled to the production of 2 moles of protons. Proton generation can be inferred from the observed changes in pH_i and taking into account (i) protons consumed by PCr degradation throughout the CK reaction (H_{CK}), (ii) protons passively buffered in the cytosol (H_{β}), (iii) protons leaving the cell (rate of net proton efflux, H_{Efflux}) and (iv) protons produced by oxidative phosphorylation (H_{Ox}): $H_{\text{Gly}} = H_{\text{CK}} + H_{\beta} + H_{\text{Efflux}} - H_{\text{Ox}}$. Calculation of H_{CK} was done from the time-dependent changes in [PCr] and with the stoichiometric coefficient $\varphi = 1/(1+10^{(\text{pH}_i-6.75)})$, which represents the number of protons

generated per mole of PCr degraded: $H_{CK} = \varphi \text{ dPCr}/dt$. Besides, H_{β} was the product of β_{total} (in Slykes, millimoles acid added per unit change in pH_i) and pH_i changes ($\Delta pH_i = pH_{observed} - pH_{rest}$): $H_{\beta} = (-\beta_{total}\Delta pH_i)$. The apparent buffering capacity (β_{total}) takes into account the buffering capacity of P_i (β_{Pi}) and the buffering capacity of muscle tissue (β_{tissue}): $\beta_{total} = \beta_{Pi} + \beta_{tissue}$, where $\beta_{Pi} = 2.3[P_i]/((1+10^{(pH_i-6.75)})(1+10^{(6.75-pH_i)}))$. It has been demonstrated that β_{tissue} varies linearly between pH 7 (16 Slykes) and pH 6 (37 Slykes) in murine gastrocnemius muscle. Accordingly, β_{tissue} was calculated as follows: $\beta_{tissue} = -21pH_i + 163$. During exercise, H_{efflux} was calculated using the proportionality constant λ (in $\mu\text{mol/s/pH unit}$) referring to the ratio between the rate of proton efflux and pH_i : $H_{efflux} = -\lambda\Delta pH_i$. This constant was determined at the start of the post-exercise recovery period as $\lambda = -V_{eff}/\Delta pH_i$. At that time, although protons are generated throughout the aerobic PCr resynthesis, pH_i recovers back to basal because of net proton efflux from the cell: H_{efflux} can then be calculated taking into account proton loads associated with CK reaction and mitochondrial ATP synthesis on the one hand and the rate of pH changes on the other hand. $H_{efflux} = H_{CK} + H_{Ox} + \beta_{total}dpH_i/dt$. The rate of aerobic proton production coupled to oxidative ATP synthesis was quantified as follows: $H_{Ox} = mVPCr_{rec}$, with $m = 0.16/(1+10^{(6.1-pH)})$.

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Conflict of interest

The authors have no conflicts of interest, financial or otherwise, to declare.

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Table 1

Metabolites levels and citrate synthase activity measured in vitro in sedentary and trained (8 weeks of regular physical activity) GK rats.

	Sedentary	Trained
<i>Plasma</i>		
Glucose (mM)	19.4 ± 10.7	19.7 ± 8.6
Non-esterified fatty acids (mM)	0.21 ± 0.06	0.20 ± 0.11
Insulin (ng/ml)	2.2 ± 0.8	0.9 ± 0.2 ^{\$}
<i>Gastrocnemius muscle</i>		
Citrate synthase activity (nmol/mg prot/min)	0.13 ± 0.03	0.19 ± 0.06*
ATP (mM)	7.1 ± 1.1	7.4 ± 0.8

Data are means ± SD. * $P < 0.05$ vs sedentary; ^{\$} $P < 0.001$ vs sedentary.

Table 2

Effect of an 8-week period of regular physical activity on gastrocnemius muscle bioenergetics assessed longitudinally and noninvasively using ^{31}P -MRS before.

	t_0	4 weeks	8 weeks
<i>Rest</i>			
[PCr]/[ATP]	3.5 \pm 0.6	3.4 \pm 0.7	3.9 \pm 0.6
[P _i]/[PCr] (x 1000)	96 \pm 8	116 \pm 17* ^{\$}	90 \pm 27
[PCr] (mM)	18.7 \pm 2.6	16.6 \pm 2.4*	19.7 \pm 3.3
[ADP] (μM)	8 \pm 1	7 \pm 1	8 \pm 1
pH _i	7.14 \pm 0.09	7.12 \pm 0.04	7.15 \pm 0.07
<i>End of the fatiguing exercise</i>			
[PCr] (mM)	6.9 \pm 1.9	3.9 \pm 0.9*	4.8 \pm 1.2*
PCr depletion (relative to rest; %)	63 \pm 7	76 \pm 5*	76 \pm 5*
ATP depletion (relative to rest; %)	30 \pm 12	36 \pm 9	29 \pm 14
[ADP] (μM)	14 \pm 5	17 \pm 5	17 \pm 4
pH _i	6.39 \pm 0.07	6.27 \pm 0.04*	6.28 \pm 0.05*
pH _i reduction (relative to rest)	0.75 \pm 0.12	0.86 \pm 0.06*	0.87 \pm 0.08*

Investigations were repeated three times for each animal (n = 10), i.e., before (t_0), in the middle (4-week) and at the end of an 8-week period of regular physical activity. Data are means \pm SD. * Significantly different from t_0 ; ^{\$} Significantly different from 8-week.

Figure legends

Figure 1. Time courses of gastrocnemius muscle force production (A) and force-generating capacity (B) throughout the 6-min fatiguing exercise performed simultaneously to the dynamic ^{31}P -MRS acquisition before (t_0), in the middle (4-week) and at the end of the 8-week period of regular physical activity. Maximal force reached during the exercise (C), extent of force reduction measured at the end of the exercise (D) and total amount of force produced during the whole exercise (E). Data are means \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2. Time courses of [PCr] (A), pH_i (B) and [ATP] (C) during the 6-min fatiguing exercise and the following 15-min recovery period. Measurements were performed noninvasively using dynamic ^{31}P -MRS acquisition before (t_0), in the middle (4-week) and at the end of the 8-week period of regular physical activity. For each panel, the first point ($t = 0$) indicates the resting value. Data are means \pm SD.

Figure 3. Time constant (A) and initial rate (B) of PCr resynthesis at the start of the post-exercise recovery period, maximal oxidative capacity (C) and proton efflux (D) assessed noninvasively using dynamic ^{31}P -MRS acquisition before (t_0), in the middle (4-week) and at the end of the 8-week period of regular physical activity. Data are means \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 4. ATP production from CK reaction (A) and glycolysis (B), total anaerobic ATP production calculated as the sum of glycolytic and CK reaction contributions (C), oxidation ATP production (D), relative contribution of oxidative process to total ATP production (E), total ATP production (F), and averaged ATP cost of contraction (G) during the whole 6-min fatiguing exercise performed before (t_0), in the middle (4-week) and at the end of the 8-week period of regular physical activity. Data are means \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Supplementary data

Figure S1. Individual value plot of plasmatic insulin level (A) and muscle citrate synthase activity (B) for sedentary (SED) and training (TR) GK rats, and muscle basal $[P_i]/[PCr]$ ratio before (t_0), in the middle (4-wk) and at the end (8-wk) of the 8-week training protocol (C).

Figure 1

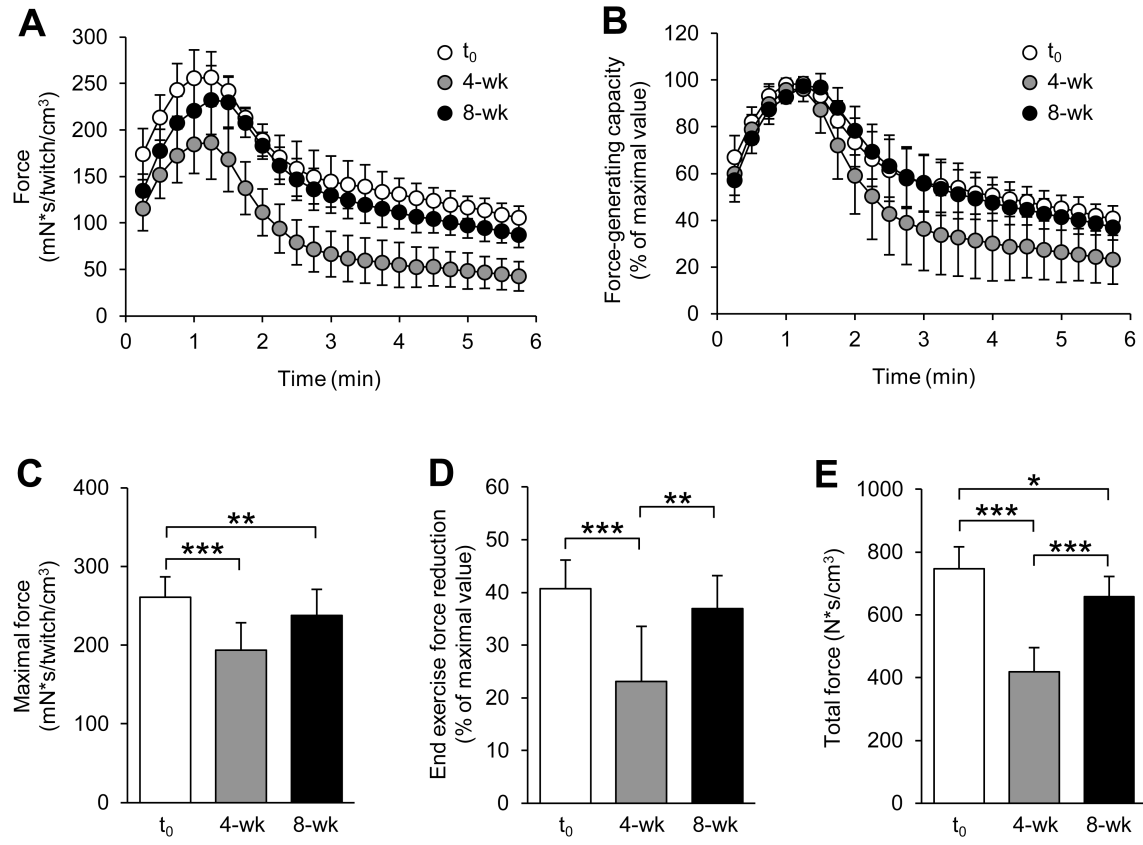


Figure 2

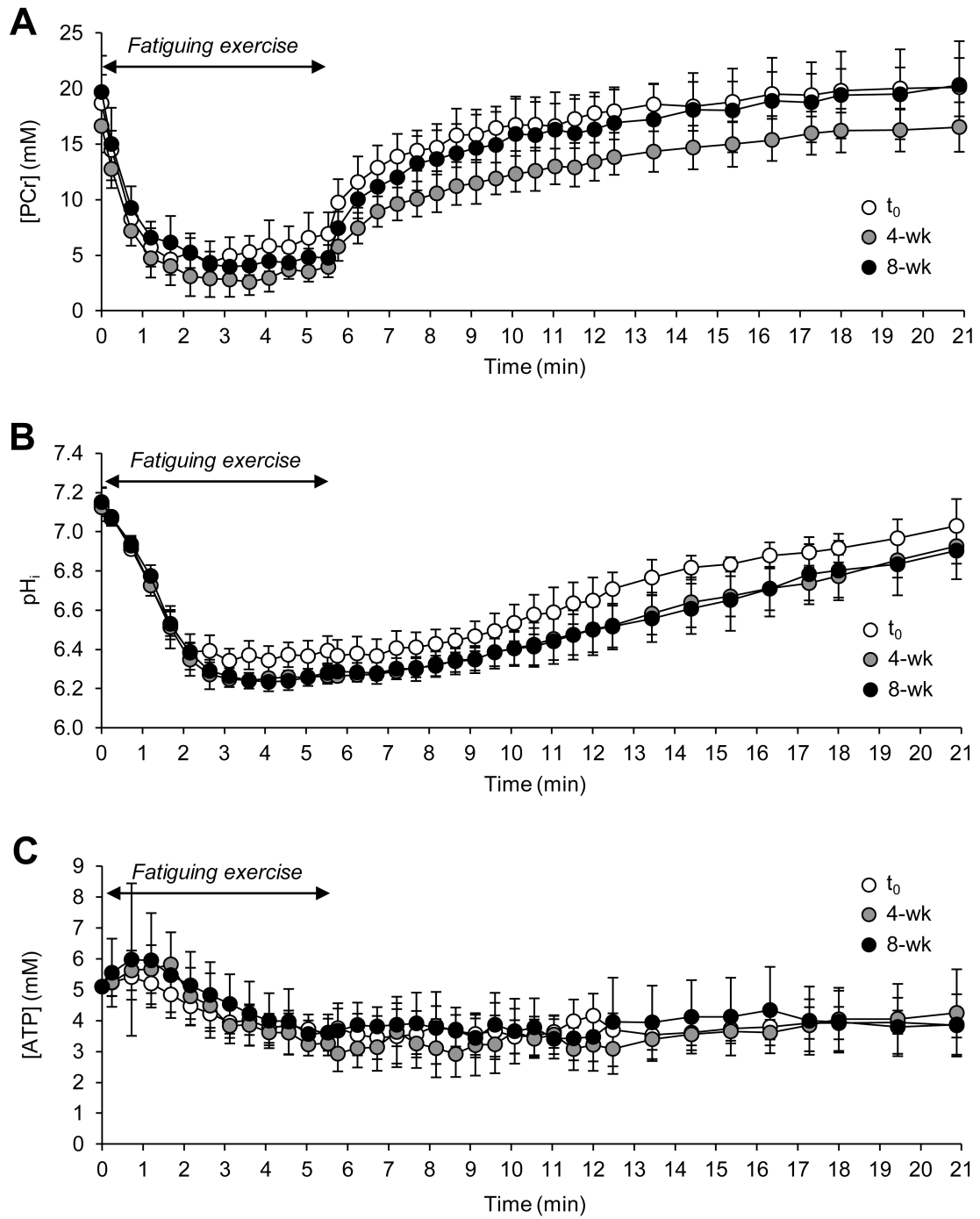


Figure 3

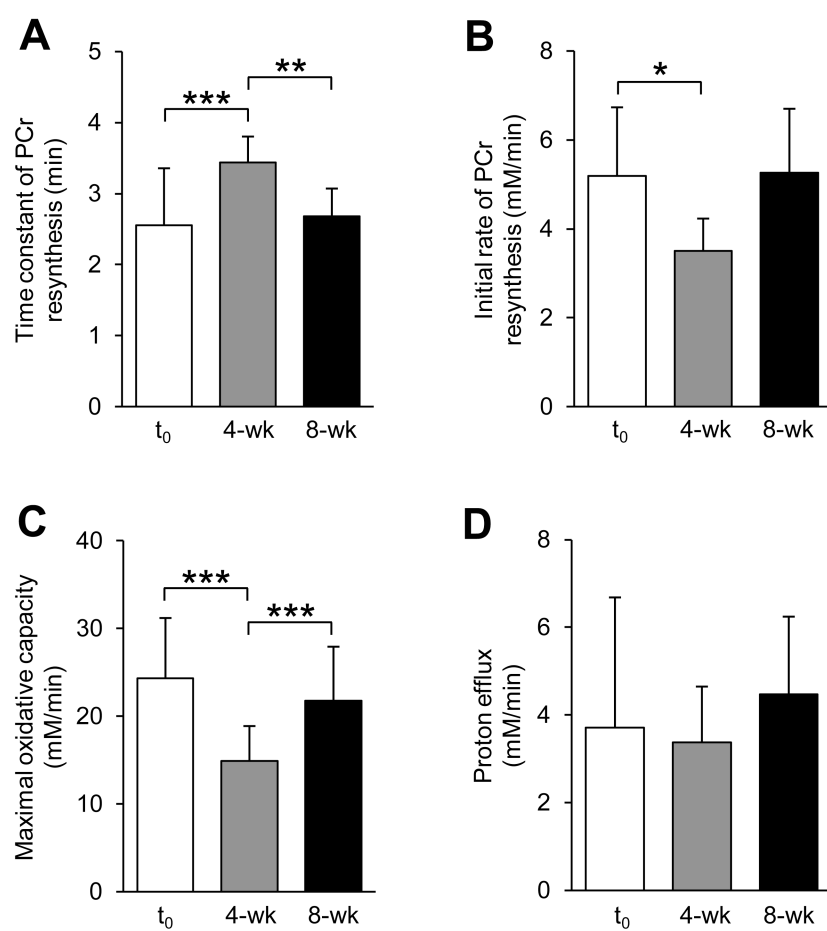


Figure 4

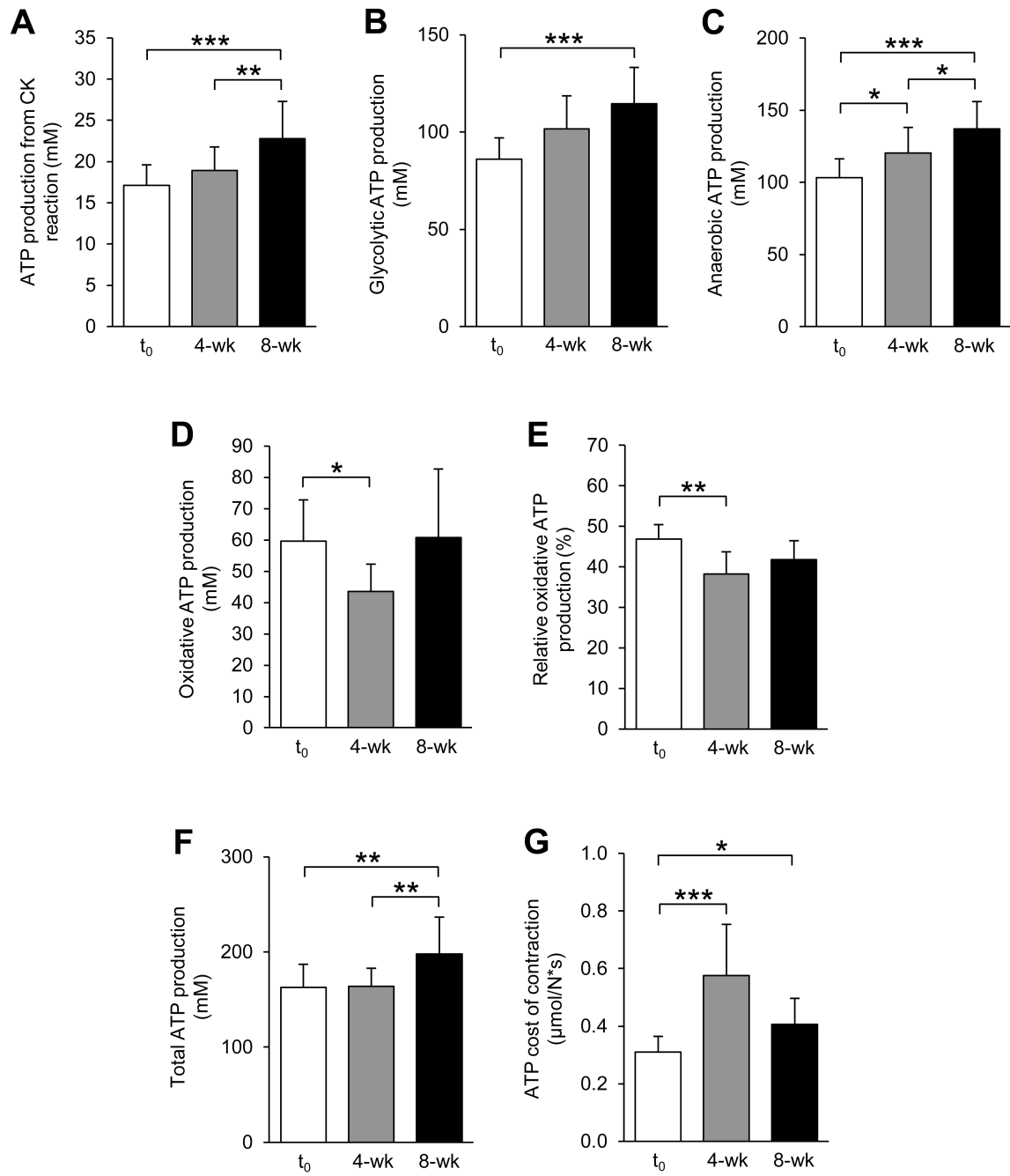


Figure S1

