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Skeletal muscle adaptations to exercise are not influenced by metformin treatment in humans: secondary analyses of two randomized, clinical trials.

Short title: Interaction between exercise and metformin in humans.

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ABSTRACT

Metformin and exercise both improve glycemic control, but in vitro studies have indicated that an interaction between metformin and exercise occurs in skeletal muscle, suggesting a blunting effect of metformin on exercise training adaptations. Two studies (a double-blind, parallel-group, randomized clinical trial conducted in 29 glucose-intolerant individuals and a double-blind, cross-over trial conducted in 15 healthy lean males) were included in this paper. In both studies, the effect of acute exercise +/- metformin treatment on different skeletal muscle variables, previously suggested to be involved in a pharmaco-physiological interaction between metformin and exercise, was assessed. Furthermore, in the parallel-group trial, the effect of 12 weeks of exercise training was assessed. Skeletal muscle biopsies were obtained before and after acute exercise and 12 weeks of exercise training, and mitochondrial respiration, oxidative stress and AMPK activation was determined. Metformin did not significantly affect the effects of acute exercise or exercise training on mitochondrial respiration, oxidative stress or AMPK activation, indicating that the response to acute exercise and exercise training adaptations in skeletal muscle is not affected by metformin treatment. Further studies are needed to investigate whether an interaction between metformin and exercise is present in other tissues, e.g. the gut.

Trial registration: ClinicalTrials.gov (NCT03316690 and NCT02951260).

Keywords

Exercise; Impaired glucose tolerance; Prediabetes, Healthy lean males; Interaction; Metformin; Training; Skeletal muscle; AMPK; ROS; Complex-1;

Abbreviations
ADP: adenosine 5’-diphosphate

AMP: adenosine 5’-monophosphate

ATP: adenosine triphosphate

AMPK: AMP-activated protein kinase

CS: citrate synthase

eGFR: estimated glomerular filtration rate

ETF: electron-transferring flavoprotein

HbA1c: hemoglobin A1c

MET: metformin

OGTT: oral glucose tolerance test

OXPHOS: oxidative phosphorylation

PLA: placebo

RER: respiratory exchange ratio

ROS: reactive oxygen species

RPE: rate of perceived exertion

VO_{2peak}: maximum volume of O_2

8-oxoGuo: 8-oxo-7,8-dihydroguanosine

8-oxodG: 8-oxo-7,8-dihydro-2’-deoxyguanosine
Novelty bullets

- Metformin does not affect exercise-induced alterations in mitochondrial respiratory capacity in human skeletal muscle
- Metformin does not affect exercise-induced alterations in systemic levels of oxidative stress nor emission of reactive oxygen species from human skeletal muscle
- Metformin does not affect exercise-induced AMPK activation in human skeletal muscle
INTRODUCTION

Metformin and exercise training both improve glycemic control and other cardiovascular risk factors in glucose-intolerant individuals, when prescribed independently (Boule et al. 2001; Piera-Mardemootoo et al. 2018; Snowling and Hopkins 2006). Several studies, however, suggest that an interaction between metformin and exercise does occur, leading to metformin blunting or even antagonizing the exercise training-induced improvements in glucose metabolism (Malin et al. 2012; Myette-Cote et al. 2016; Pilmark et al. 2021b; Sharoff et al. 2010). A pharmaco-physiological interaction would be of great clinical relevance given that metformin and exercise training are both part of the first line treatment of type 2 diabetes (2020).

Previous studies have suggested that a mechanistic basis for an interaction between metformin and exercise may be found in skeletal muscle (Konopka et al. 2019; Sharoff et al. 2010). Special attention has been directed towards the potential role of the mitochondrial complexes (especially Complex I), AMP-activated protein kinase (AMPK) and oxidative stress, which are all involved in the metabolic responses to exercise and suggested to be part of the cellular mechanisms of metformin. Mitochondrial Complex I-linked respiration is increased during an acute bout of exercise to facilitate ATP-production in the exercising muscle (Tonkonogi et al. 1998; Tonkonogi et al. 1999). Metformin treatment has been suggested to inhibit Complex I (Foretz et al. 2019; Owen et al. 2000) resulting in lower oxidative phosphorylation (OXPHOS) (Wessels et al. 2014) and lower H₂O₂ production during exercise (Kane et al. 2010), both potentially hampering adaptations to exercise training. Moreover, a metformin-induced inhibition of Complex I may lead to increased basal activity of AMPK by an increased AMP/ATP ratio (Rena et al. 2017) with potential less exercise-induced increments in AMPK activation and concomitant reduced adaptations to exercise training (Kjobsted et al. 2018; Steinberg and Kemp 2009). While these speculations are intriguing, it must be noted that most of the evidence is based on in vitro or animal studies with supra-
pharmacological concentrations of metformin (Fontaine 2018; Owen et al. 2000; Vial et al. 2019), and the available data may therefore not be directly translatable to a clinical setting.

In vivo, metformin is transported into skeletal muscle cells by organic cation transporter 3 (OCT3) (Gong et al. 2012), but until recently, little was known about to what extent metformin was taken up by skeletal muscle. In 2016, a PET-study with \(^{11}\)C-labelled metformin showed only discrete uptake of metformin in human skeletal muscle in vivo (Gormsen et al. 2016). Moreover, it was recently shown that metformin concentrations in skeletal muscle were considerably lower than concentrations in plasma (Kristensen et al. 2019). As such, during treatment with pharmacological doses of metformin, it may be questioned if the intramuscular concentrations of metformin, especially in the mitochondria (Foretz et al. 2019), are high enough to affect mitochondrial respiration during exercise (Martin-Rodriguez et al. 2020). In this context, while some studies have shown that metformin inhibits maximal Complex I-linked respiration (El-Mir et al. 2000; Owen et al. 2000) and reduces OXPHOS (Wessels et al. 2014), these studies have typically used supra-therapeutic concentrations of metformin (~5 mM). Conversely, studies which have used clinically achievable drug concentrations (<100 \(\mu\)M) have not reported these effects (Fontaine 2018; Vial et al. 2019; Wang et al. 2019). Therefore, the aim of this study was to assess the mechanisms behind a potential interaction between metformin and exercise in human skeletal muscle in response to in vivo metformin treatment. To fulfil this, we present data from two randomized studies evaluating the effect of an acute bout of exercise and exercise training with metformin or placebo treatment on different variables suggested to be involved in a potential metformin-exercise interaction (i.e. Complex I-linked respiration, OXPHOS, oxidative stress and AMPK activation).

METHODS
This manuscript includes data from two different studies: **Metex1**, including healthy, normal-weight males and **Metex2**, including overweight/obese, glucose-intolerant individuals (2-hour oral glucose tolerance test (OGTT) glucose concentrations of 7.8-11.0 mmol/l and/or HbA1c of 5.7-6.5 % (39-47 mmol/mol)). Exclusion criteria for both studies (among others) were: prior or current metformin treatment, contraindications to increased levels of physical activity (Pedersen and Saltin 2015), liver cell damage, prior history of lactic acidosis and eGFR<60 ml/min. For detailed description of these criteria, please see (Pilmark et al. 2021b) and (Pilmark et al. 2021a). All individuals underwent a screening, consisting of a medical interview and examination, and a maximal oxygen uptake (VO\textsubscript{2peak}) test on a cycle ergometer (Monark 739E, Varberg, Sweden) using indirect calorimetry (Cosmed Quark, Rome, Italy) as previously described (Pilmark et al. 2021a). Written informed consent was obtained from all participants and the studies were approved by the Ethical Committee of the Capital Region of Denmark (**Metex1**: H-16032037 **Metex2**: H-17012307) and registered at [www.ClinicalTrials.gov](http://www.ClinicalTrials.gov) (**Metex1**: NCT02951260, **Metex2**: NCT03316690).

**Study designs and interventions**

**Metex1** was a double-blind, cross-over, counter-balanced study with two treatment periods performed in randomized order (Figure 1a). The two treatment periods, each lasting 17 days, were identical beside the following treatment: placebo (PLA) or metformin (MET). To minimize gastrointestinal side-effects of metformin and thereby maintaining blinding, MET/PLA treatment was gradually increased until the final dose (1000 mg x 2) was reached after 9 days. This dose was then maintained throughout the remaining treatment period. On the last day of each treatment period (day 17), participants underwent an experimental day including an acute bout of exercise.
Between the two treatment periods, a 4-day washout period was applied before initiation of the next
treatment period.

Metex2 was a double-blind, parallel group, randomized clinical study with two arms (PLA and
MET; Figure 1b). Participants reported to the laboratory for 4 experimental days: BASELINE:
where randomization to PLA/MET was performed, MEDICATION: after three weeks of PLA/MET
treatment (same dose escalation regimen as Metex1), EXERCISE: one week after MEDICATION,
where an acute bout of exercise was performed on MET/PLA treatment, and TRAINING: which
was conducted after 12 weeks of exercise training performed on MET/PLA treatment. The 12
weeks training intervention was initiated immediately after the EXERCISE experimental day. The
exercise training intervention consisted of supervised bouts of interval-type exercise (ergometer
cycling) for 45 min at ~64% of watt\textsubscript{max} (defined as the maximal intensity reached at the baseline
VO\textsubscript{2peak} test) 4 times/week for 12 weeks. Other data from this study have previously been published
(Pilmark et al. 2021b). In both Metex1 and Metex2, participants refrained from any exercise 48 h
prior to each experimental day.

Experimental days

At each experimental day, participants arrived at the laboratory following a standardized breakfast
ingested 1-2 hours before arrival (60 g bun with 20 g cheese (220 kcal: fat 8.8 g, carbohydrates 24.7
g, protein 9.9 g) during which the morning MET/PLA treatment was taken.

Following 30-90 minutes of rest, a skeletal muscle biopsy from m. quadriceps femoris, vastus
lateralis was obtained, see detailed description below (In Metex2, biopsies were only obtained at
EXERCISE and TRAINING).
The acute bout of exercise performed in Metex1 and the EXERCISE experimental day in Metex2 was initiated after the resting skeletal muscle biopsy was obtained. The intended intensity of the exercise bouts was 70% of VO2peak in Metex1 and 60% of Wattpeak in Metex2, both of which were considered to be intense enough to ensure AMPK activation (Kjobsted et al. 2016). The exercise bout lasted for 50 minutes, starting with a 5 minutes warm-up period. At the end of each exercise bout, participants were asked to evaluate the overall rate of perceived exertion (RPE) (Borg et al. 1985). VO2 and respiratory exchange ratio (RER) were assessed continuously during the exercise bouts by indirect calorimetry using a mask and breath-by-breath measurements (Cosmed Quark). External work performed (Joule) was calculated by multiplying intensity (watt) with time (seconds) spend at each intensity. A second muscle biopsy was obtained within 1 minute after termination of the exercise bout while the participant was still sitting on the ergometer cycle.

Furthermore, in Metex2 a VO2peak test and measurement of body weight was conducted at BASELINE, MEDICATION and TRAINING. During the same experimental days, a 24 h collection of urine was performed (from arrival in the laboratory and until next morning) in a cooled urine container. Upon completion, participants returned the urine container, urine volume was measured, and a sample for analysis of markers of systemic oxidative stress was collected and stored at -80°C until analyses.

Skeletal muscle biopsies

Muscle biopsies were obtained using local anesthesia (lidocaine 2%, 5ml) intradermally and subcutaneously to the muscle fascia. At the acute exercise days, anaesthesia for both biopsies was administered before initiation of the exercise bout and a small incision was made in preparation for the second muscle biopsy. The incision was closed with strips and bandaged.
All biopsies were obtained from the left leg using a Bergström needle with suction (Bergström et al. 1967). The biopsy was immediately dissected free of fat and connective tissue. One portion of the muscle tissue was snap-frozen in liquid nitrogen for analysis of AMPK activity and Citrate Synthase (CS) activity (only in Metex2), and afterwards stored at -80°C. Another portion was placed in ice-cold biopsy preservation solution (BIOPS) for respirometry and H$_2$O$_2$ measurements performed on the same day. The measurement of high-resolution respirometry and skeletal muscle H$_2$O$_2$ emission were performed at two distinct laboratories (Metex1: affiliation 2, Metex2: affiliation 1).

**High-resolution respirometry**

**Permeabilized muscle fiber preparation**

After biopsy collection, muscle tissue (20–30 mg) was immediately transferred to ice-cold BIOPS buffer (10 mM Ca-EGTA, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl$_2$, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1). Muscle fibers were mechanically separated into small fiber bundles with fine forceps to maximize surface area and minimize diffusion limitations. Permeabilization of fiber bundles was ensured by 30 min saponin treatment (40 µg/mL in BIOPS) with gentle agitation at 4°C. After permeabilization, fiber bundles were washed 10 min in mitochondrial respiration medium 05 (MiR05) (0.5 mM EGTA, 3 mM MgCl$_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH$_2$PO$_4$, 20 mM Heps, 110 mM sucrose, 1 g/L BSA, pH 7.1) before analyses (Pesta and Gnaiger 2012).

**High-resolution respirometry**
The methodology is described in detail elsewhere (Perry et al. 2013; Pesta and Gnaiger 2012).

Briefly, mitochondrial respiration was measured in duplicate in permeabilized muscle fiber bundles under hyperoxic conditions ([O2] ~200-400 nmol/ml) at 37°C in MiR05 in Metex1 and in MiR06Cr ((MiR05+20 mM creatine + 280 U/mL catalase) in Metex2 using the Oxygraph-2k system (Oroboros Instruments, Innsbruck, Austria). In Metex1, Complex I-supported respiration was measured after simultaneous addition of 5mM pyruvate, 10mM glutamate, 2mM malate and 4mM ADP, while Complex I+II supported maximal respiration (OXPHOS) was measured after addition of 10mM succinate. In the Metex2 study, Complex I and electron-transferring flavoprotein-supported respiration (Complex I+ETF) was measured with the combination of 5mM pyruvate, 10mM glutamate, 2mM malate, 0.25mM octanoyl-carnitine and 5mM ADP. Subsequently 10mM succinate was added to assess Complex I+II+ETF supported OXPHOS (OXPHOS_{ETF}). Mitochondrial outer membrane was assumed compromised if oxygen flux increased > 15% following the titration of 10 µM cytochrome c. Respiratory data which exhibited higher increase were not included in data analysis (11% of all determinations). In both studies mitochondrial oxygen flux was expressed relative to muscle wet weight.

Oxidative stress

Skeletal muscle H2O2 emission

Mitochondrial emission of H2O2 (H2O2 escaping the mitochondrial matrix) was measured in parallel with oxygen flux using the O2k-Fluo LED2-Module (Oroboros Instruments) in buffer Z after addition of 25 µM blebbistatin (Perry et al. 2011) in Metex2 and in MiR05 in Metex1. Horseradish peroxidase (Metex1:4 U/mL, Metex2: 6 U/mL) and Amplex Red (Metex1:10 µM, Metex2:20 µM) were added to the respiration chambers and the H2O2-mediated conversion of Amplex Red to
Resorufin was tracked fluorometrically by excitation/emission at 565/600 nm. Superoxide dismutase (\textbf{Metex1}:30 U/mL, \textbf{Metex2}:45 U/mL) was added to ensure full conversion of superoxide to H$_2$O$_2$.

To correct for nonlinearity of the resorufin fluorescence signal, sensor calibrations were performed by adding 0.1 µM H$_2$O$_2$ between each step in the protocol. In the \textbf{Metex1} study, H$_2$O$_2$ emission is reported in the presence of 2mM malate, 5mM pyruvate, 10mM glutamate and 10mM succinate, while in the \textbf{Metex2} study, H$_2$O$_2$ emission was measured in the presence of 2mM malate, 5mM pyruvate and 10mM succinate (Larsen et al. 2018). In both studies mitochondrial H$_2$O$_2$ emission rates were expressed relative to muscle wet weight.

\textbf{Systemic oxidative stress}

The urine samples were stored at -80°C until analyzed for RNA and DNA oxidative stress markers (8-oxo-7,8-dihydroguanosine (8-oxoGuo) and 8-oxo-7,8-dihydro-2’-deoxyguanosine (8-oxodG)) using a ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) system as previously described (Rasmussen et al. 2016). Urinary excretion of 8-oxoGuo and 8-oxodG was normalized to urine creatinine.

\textbf{AMPK activity}

AMPK trimer specific activity was measured on immunoprepicipitates from 250 and 200µg of muscle lysate protein in \textbf{Metex1} and \textbf{Metex2} respectively as previously described in detail (Birk and Wojtaszewski 2018). All antibodies used were custom-made: Gamma3 and Alfa2 antibodies from MRC PPU Reagents and Services, University of Dundee Scotland, UK and Alfa1, Genscript USA.
Citrate synthase activity

Freeze-dried skeletal muscle tissue (~3 mg ww) was dissected and homogenized (Tissuelyser; Qiagen) in freshly prepared MG homogenization buffer (Meinild Lundby et al. 2018). Samples were then rotated end over end for 1 hour at 4 °C and centrifuged for 30 min at 12,000 rpm, and the lysate was used for further analysis. Total protein concentrations were determined by BCA assay (Pierce, Rockford, IL USA) and CS activity colorometrically by a commercial available kit (C3260, St. Louis, MO, Sigma-Aldrich). All activities were normalized to mg/total protein.

Statistics

Differences in baseline characteristics/acute exercise variables between studies and between treatments within each study were compared using two-tailed Student’s t tests; paired within Metex1 and unpaired between studies and within Metex2. Besides this, no between-study comparisons were performed. A two-way repeated measures ANOVA with the outcome as dependent variable and treatment (two levels) and time (two levels) as independent variables (fixed effects) and with the unique patient identifier as random effect was performed for all variables. Standard model diagnostics were used to assess the adequacy of the model. The within-treatment differences are presented as least square means with Bonferroni-corrected (2 comparisons according to the different treatments) 95% CIs and p-values. Between-treatment difference in change (post-intervention minus pre-intervention) was assessed by the treatment*time interaction, but since no significant treatment*time interactions were found for any variable, quantitative data is not reported.

All statistical analyses were performed by Prism version 8 (GraphPad). Statistical significance was accepted with $p<0.05$ (two-sided).
RESULTS

Participants:

Metex1: Fifteen participants were included and completed the study. Seven participants had PLA as their first treatment, whereas eight had MET as their first treatment. The study procedures took place from October 2016 to April 2017 (Pilmark et al. 2021a).

Metex2: Thirty-four participants were included in the study. Five participants did not complete (4 in MET, 1 in PLA), resulting in 29 participants completing the study (15 PLA, 14 MET). The compliance to both medicine (PLA 99.6 ± 0.6%, MET 99.3 ± 1.1%, p = 0.4) and the training intervention (PLA 98.5 ± 5.4%, MET 97.8 ± 8.1%, p = 0.8) was high in both groups.

The study procedures took place from October 2017 to September 2018 (Pilmark et al. 2021b).

As expected, based on the differential in- and exclusion criteria, baseline differences were encountered between the two studies, Table 1.

Acute exercise bouts:

In both studies the acute exercise bouts were performed close to the intended 70% of VO$_2$peak, with no differences between treatments. Moreover, no differences in VO$_2$, external work, mean watt, heart rate (HR) or RER were seen between treatments. In Metex1, RPE was significantly higher in MET than in PLA, as previously reported (Pilmark et al. 2021a). In Metex2, no difference in RPE was seen between treatments (Table 2). A significant between-study difference was seen for all variables except RER, with the Metex1 exercise bout being performed with higher mean VO$_2$, external work, HR and lower RPE and percent of VO$_2$peak compared to the Metex2 exercise bout (Table 2).
**Metex2 Exercise training:**

The training compliance (amount of training volume performed relative to prescribed) was high (98.1±1.2%) and did not differ between treatments. The training intervention was performed close to the intended 60-65% of Watt$_{\text{max}}$, 63.7±0.4 watt) with no difference between treatments. No difference between treatments was seen in mean HR or RPE (Table 3+4). As previously reported, an average increase in VO$_2$peak of 15% (4.6 [95% CI 3.3;5.9] ml·kg$^{-1}$·min$^{-1}$, $p<0.0001$), with no between-group difference ($\Delta$PLA 4.8 [3.0;6.6] ml/kg/min and $\Delta$MET 4.3 [2.5;6.2] ml/kg/min), was observed after the training intervention (Pilmark et al. 2021b). Furthermore the training intervention resulted in a solid weight reduction $\Delta$PLA -3.7 [-5.9;−1.5] kg $p=0.001$, $\Delta$MET -3.5 [-6.0;−0.9] kg, $p=0.008$, with no between-group difference (0.2 [-2.3;2.7] kg, $p=0.8$).

**High resolution respirometry**

**Metex1**: No difference between treatment periods was present at rest (Complex I-linked respiration: $P=0.8$; OXPHOS: $P=0.8$) and no within-treatment changes were seen in either Complex I-linked respiration ($\Delta$PLA: 0.1 [-3.7;4.0], $P>0.99$; $\Delta$MET: -0.2 [-4.1;3.7] pmol/(s·mg, $P>0.99$) or OXPHOS ($\Delta$PLA: 0.6 [-6.7;7.9], $P>0.99$, $\Delta$MET: -3.6 [-10.9;3.7], $P=0.5$) pmol/(s·mg) following acute exercise, nor were any significant treatment*time interactions seen (Complex I-linked respiration: $P=0.9$; OXPHOS: $P=0.1$) (Figure 2, a+b).

**Metex2**: No difference between treatments was present at rest (Complex I+ETF-linked respiration: $P=0.3$; OXPHOS$_{\text{ETF}}$: $P=0.6$). No changes in either Complex I+ETF-linked respiration ($\Delta$PLA: 6.0 [-2.9;14.8], $P=0.2$; $\Delta$MET: -0.3 [-10.9;10.4] pmol/(s·mg), $P>0.99$) or OXPHOS$_{\text{ETF}}$ ($\Delta$PLA: 9.2 [-4.9;23.3], $P=0.3$; $\Delta$MET: 8.4 [-8.5;25.4] pmol/(s·mg), $P=0.5$) were seen with exercise training.
(Figure 2, panel c+d), nor were any significant treatment*time interactions present (Complex I+ETF-linked respiration: $P=0.3$; OXPHOS$_{ETF}$: $P=0.9$).

**Skeletal muscle H2O2 emission**

**Metex1**: No difference between treatment periods were present at rest ($P=0.14$). A significant decrease in H$_2$O$_2$ emission with an acute exercise was seen in PLA ($\Delta$PLA: $-0.2 [-0.4;-0.1]$ pmol/(s·mg ww), $P=0.002$), whereas no significant difference was seen in MET ($\Delta$MET: $-0.1 [-0.2;0.0]$ pmol/(s·mg ww, $P=0.3$) (Figure 3, panel a). A significant effect of time ($P=0.001$), but no significant treatment*time interaction was present ($P=0.1$).

**Metex2**: No difference between treatments was seen at rest ($P=0.4$). No changes in H$_2$O$_2$ emission was seen with acute exercise ($\Delta$PLA: $-0.0 [-0.2;0.1]$, $P=0.99$; $\Delta$MET: $-0.1 [-0.2;0.1]$ pmol/(s·mg ww), $P=0.3$) (Figure 3, panel b) or with exercise training ($\Delta$PLA: $0.0 [-0.2;0.2]$, $\Delta$MET: $0.0 [-0.2;0.2]$ pmol/(s·mg ww), $P>0.9$ (Figure 3, panel c), and no significant treatment*time interactions were seen (acute exercise: $P=0.4$; exercise training: $P>0.99$).

**Systemic oxidative stress**

**Metex2**: No differences between treatments were seen at rest (8-oxoGuo: $P=0.6$; 8-oxo-dG: $P=0.8$). No changes were seen with exercise training for either 8-oxoGuo ($\Delta$PLA: $-0.1 [-0.4;0.1]$, $P=0.3$; $\Delta$MET: $0.1 [-0.1;0.3]$ nmol/mmol, $P=0.6$) or 8-oxodG ($\Delta$PLA: $-0.1 [-0.3;0.1]$, $P=0.4$; $\Delta$MET: $-0.1 [-0.3;0.1]$ nmol/mmol, $P=0.3$), and no significant treatment*time interaction was present (8-oxoGuo: $P=0.1$; 8-oxo-dG: $P=0.9$) (Figure 3 d+e).
Citrate synthase activity

Metex2: No difference between treatments was present at rest ($P=0.4$). An increase in CS activity was seen following exercise training ($\Delta$PLA: 160 [88;231], $P<0.001$; $\Delta$MET: 86 [11;161] mIU/mg protein, $P=0.02$), with a significant effect of time $P<0.001$, but with no significant treatment*time interaction ($P=0.10$) (Figure 2, panel e). No changes within- or between treatments in either Complex I+ETF-linked respiration or OXPHOS$_{ETF}$ were seen when data were normalized to CS activity (please see supplementary material S1).

AMPK activity

Metex1: No differences between treatments were seen at rest (AMPK $\alpha_1\beta_2\gamma_1$: $P=0.9$; AMPK $\alpha_2\beta_2\gamma_1$: $P=0.2$; AMPK $\alpha_2\beta_2\gamma_3$: $P=0.9$). No difference in AMPK $\alpha_1\beta_2\gamma_1$ ($\Delta$PLA: -0.1 [-0.2;0.1], $P>0.99$; $\Delta$MET: -0.1 [-0.2;0.1] pmol·mg$^{-1}$·min$^{-1}$, $P>0.99$) or AMPK $\alpha_2\beta_2\gamma_1$ ($\Delta$PLA: 0.1 [-0.1;0.2] pmol·mg$^{-1}$·min$^{-1}$, $P=0.5$; $\Delta$MET: -0.0 [-0.2;0.1] pmol·mg$^{-1}$·min$^{-1}$, $P>0.99$) was seen following acute exercise. Conversely, a significant increase in AMPK $\alpha_2\beta_2\gamma_3$ ($\Delta$PLA: 2.8 [1.4;4.2] pmol·mg$^{-1}$·min$^{-1}$, $P<0.001$, $\Delta$MET: 2.2 [0.8;3.6] pmol·mg$^{-1}$·min$^{-1}$, $P=0.002$) was observed. A significant effect of time in AMPK $\alpha_2\beta_2\gamma_3$ was observed ($P<0.0001$), whereas no significant treatment*time interactions were seen (AMPK $\alpha_1\beta_2\gamma_1$: $P>0.99$; AMPK $\alpha_2\beta_2\gamma_1$: $P=0.2$; AMPK $\alpha_2\beta_2\gamma_3$: $P=0.4$). (Figure 4, panel a-c).

Metex2: While MET had higher AMPK $\alpha_1\beta_2\gamma_1$ at rest than PLA (PLA 0.4±0.2, MET 0.6±0.3 pmol·mg$^{-1}$·min$^{-1}$, $P=0.03$), no differences between treatments were seen in AMPK $\alpha_2\beta_2\gamma_1$ ($P=0.2$) or AMPK $\alpha_2\beta_2\gamma_3$ ($P=0.8$) at rest (Figure 4, panel d-f).

No changes in AMPK $\alpha_1\beta_2\gamma_1$ was observed with acute exercise in PLA ($\Delta$PLA: -0.1 [-0.3;0.1] pmol·mg$^{-1}$·min$^{-1}$, $P=0.3$), whereas a significant decrease was seen in MET ($\Delta$MET: -0.3 [-0.4;-0.1] pmol·mg$^{-1}$·min$^{-1}$, $P=0.006$). No changes in AMPK $\alpha_2\beta_2\gamma_1$ ($\Delta$PLA: -0.1 [-0.3;0.2] pmol·mg$^{-1}$·min$^{-1}$,
$P>0.99$; $\Delta\text{MET}: -0.2 \,[{-0.5};0.0] \,\text{pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}, P=0.1$) was seen with acute exercise. A significant increase in AMPK $\alpha_2\beta_2\gamma_3$ was observed with acute exercise independent of treatment ($\Delta\text{PLA}: 2.5\,[1.0;4.0] \,\text{pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}, P<0.001$; $\Delta\text{MET}: 1.6 \,[0.2;3.0] \,\text{pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}, P=0.003$). A significant effect of time in AMPK $\alpha_1\beta_2\gamma_1$, $P=0.003$ and $\alpha_2\beta_2\gamma_3 \,P<0.0001$ was observed, whereas no significant treatment*time interactions were seen ($\Delta\text{PLA}: 0.2 \,\text{pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}, P=0.3$; $\Delta\text{MET}: 0.2 \,\text{pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}, P=0.2$) (Figure 4, panel f).

When investigating the alterations in exercise-induced AMPK, a significant increase in AMPK $\alpha_1\beta_2\gamma_1$ was observed ($\Delta\text{PLA}: 0.4 \,[0.2;0.7] \,\text{pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}, P<0.001$, $\Delta\text{MET}: 0.4 \,[0.2;0.7] \,\text{pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}, P<0.001$), whereas no changes were seen in either AMPK $\alpha_2\beta_2\gamma_1$ ($\Delta\text{PLA}: 0.1 \,[-0.2;0.4], P=0.7$; $\Delta\text{MET}: 0.0 \,[-0.3;0.3] \,\text{pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}, P>0.99$) or AMPK $\alpha_2\beta_2\gamma_3$ ($\Delta\text{PLA}: 0.0 \,[-0.3;0.3] \,\text{pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}, P>0.99$; $\Delta\text{MET}: 0.1 \,[-0.2;0.4] \,\text{pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}, P>0.99$). Moreover, a significant effect of time in AMPK $\alpha_1\beta_2\gamma_1$, $P<0.0001$ was observed, but no treatment*time interactions were seen ($\Delta\text{PLA}: 0.9 \,\text{pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}, P=0.8$; $\Delta\text{MET}: 0.3 \,\text{pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}, P=0.3$; $\Delta\text{MET}: 0.2 \,\text{pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}, P=0.2$) (Figure 4, panel g-i).

**DISCUSSION**

In this study, no indications of a pharmaco-physiological interaction between metformin and exercise in human skeletal muscle was seen. As such, metformin did not have additive or detrimental effects of acute exercise or exercise training on mitochondrial respiratory capacity, oxidative stress or AMPK activation. The inclusion of two different studies with different populations but still with comparable findings increases the strength of the results. Since previously published data from the included studies have suggested a pharmaco-physiological interaction between metformin and exercise (Pilmark et al. 2021b) it may be speculated that the mechanistic
basis for this interaction should be sought elsewhere than skeletal muscle, something which should be addressed in future studies.

To our knowledge, only one other study assessing a metformin-exercise interaction on mitochondrial respiration after metformin treatment has been conducted in humans. In that study (Konopka et al. 2019), MET hampered the 12 weeks exercise training-induced increases in Complex I-linked submaximal respiration compared to PLA. Furthermore, a numerical increase in PLA, but not in MET, was found when assessing maximal Complex I-linked respiration after 12 weeks of exercise training. Whereas we did not measure submaximal respiration in our study, maximal Complex I+ETF-linked respiration showed a similar numerical increase in PLA but no changes in MET after 12 weeks of exercise training (Fig 2, panel c).

Unexpectedly, no statistically significant increases in OXPHOS$_{ETF}$ were seen following 12 weeks of exercise training despite the significant increase in CS-activity. When mitochondrial respiration parameters were normalized to CS activity, there were still no significant changes in mitochondrial respiration parameters. Although this lack of robust increase in OXPHOS$_{ETF}$ with exercise training cannot readily be explained, the finding is consistent with data from previous studies (Konopka et al. 2019; Meinild Lundby et al. 2018). Given that assessments of mitochondrial respiration typically have considerable variation, however, we cannot rule out that the lack of improvements represents a statistical type 2 error.

No matter the reason, the lack of effects of exercise training on mitochondrial respiratory capacity in the placebo group in Metex2 complicates the interpretation of data, given that a potential hampering effect of metformin on training adaptations cannot be shown. Since the training intervention was highly controlled, quite intense and with great adherence, revealing robust exercise training-induced improvements in other variables than mitochondrial respiration, however, we
believe that the lack of robust changes in mitochondrial respiration after exercise training in the
placebo group is a correct finding which could indicate that the mechanistic basis for a potential
interaction between metformin and exercise should be sought elsewhere than in skeletal muscle.

Skeletal muscle H$_2$O$_2$ emission after acute exercise and exercise training was not affected by
metformin treatment neither in healthy lean males nor in glucose-intolerant individuals. Whereas an
inhibitory effect of metformin on H$_2$O$_2$ emission has been found in vitro (Batandier et al. 2006;
Kelly et al. 2015) and a blunting of exercise-induced increases in H$_2$O$_2$ emission with metformin
treatment has been suggested (Malin and Braun 2016), no human in vivo studies have, to our
knowledge, addressed this until now. Because there was no blunting of whole-body oxidative stress
with metformin treatment, a metformin-exercise interaction on H$_2$O$_2$ emission in vivo seems
unlikely. The above-mentioned differences between metformin concentration used for treatment in
human studies and in vitro may potentially explain the conflicting results.

The effect of acute exercise on skeletal muscle H$_2$O$_2$ emission differed between populations. While
no effects were seen in glucose-intolerant individuals, a decrease in H$_2$O$_2$ emission in response to
acute exercise was seen in healthy males. Although unknown, we speculate that younger,
metabolically healthier individuals have a greater intracellular antioxidative response to exercise,
and that this may explain the differences observed (Ji 2001). Alternatively, differences in the
methods applied may be causative.

A significant difference in baseline AMPK α1β2γ1 activity was seen in glucose intolerant
individuals after 3 weeks of metformin or placebo treatment, suggesting that metformin might lead
to activation of AMPK α1β2γ1. However, given that no difference in baseline AMPK α1β2γ1 was
seen in healthy lean males after metformin vs. placebo treatment, we cannot rule out that this is a
random finding.
A robust exercise-induced increase in AMPK α2β2γ3 activation was seen in both healthy lean males and glucose-intolerant individuals but metformin did not affect this AMPK activation. Furthermore, a training-induced increase in AMPK α1β2γ1 activity was seen in glucose-intolerant individuals independent of treatment. The training-induced increase in AMPK activation is consistent with existing literature (Kjobsted et al. 2016), whereas the importance of metformin is less well agreed upon. Hence, a study by Kristensen et al (Kristensen et al. 2019) reported no effect of short term metformin treatment on AMPK activation after acute exercise in healthy males, whereas a study by Sharoff et al (Sharoff et al. 2010) reported a reduced increase in AMPK activity with exercise when combined with metformin treatment. Importantly, in the Sharoff study, AMPK activity was assessed as the sum of AMPK α2β2γ3 activity and AMPK α2β2γ1 activity (termed AMPK α2). When the same analysis (AMPK α2) was performed in glucose-intolerant individuals (Metex2), a similar (although not as pronounced) response was seen, meaning that metformin blunted the increased AMPK α2 activity seen with acute exercise. This was not the case in healthy, young males (Metex1) (data not shown). Whether this is due to differences in populations, and whether an even greater attenuation would have been seen if patients had been even more glucose-intolerant needs further investigation.

Strengths and limitations

Important strengths of this study include the human in vivo setting and the use of clinically relevant doses of metformin. Another important strength is the highly controlled exercise/exercise training interventions which were comparable between MET and PLA. Finally, the inclusion of two different populations increases the robustness of the findings.
A limitation of the study is the different respirometry and H\textsubscript{2}O\textsubscript{2} protocols applied. Specifically, creatine was added to the respiration medium in Metex2, but not in Metex1, which may have resulted in a higher respiratory capacity in the former (Walsh et al. 2001). Similarly, in Metex2, octanoyl-carnitine, supplying electrons into the Q-junction via not only Complex I but also via electron-transferring flavoprotein, was added, which may also have led to a higher respiratory capacity as compared to Metex1. Apart from that, the concentration of added ADP was 4mM in Metex1 and 5mM in Metex2, which, however, has most likely not influenced the respiratory results as both concentrations exceed a saturating ADP concentration (Perry et al. 2011). Another limitation of the present study is the short-term metformin treatment regime, which prevents the evaluation of potential long-term effects of metformin treatment on exercise training-induced adaptations in skeletal muscle markers.

When considering other tissues where a potential interaction between metformin and exercise might be present, the liver has traditionally been considered central for the effects of metformin. In the recent years, increasing evidence points towards the gut being important for the metformin effects on glucose metabolism (Bailey et al. 1994; Buse et al. 2016; McCreight et al. 2016; Wu et al. 2017), and we have previously suggested that a potential interaction between metformin and physical activity may be found here (Pilmark et al. 2021b). As such, this should be explored in future studies.

In summary, metformin does not seem to have any additive or detrimental effect on exercise-induced alterations in mitochondrial markers, H\textsubscript{2}O\textsubscript{2} emission or AMPK activation in human skeletal muscle. Further studies are needed to investigate whether an interaction between metformin and physical activity is present in other tissues, e.g. the gut.
Acknowledgements: The authors would like to thank the participants involved in both studies for great commitment, Lene Foged, Anne Jørgensen and Ida Holm, Centre for Physical Research Activity (CFAS), Copenhagen, for technical assistance and Jesper Christensen and Mathias Ried-Larsen (CFAS) for statistical guidance. Furthermore, we want to thank Marc Donath, Basel Universitäts hospital, Switzerland for input to study design and analysis and Carsten Lundby for help in designing the training intervention.

Data availability: Data from the study are available from the corresponding author on reasonable request, if this does not interfere with the regulations from the Danish Data Protection Agency.

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Authors’ relationships and activities: The authors declare that there are no relationships or activities that might bias, or be perceived to bias, their work.

Contribution statement: NSP and KK wrote the manuscript. NSP and KK performed the statistical analysis. KK, NSP, JFH, JMK and AKML conceptualized and designed the analysis. NSP and KK obtained funding. NSP, LO, CPB, IE, ML, GE, CS and NFRH contributed to data collection, data analysis/processing and/or data quality control procedures. JFH, JMK, JB, AKML, JW and HP contributed to data analysis/processing and/or data quality control procedures of skeletal muscle biopsy variables. EL and HEP contributed to data analysis/processing and/or data quality control procedures of systemic oxidative stress measurements. All authors approved the final version of the manuscript. All authors accept responsibility for all aspects of the work insofar
as ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved. KK is responsible for the integrity of the work as a whole.


Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>Metex1 PLA</th>
<th>Metex1 MET</th>
<th>Metex2</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>15</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>15/0</td>
<td>5/10</td>
<td>9/5</td>
</tr>
<tr>
<td>Age (years)</td>
<td>23.7±0.6</td>
<td>51±13</td>
<td>48±7</td>
</tr>
<tr>
<td><strong>Physical fitness (VO_{2peak})</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO_{2peak} absolute (l/min) $^\dagger$</td>
<td>3.5±0.6</td>
<td>2.6±0.5</td>
<td>3.0±0.6</td>
</tr>
<tr>
<td>VO_{2peak} relative (ml/kg$^{-1}$ min$^{-1}$) $^\dagger$</td>
<td>46.5±4.9</td>
<td>24±4</td>
<td>28±6</td>
</tr>
<tr>
<td><strong>Body composition</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (kg) $^\dagger$</td>
<td>75.3±9.4</td>
<td>109±15</td>
<td>108±16</td>
</tr>
<tr>
<td>Height (m) $^\dagger$</td>
<td>1.84±0.1</td>
<td>1.72±2</td>
<td>1.77±2</td>
</tr>
<tr>
<td>BMI (kg/m$^2$) $^\dagger$</td>
<td>22.3±2.0</td>
<td>37±4</td>
<td>34±5</td>
</tr>
<tr>
<td><strong>Glycemic control</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting glucose (mmol/l) $^\dagger$</td>
<td>4.6±0.3</td>
<td>5.7±0.9</td>
<td>5.9±1.0</td>
</tr>
<tr>
<td>2 h OGTT glucose (mmol/l) $^\dagger$</td>
<td>5.6±1.1</td>
<td>9.5±2.3</td>
<td>8.3±3.0</td>
</tr>
<tr>
<td>HbA$_{1c}$ (mmol/mol) $^\dagger$</td>
<td>32.7±3.0</td>
<td>40.6±4.4</td>
<td>39.7±5.0</td>
</tr>
<tr>
<td>HbA$_{1c}$ (%) $^\dagger$</td>
<td>5.1±0.3</td>
<td>5.9±0.4</td>
<td>5.7±0.5</td>
</tr>
</tbody>
</table>

Baseline characteristics are presented as mean±SD. Abbreviations: BMI, body mass index; VO$_{2peak}$, maximal oxygen consumption; HbA$_{1c}$, hemoglobin A1c; OGTT, oral glucose tolerance test; $^\dagger$ P<0.05 between-study difference. No between-group differences were seen between PLA and MET in Metex2.
Table 2. Characterization of the acute exercise bouts

<table>
<thead>
<tr>
<th></th>
<th>Metex1</th>
<th>Metex2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PLA</td>
<td>MET</td>
</tr>
<tr>
<td>EXERCISE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean watt(^b)</td>
<td>166±20</td>
<td>169±19</td>
</tr>
<tr>
<td>Mean VO(_2) (l/min)(^b)</td>
<td>2.6±0.1</td>
<td>2.6±0.1</td>
</tr>
<tr>
<td>% of screening VO(_{2peak})(^b)</td>
<td>71.3±7.1</td>
<td>72.2±6.2</td>
</tr>
<tr>
<td>External work (kJ)(^b)</td>
<td>433±47</td>
<td>442±57</td>
</tr>
<tr>
<td>RER</td>
<td>0.89±0.03</td>
<td>0.90±0.05</td>
</tr>
<tr>
<td>Mean heart rate (bpm)(^b)</td>
<td>158±15</td>
<td>164±14</td>
</tr>
<tr>
<td>Mean RPE(^b)(^*)</td>
<td>14.0±1.1</td>
<td>14.8±1.3</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD. Abbreviations: VO\(_{2peak}\), peak oxygen consumption rate; RER, respiratory exchange ratio; RPE, rate of perceived exertion. \(^b\) indicates between-study difference (P<0.05); \(^*\) indicates between-treatment difference in Metex1 (P<0.05). No significant between-treatment differences were seen in Metex2.
Table 3. Characterization of the training intervention in Metex2

<table>
<thead>
<tr>
<th>TRAINING INTERVENTION</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Week</td>
<td>1-2</td>
<td>3-5</td>
<td>6-12</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>PLA</td>
<td>MET</td>
<td>PLA</td>
<td>MET</td>
</tr>
<tr>
<td>Mean % of Watt\textsubscript{max}</td>
<td>57.7±2.9</td>
<td>56.6±2.5</td>
<td>64.0±2.8</td>
<td>63.3±3.3</td>
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<tr>
<td>HR (bpm)</td>
<td>144.2±9.7</td>
<td>149.4±11.6</td>
<td>142.2±10.4</td>
<td>150.8±11.5</td>
</tr>
<tr>
<td>RPE</td>
<td>15.3±1.6</td>
<td>15.2±1.1</td>
<td>16.1±1.2</td>
<td>16.3±1.3</td>
</tr>
<tr>
<td>Compliance (%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD. Abbreviations: HR, heart rate; RPE, rate of perceived exertion. Compliance is calculated as % of completion the total planned exercise volume (48 training sessions/participant).
Table 4 Effects of training intervention in Metex2.

<table>
<thead>
<tr>
<th></th>
<th>PRE</th>
<th></th>
<th>POST</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>PLA</td>
<td>MET</td>
<td>PLA</td>
<td>MET</td>
</tr>
<tr>
<td>VO_{peak} (l/min)</td>
<td>2.6±0.6</td>
<td>2.9±0.6</td>
<td>3.0±0.5</td>
<td>3.3±0.8*</td>
</tr>
<tr>
<td>VO_{peak} (ml kg(^{-1}) min(^{-1}))</td>
<td>23.5±4.8</td>
<td>27.5±6.9</td>
<td>28.3±4.3</td>
<td>31.9±8.1*</td>
</tr>
<tr>
<td>Watt_{max} (watt)</td>
<td>195.0±54</td>
<td>233.4±58.1</td>
<td>247.1±51.5</td>
<td>279.1±73.9*</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>108.6±15</td>
<td>107.7±16</td>
<td>105.0±14*</td>
<td>103.4±15*</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD. *P<0.05 Pre-post difference. No between-treatment differences were observed.
Figure 1 Study designs


Figure 2 Respirometry and CS activity

Markers related to mitochondrial respiration were measured in skeletal muscle biopsies obtained before and after acute exercise (panel a-b) and before and after an exercise training intervention (panel c-e).

Panel a: Oxygen consumption rate with substrates supporting Complex 1-linked respiration (malate, glutamate, pyruvate, ADP) before and after the acute bout of exercise in Metex1. Panel b: with substrates supporting OXPHOS (malate, glutamate, pyruvate, ADP and succinate) before and after the acute bout of exercise in Metex1. Panel c: with substrates supporting Complex 1 and ETF-linked respiration (malate, octanoyl-carnitine, glutamate, pyruvate and ADP) before and after the 12 weeks training intervention in Metex2. Panel d: with substrates supporting OXPHOS+ETF (malate, octanoyl-carnitine, glutamate, pyruvate, ADP and succinate) before and after the 12 weeks training intervention in Metex2. Panel e: CS activity before and after the 12 weeks training intervention in Metex2. All data are presented as means ± SEM. Abbreviations: CS, citrate synthase; ETF, electron-transferring flavoprotein; JO₂, oxygen consumption; OXPHOS, oxidative phosphorylation; All statistical significant findings are indicated in the figures. *indicates p<0.05 within group. For detailed statistical analysis see text.

Figure 3. Oxidative stress
Markers related to mitochondrial oxidative stress were measured in skeletal muscle biopsies obtained before and after acute exercise (panel a-b) and before and after an exercise training intervention (panel c). Markers related to systemic oxidative stress were measured before and after an exercise training intervention (panel d-e) Panel a: Mean mitochondrial H$_2$O$_2$ emission before and after the acute bout of exercise in Metex1. Panel b: mean mitochondrial H$_2$O$_2$ emission before and after the acute bout of exercise in Metex2. Panel c: mean mitochondrial H$_2$O$_2$ emission before and after the 12 weeks training intervention in Metex2. Panel d: mean urine 8-oxoGuo/creatinine before and after the 12 weeks training intervention in Metex2. Panel e: mean urine 8-oxodG/creatinine before and after the 12 weeks training intervention in Metex2. All data are presented as means ± SEM. H$_2$O$_2$: Hydrogen peroxide. All statistically significant findings are included in the figure. *indicates p<0.05 within group. For detailed statistical analysis see text.

**Figure 4. AMPK activity**

Markers related to AMPK activity were measured in skeletal muscle biopsies obtained before and after acute exercise (panel a-f) and before and after an exercise training intervention (panel g-i).

Panel a: AMPK α1β2γ1 activity before and after the acute bout of exercise in Metex1. Panel b AMPK α2β2γ1 activity before and after the acute bout of exercise in Metex1. Panel c: AMPK α2β2γ3 activity before and after the acute bout of exercise in Metex1. Panel d: AMPK α1β2γ1 activity before and after the acute bout of exercise in Metex2. Panel e: AMPK α2β2γ1 activity before and after the acute bout of exercise in Metex2. Panel f: AMPK α2β2γ3 activity before and after the acute bout of exercise in Metex2. Panel g: AMPK α1β2γ1 activity before and after the 12 weeks training intervention in Metex2. Panel h: AMPK α2β2γ3 activity before and after the 12 weeks training intervention in Metex2. Panel i: AMPK α2β2γ3 activity before and after the 12 weeks training intervention in Metex2.
weeks training intervention in **Metex2**. All data are presented as means ± SEM. All statistically significant findings are included in the figure. *indicates p<0.05 within group. † indicates p<0.05 between groups at baseline. For detailed statistical analysis see text.
Markers related to mitochondrial respiration were measured in skeletal muscle biopsies obtained before and after acute exercise (panel a-b) and before and after an exercise training intervention (panel c-e).

Panel a: Oxygen consumption rate with substrates supporting Complex 1-linked respiration (malate, glutamate, pyruvate, ADP) before and after the acute bout of exercise in Metex1. Panel b: with substrates supporting OXPHOS (malate, glutamate, pyruvate, ADP and succinate) before and after the acute bout of exercise in Metex1. Panel c: with substrates supporting Complex 1 and ETF-linked respiration (malate, octanoyl-carnitine, glutamate, pyruvate and ADP) before and after the 12 weeks training intervention in Metex2. Panel d: with substrates supporting OXPHOS+ETF (malate, octanoyl-carnitine, glutamate, pyruvate, ADP and succinate) before and after the 12 weeks training intervention in Metex2. Panel e: CS activity before and after the 12 weeks training intervention in Metex2. All data are presented as means ± SEM.

Abbreviations: CS, citrate synthase; ETF, electron-transferring flavoprotein; JO2, oxygen consumption; OXPHOS, oxidative phosphorylation; All statistical significant findings are indicated in the figures. *indicates p<0.05 within group. For detailed statistical analysis see text.
Markers related to mitochondrial oxidative stress were measured in skeletal muscle biopsies obtained before and after acute exercise (panel a-b) and before and after an exercise training intervention (panel c).

Markers related to systemic oxidative stress were measured before and after an exercise training intervention (panel d-e) Panel a: Mean mitochondrial H2O2 emission before and after the acute bout of exercise in Metex1. Panel b: mean mitochondrial H2O2 emission before and after the acute bout of exercise in Metex2. Panel c: mean mitochondrial H2O2 emission before and after the 12 weeks training intervention in Metex2. Panel d: mean urine 8-oxoGuo/creatinine before and after the 12 weeks training intervention in Metex2. Panel e: mean urine 8-oxodG/creatinine before and after the 12 weeks training intervention in Metex2. All data are data are presented as means ± SEM. H2O2: Hydrogen peroxide. All statistically significant findings are included in the figure. *indicates p<0.05 within group. For detailed statistical analysis see text.
Markers related to AMPK activity were measured in skeletal muscle biopsies obtained before and after acute exercise (panel a-f) and before and after an exercise training intervention (panel g-i). Panel a: AMPK α1β2γ1 activity before and after the acute bout of exercise in Metex1. Panel b: AMPK α2β2γ1 activity before and after the acute bout of exercise in Metex1. Panel c: AMPK α2β2γ3 activity before and after the acute bout of exercise in Metex1. Panel d: AMPK α1β2γ1 activity before and after the acute bout of exercise in Metex2. Panel e: AMPK α2β2γ1 activity before and after the acute bout of exercise in Metex2. Panel f: AMPK α2β2γ3 activity before and after the acute bout of exercise in Metex2. Panel g: AMPK α1β2γ1 activity before and after the 12 weeks training intervention in Metex2. Panel h: AMPK α2β2γ3 activity before and after the 12 weeks training intervention in Metex2. Panel i: AMPK α2β2γ3 activity before and after the 12 weeks training intervention in Metex2. All data are data are presented as means ± SEM. All statistically significant findings are included in the figure. *indicates p<0.05 within group. † indicates p<0.05 between groups at baseline. For detailed statistical analysis see text.