1 **TITLE:** Semaglutide-induced weight loss improves mitochondrial energy efficiency in skeletal

2 muscle.

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28   **WORD COUNT:** 4824 words (Max 4000 words)

29

30   **KEYWORDS:** semaglutide, weight loss, obesity, energy efficiency, OXPHOS, mitochondria

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32   **RUNNING TITLE:** The effect of semaglutide on muscle energy efficiency

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34   **DISCLOSURE:** The authors declared no conflict of interest.

35

36   **AUTHOR CONTRIBUTIONS**

37   R.H.C, T.K, A.C, and K.F. designed the study. R.H.C, T.K, C.A.M, K.H.-F-W performed the

38   mouse and mitochondrial experiments, and analyzed data. J.A.M. and J.E.C conducted

39   lipidomics analyses. A.M, J.E.C, K.H.F-W conducted proteomic analysis. L.S.N assisted with

40   electron microscopic images. R.H.C and K.F wrote the manuscript with edits from all authors.

41

42

43 **What is already known about this subject?**

44 -GLP-1 receptor agonists successfully reduce adiposity in mice and in humans.

45 -In rodents, dietary intervention-induced weight loss improves skeletal muscle OXPHOS energy

46 efficiency.

47 **What are the new findings in your manuscript?**

48 -Semaglutide-induced weight loss also increased muscle mitochondrial OXPHOS efficiency in

49 mice.

50 - Semaglutide-induced increase in mitochondrial efficiency was only observed in permeabilized

51 muscle fibers but not in isolated mitochondria.

52 **How might your results change the direction of research or the focus of clinical practice?**

53 - GLP-1 receptor agonists likely promote reduced energy expenditure in a similar fashion to

54 dietary intervention-induced weight loss. This may influence their propensity for weight regain or

55 other beneficial effects of increasing energy expenditure.

56 **Abstract**

57 **Objective:** Glucagon-like peptide 1 receptor agonists (e.g. semaglutide) potently induce weight

58 loss and thereby reducing obesity-related complications. However, weight regain occurs when

59 treatment is discontinued. An increase in skeletal muscle oxidative phosphorylation (OXPHOS)

60 efficiency upon diet-mediated weight loss has been described, which may contribute to reduced

61 systemic energy expenditure and weight regain. We set out to determine the unknown effect of

62 semaglutide on muscle OXPHOS efficiency.

63 **Methods:** C57BL/6J mice were fed a high-fat diet for 12 weeks before receiving semaglutide or

64 vehicle for 1 or 3 weeks. The rate of ATP production and O_2 consumption were measured by a

65 high-resolution respirometry and fluorometry to determine OXPHOS efficiency in skeletal

66 muscle at these 2 timepoints.

67 **Results:** Semaglutide treatment led to significant reductions in fat and lean mass. Semaglutide

68 improved skeletal muscle OXPHOS efficiency, measured as ATP produced per O_2 consumed

69 (P/O) in permeabilized muscle fibers. Mitochondrial proteomic analysis revealed changes

70 restricted to two proteins linked to complex III assembly (Lyrm7 and Ttc1, $p < 0.05$ without

71 multiple corrections) without substantial changes in the abundance of OXPHOS subunits.

72 **Conclusions:** These data indicate that weight loss with semaglutide treatment increases

73 skeletal muscle mitochondrial efficiency. Future studies could test whether it contributes to

74 weight regain.

75

Introduction

Obesity predisposes individuals to a broad spectrum of diseases, including type 2 diabetes and cancer. Modest (5-10%) weight loss improves clinical parameters associated with obesity-related diseases (1). Lifestyle interventions can successfully induce ≥5% weight loss from an overweight state; however, maintaining a weight-reduced state has been extremely challenging. Almost 80% of people who have undergone weight loss by various methods is expected to undergo a weight regain phase over the next 5 years (2). Combating weight rebound for sustainable weight management remains a primary challenge, and is compounded by environmental, behavioral, and physiological factors that promote regain.

Weight loss from an overweight state increases energy efficiency thereby lowering the fuel cost during low levels of physical activity (3-5). This metabolic adaptation persists for several years following weight loss and makes individuals susceptible to significant weight regain (3, 6). It has been demonstrated that increased skeletal muscle work efficiency during as well as after weight loss contributes to decreased energy expenditure (4). Potential mechanisms underlying metabolic adaptations in the weight-reduced state include decreased glycolytic enzymatic activity, such as phosphofructokinase, lower bioactive thyroid hormones, increased activity of sarco/endoplasmic reticular Ca^{2+}-dependent ATPase (SERCA), shift in myosin heavy chain (MHC) isoforms toward MHC I—characterized as more efficient and oxidative—that may enhance skeletal muscle work efficiency in a weight-reduced state by altering myofibrillar ATPases (4, 5, 7). Similar changes in metabolic rates can be found in the context of exercise training and high-altitude adaptation (8, 9).

Recently, we showed that dietary intervention-induced weight loss in overweight mice increased mitochondrial oxidative phosphorylation (OXPHOS) efficiency of skeletal muscle, quantified by ATP production per O_2 consumption (P/O) (10). Those changes were not associated with

102   changes in SERCA abundance or efficiency compared to lean controls and overweight groups.

103   These results suggest that increased energy efficiency in a weight-reduced state may be due to

104   alteration of mitochondrial OXPHOS efficiency, which requires less O_2 to produce equimolar of

105   ATP production.

106

107   In recent years, glucagon-like peptide-1 (GLP-1) receptor agonists, such as semaglutide, have

108   shown striking efficacy for weight loss, inducing 10% to 15% reduction in body weight within one

109   year. In STEP and SELECT trials lasting 68 weeks and 208 weeks respectively, weekly

110   subcutaneous injection of semaglutide (2.4 mg) decreased body weight by approximately 10%

111   to 15% within one year, which was successfully sustained for the experimental duration (11, 12).

112   Importantly, individuals who stopped taking GLP-1 receptor agonists regained 10% of their lost

113   weight within a year (13). While changes in food intake likely largely contribute to weight regain,

114   there are limited studies on the contribution of energy efficiency/expenditure on the propensity

115   for weight regain during or after GLP-1 receptor agonists treatment. Furthermore, neither the

116   duration of changed energy efficiency during and/or after weight loss, as well as the outcomes

117   of sustained increase in energy efficiency have been fully described. As mentioned above,

118   increased energy efficiency from weight loss is like that observed with exercise training, thus

119   increased energy efficiency in the weight-reduced state could have potential benefits on the

120   health, such as improving glycemic controls and reducing oxidative stress (14, 15).

121

122   In this study, we set out to determine the influence of GLP-1 receptor agonist-induced weight

123   loss on skeletal muscle mitochondrial energy efficiency. We hypothesized that semaglutide

124   treatment will increase energy efficiency of skeletal muscle mitochondrial ATP production,

125   similarly to our observation with dietary intervention-induced weight loss (10).

126

Materials and Methods

Animals

Male C57BL/6J (RRID:IMSR_JAX:000664) mice were purchased from Jackson Laboratory at

20 weeks of age. Mice were maintained on a high-fat diet (42% calories from fat, Inotiv

TD88137) for the duration of the experiments. Body composition was determined by Bruker

Minispec NMR (Bruker, Germany) before and after the interventions. At 12 weeks of HFD

feeding, mice were divided into two groups: vehicle (phosphate buffered saline, PBS) and

semaglutide (Novo Nordisk, s.c. 3 nmol/kg). Reagents were administered once daily for either 1-

week or 3-week. Mice were housed in a 12-h light/dark cycle and provided access to food and

water *ad libitum*. For terminal experiments, mice were injected intraperitoneally with ketamine

(80 mg/kg) and xylazine (10 mg/kg) after which tissues were collected. All protocols were

approved by Institutional Animal Care and Use Committees at the University of Utah.

Preparation of permeabilize muscle fiber bundles (PmFB)

A small portion of mouse red gastrocnemius muscle was dissected and placed in buffer X [7.23

mM K_2EGTA, 2.77 mM CaK_2EGTA, 20 mM imidazole, 20 mM taurine, 5.7 mM ATP, 14.3 mM

phosphocreatine, 6.56 mM $MgCl_2 \cdot 6H_2O$, and 50 mM 2-(N-Morpholino) ethanesulfonic acid

potassium salt (K-MES) (pH7.4)]. Fiber bundles were separated and permeabilized for 30 min at

4°C with saponin (30 µg/ml). Immediately after permeabilization, fiber bundles were washed in

buffer Z [105 mM K-MES, 30 mM KCl, 10 mM K_2HPO_4, 5 mM $MgCl_2 \cdot 6H_2O$, 0.5 mg/mL BSA, and

1 mM EGTA, pH 7.4] with 0.5 mM pyruvate and 0.2 mM malate for 15 min. After washing, fiber

bundles were placed in buffer Z until analysis.

Mitochondria isolation from mouse skeletal muscle

Mouse gastrocnemius muscle was freshly dissected and used to isolate mitochondria as

previously described (16). Briefly, the tissue was minced in ice-cold mitochondrial isolation

153   medium (MIM) buffer [0.3 M sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

154   (HEPES), 1 mM ethylene glycol tetraacetic acid (EGTA), pH 7.1] with bovine serum albumin

155   (BSA; 1 mg/ml) and homogenized using a TH-01 homogenizer (Omni International). The

156   homogenate was then centrifuged at 800 g for 10 min at 4°C. The supernatant was transferred

157   to a new tube then centrifuged at 12,000 g for 10 min at 4°C. The supernatant was removed and

158   washed the crude mitochondrial pellet in 1 ml MIM buffer. Subsequently the pellet was

159   centrifuged again at 12,000 g for 10 min at 4°C, and the pellet was resuspended in 100 µl MIM

160   buffer.

161

162   ***High-resolution respirometry and fluorometry***

163   PmFB and isolated mitochondria were used to determine oxygen consumption and ATP

164   production as previously described (10). Respiration was measured using the Oroboros

165   Oxygraph-2K (Oroboros Instruments). ATP production was determined by enzymatically

166   coupling ATP production to NADPH synthesis using Horiba Fluorolog-QM (Horiba Scientific), as

167   previously described (17). Both experiments were performed in buffer Z in the presence of 0.5

168   mM malate, 5 mM pyruvate, 5 mM glutamate, and 10 mM succinate. Respiration and ATP

169   production was recorded with a subsequent ADP titration (20, 200, and 2000 µM for PmFB; 2,

170   20, and 200 µM for isolated mitochondria). In PmFB experiments, 20 mM creatine monohydrate

171   and 10 M blebbistatin were added to buffer Z to inhibit myosin adenosine triphosphatases. The

172   P/O ratio was analyzed by dividing the rate of ATP production by the rate of atomic O_2

173   consumed (17).

174

175   ***Sample preparation and nLC-MS/MS label free proteomic analysis***

176   Mitochondria from mouse skeletal muscle were isolated and prepared for label free proteomic

177   analysis as previously described with some modifications (10). Isolated mitochondria

178   resuspended in MIM buffer was added an ice-cold acetone and incubated for 10 min to

179   precipitate protein. The samples were vortexed then centrifuged at 12,000 g for 10 min at 4°C .

180   The pellet was processed following the manufacturer's instructions for S-Trap™ (Protifi). Protein

181   concentration was determined by BCA protein assay kit (ThermoFisher Scientific). Final

182   peptides were resuspended in 0.1% formic acid for mass spectrometry analysis. Proteomic

183   analysis was performed at the University of Proteomics Core.

184

185   Reversed-phase nano-LC-MS/MS was performed on a nanoElute 2 (Bruker Daltonics) coupled

186   to a Bruker timsTOF Pro2 mass spectrometer equipped with a nanoelectrospray source. 150 ng

187   of each sample was injected directly onto the liquid chromatograph reverse-phase C18 15 cm x

188   150 μm PepSep™ nanocolumn (Bruker Daltonics) heated to 50°C. The peptides were eluted

189   with a gradient of reversed-phase buffers (Buffer A: 0.1% formic acid in 100% water; Buffer B:

190   0.1% formic acid in 100% acetonitrile) at a flow rate of 0.5 μL/min. The LC run lasted for 45

191   minutes with a starting concentration of 5% buffer B increasing to 28% buffer B over 40 minutes,

192   up to 32% buffer B over 2 minutes and held at 95% B for 3 minutes. The separation column is

193   equilibrated with 4 column volumes at 800 bar following each run. The timsTOF Pro2 was

194   operated in PASEF data-dependent acquisition (DDA) MS/MS scan mode to generate the

195   custom peptide library. The TIMS section was operated with a 120 ms ramp time at a rate of

196   7.93 Hz and an ion mobility scan range of 0.6-1.4 V·s/cm^2. MS and MS/MS spectra were

197   recorded from 100 to 1,700 m/z. A polygon filter was applied to select against singly charged

198   ions. The quadrupole isolation width was set to 3 Da. The mass spectrometer was operated in

199   PASEF data-independent acquisition (DIA) MS/MS scan mode to analyze each experimental

200   sample. The TIMS section was operated with a 120 ms ramp time at a rate of 7.93 Hz and an

201   ion mobility scan range of 0.6-1.4 V·s/cm^2. dia-PASEF window parameters were set to mass

202   width of 25 Da and 47 mass steps/ cycle. MS and MS/MS spectra were recorded from 147 to

203   1,322.6 m/z. The custom peptide library was created using Fragpipe v20.0 software

204   (downloaded 4-7-23) against the uniprot_ref_mouse_database (downloaded 12-7-2023 with

205   17,191 proteins) as well as MitoCarta 3.0 (Rath 2021). Protein abundances based on peak

206   intensity were calculated using DIA-NN software version 1.8.1. An allowance was made for 1

207   missed cleavage following trypsin/Lys C digestion. No fixed modifications were considered. The

208   variable modifications of methionine oxidation, N-terminal acetylation and cysteine

209   carbamidomethylation (IAA) were considered with a mass tolerance of 15 ppm for precursor

210   ions and a mass tolerance of 10 ppm for fragment ions. The results were filtered with a false

211   discovery rate of 0.01 for both proteins and peptides. Mitochondrial enrichment factor was

212   determined by comparing mitochondrial protein abundance to total protein abundance.

213

214   ***Mitochondrial lipid mass spectrometry***

215   Mitochondrial lipidome was analyzed from isolated mitochondria from mouse gastrocnemius

216   muscle. Lipids were extracted as described previously (10). Briefly, a mixture of ice-cold methyl-

217   tert-butyl ether (MTBE), methanol, and internal standards (EquiSPLASH, Avanti, Cat #: 330731

218   and Cardiolipin Mix I, Avanti, Cat #: LM6003) was added to 50 µg of mitochondrial protein. The

219   samples were vortexed and sonicated for 1 min. The samples were then incubated on ice for 15

220   min and vortexed every 5 min. Phase separation was induced by adding H_2O to the samples,

221   then the 15-min incubation with vortex every 5 min was performed. The samples were

222   centrifuged at 12,000 g for 10 min. The organic phase was collected to a new tube while the

223   aqueous phase was used for second extraction with 1 ml of 10:3:2.5 (v/v/v)

224   MTBE/methanol/H_2O. The organic layers were combined and dried under Genevac™ miVac

225   centrifugal evaporation system. The dried lipids were reconstituted in 300 ul of 8:2:2 (v/v/v)

226   IPA/ACN/H_2O. Mitochondrial lipidome profiles were obtained using targeted LC-MS/MS

227   platforms in operation at the University of Utah Metabolomics Core. Lipids contents were

228   normalized to mitochondrial protein levels.

229

230   ***Western blot***

231   Proteins from isolated mitochondria and whole frozen muscle were utilized for the analysis.

232   Frozen gastrocnemius muscle was homogenized using TH-01 homogenizer in ice-cold RIPA

233   buffer (ThermoFisher Scientific, Cat #: 89901) supplemented with protease inhibitor cocktail

234   (ThermoFisher Scientific, Cat #: 78446). Protein concentration was determined by BCA protein

235   kit (ThermoFisher Scientific). Equal amounts of proteins were analyzed with 4-20% gradient

236   SDS-polyacrylamide gel (Bio-Rad) for electrophoresis and transferred to polyvinylidene fluoride

237   membranes (ThermoFisher Scientific). The membranes were blocked with 3% skim milk in Tris-

238   buffered saline containing 0.1% Tween-20 (TBST) for 1 hr at room temperature, followed by

239   incubation of primary antibodies, OXPHOS (Abcam, MS604-300), at 4°C overnight. After

240   overnight, the membranes were washed with TBST and incubated with secondary antibody

241   (1:5,000) in 1% skim milk for 1 hr at room temperature. Following three washes in TBST and

242   one in TBS, the membranes were incubated with Western Lightning Plus-ECL (PerkinElmer)

243   and detected by a ChemiDoc Imaging System (Bio-Rad) and quantified with Image Lab

244   Software (Bio-Rad).

245

246   ***Native PAGE***

247   Mitochondria supercomplex analysis was performed as previously described with modification

248   (18). Isolated mitochondria (25 µg) suspended in MIM were pelleted at 12,000 x g for 10 min

249   and subsequently solubilized in 20 µL sample buffer (5 µL of 4x Native PAGE Sample Buffer, 2

250   µL 10% digitonin, 13 µL ddH$_2$O per sample) for 20 min on ice and then centrifuged at 18,000 x g

251   for 10 min at 4°C. The supernatant (15 µL) was collected and placed into a new tube and mixed

252   with 1 µL of G-250 sample additive (Thermo Fisher Scientific). The samples and protein ladder

253   were loaded onto a native PAGE 3-12% Bis-Tris Gel (Thermo Fisher Scientific). Dark blue

254   cathode buffer (0.02% Coomassie Brilliant Blue dissolved in Native Page running buffer) was

255   carefully added to the front of gel box and Native PAGE running buffer (anode buffer) was

256   carefully added to the back of the gel box making sure to not mix. Electrophoresis was

257   performed at 150 V for 45 min at 4°C. The dark blue cathode buffer was then replaced with light

258   blue cathode buffer (Dark blue cathode buffer and anode buffer 1:9) and run at 15 mA for 90 min

259   at 4°C. Gels were subsequently washed with transfer buffer (50 mM tricine, 7.5 mM imidazole)

260   for 30 min and transferred to PVDF membrane at 100 V for 60 min. The membranes were

261   stained with Coomassie Brilliant Blue staining solution (0.05% Coomassie Brilliant Blue, 50%

262   methanol, 10% acetic acid) and washed destaining solution (50% methanol, 7% acetic acid) to

263   visualize the membrane bound protein and ladder. The detection of OXPHOS protein was

264   performed with the same procedure described in the Western blot section.

265

266   ***Electron microscopy***

267   Samples preparation for electron microscopy was performed as previously described (16).

268   Briefly, freshly dissected red gastrocnemius muscles from 3 weeks of vehicle and semaglutide

269   were placed in fixative solution (1% glutaraldehyde, 2.5% paraformaldehyde, 100 mM

270   cacodylate buffer pH 7.4, 6 mM $CaCl_2$, 4.8% sucrose) and cut into sections ~2 mm then stored

271   at 4°C for 48 hours. Samples were prepared by the Electron Microscopy Core at University of

272   Utah. The tissues underwent 3 × 10-minute washes in 100 mM cacodylate buffer (pH 7.4) prior

273   to secondary fixation (2% osmium tetroxide) for 1 hour at room temperature. Osmium tetroxide

274   as a secondary fixative has the advantage of preserving membrane lipids, which are not

275   preserved using aldehyde, alone. After secondary fixation, samples were subsequently rinsed

276   for 5 minutes in cacodylate buffer and distilled H_2O, followed by prestaining with saturated

277   uranyl acetate for 1 hour at room temperature. After prestaining, each sample was dehydrated

278   with a graded ethanol series (2 × 15 minutes each: 30%, 50%, 70%, 95%; then 3 × 20 minutes

279   each: 100%) and acetone (3 × 15 minutes) and were infiltrated with EPON epoxy resin (5 hours

280   30%, overnight 70%, 3 × 2-hour 40 minute 100%, 100% fresh for embed). Samples were then

281   polymerized for 48 hours at 60°C. Ultracut was performed using Leica UC 6 ultratome with

282   sections at 70 nm thickness and mounted on 200 mesh copper grids. The grids with the

283   sections were stained for 20 minutes with saturated uranyl acetate and subsequently stained for

284   10 minutes with lead citrate. The sections were imaged at 120 kV using JEOL-JEM 1400plus

285   (Tokyo, Japan) transmission electron microscope and images were acquired on a Gatan 2kx2k

286   digital camera (Gatan, Inc.)

287

288   ***Statistical analysis***

289   Statistical analysis was performed using GraphPad Prism 10.2.3 software. Two-way ANOVA

290   followed by Tukey's multiple comparisons test or unpaired t-test were performed for group

291   comparison. All data are presented as mean±SEM, and statistical significance was set at p

292   ≤0.05.

293

Results

Semaglutide treatment induces weight loss in overweight mice.

Wildtype C57BL/6J mice were fed a high-fat-diet (HFD, 42% kcal from fat) for 12 weeks before being treated with either vehicle (PBS) or semaglutide (3 nmol/kg body weight/day, subcutaneous injection) for 1 week or 3 weeks (Fig. 1A). During the intervention, mice were maintained on the HFD. Mice treated with semaglutide lost about 12% ± 1.7 of body weight after 1 week of treatment and about 23 % ± 1.66 after 3 weeks (Fig. 1B and 1C). Loss in body weight was due to disproportionately more loss in fat mass than lean mass. Fat mass was significantly decreased by 26% ± 2.23 after 1 week and by 42% ± 3.66 after 3 weeks of semaglutide treatment compared to the vehicle groups (Fig. 1D and 1E). Lean mass was significantly reduced by 9% ± 1.5 both after 1 week and 3 weeks (Fig. 1F and 1G) and this similarly reflected in gastrocnemius muscle mass (Fig. 1H). Thus, semaglutide intervention significantly reduced body weight, fat mass, and lean mass.

Semaglutide-induced weight loss increases skeletal muscle OXPHOS energy efficiency.

To test whether semaglutide-induced weight loss increases the energy efficiency for mitochondrial ATP synthesis, we first quantified the rates of ATP production, O_2 consumption, and P/O in permeabilized red gastrocnemius muscle fiber bundles harvested from 1-week and 3-week vehicle and semaglutide-treated groups. The rate of ATP production (JATP) showed a trend toward an increase after 1 week of semaglutide treatment, with a more pronounced effect observed after 3 weeks of treatment (Fig. 2A and 2B). Additionally, while O_2 consumption (JO$_2$) did not change significantly after 1 week, it was markedly decreased after 3 weeks of semaglutide treatment (Fig. 2C and 2D). When calculating the P/O ratio to determine energy efficiency, semaglutide tended to increased P/O after 1 week, with a significant improvement after 3 weeks (Fig. 2E and 2F). This improved P/O was not associated with changes in OXPHOS subunits levels (Fig. 2G and 2H). Overall, these results indicate that 3 weeks of

320   semaglutide treatment robustly improved mitochondrial OXPHOS efficiency in skeletal muscle

321   by reducing JO$_2$ without affecting OXPHOS subunits.

322

323   ***Semaglutide-induced increase in mitochondrial OXPHOS efficiency is NOT observed in***

324   ***isolated mitochondria from skeletal muscle.***

325   Mitochondrial bioenergetic phenotyping in permeabilized muscle fiber bundles enables

326   assessment of respiratory responses in situ, better preserving the native conformation of

327   mitochondrial reticulum and membrane structures. However, these preparations also contain

328   other subcellular structures, including other ATP synthases and ATPases. While respiratory

329   assay buffers contain inhibitors for some of these systems, we cannot rule out the possibility

330   that non-mitochondrial mechanisms could influence ATP fluorometry (less concerning for O_2

331   consumption) and thereby affect P/O measurements.

332

333   To address this, we conducted the same bioenergetic phenotyping in isolated mitochondria from

334   gastrocnemius muscles. In isolated mitochondria, neither JATP, JO$_2$, nor P/O were significantly

335   changed by semaglutide (Fig. 3A-F), and OXPHOS subunits remained unchanged (Fig. 3G and

336   3H). We observed that JATP responses stopped after the second dose of ADP (20 µM) (Fig. 3A

337   and 3B), unlike what was observed in permeabilized muscle fibers (Fig. 2A and 2B), while JO$_2$

338   continued to respond to each ADP titration (Fig. 3C and 3D). This led to a decrease in P/O at

339   the highest ADP compared to the second dose (Fig. 3E and 3F). These results suggest that

340   cellular compartments associated with mitochondrial network preserved in permeabilized

341   muscle fiber bundles may play a critical role in the improvement of semaglutide-induced

342   OXPHOS efficiency in skeletal muscle.

343

344   ***Semaglutide treatment does not alter skeletal muscle mitochondrial supercomplexes***

345   ***assembly.***

346   Mitochondrial respiratory complexes are thought to have supercomplexes that have improved

347   efficiency for electron transport compared to free complexes (19, 20). However, semaglutide

348   treatment did not appear to influence mitochondrial OXPHOS supercomplex assembly (Fig. 4A-

349   F and S2A-F). Mitochondrial morphology, including shape and size of individual mitochondrion

350   as well as those of cristae have a strong influence on OXPHOS (21). Mitochondrial morphology

351   analyzed by electron microscopy (EM) however did not show any noticeable differences

352   between vehicle and semaglutide treatment (Fig. 4G). Previously we have shown that

353   mitochondrial lipidome can also influence OXPHOS (10, 16, 22), but semaglutide did not

354   influence the mitochondrial lipidome (Fig. 5A and S1A-I). These findings indicate that alterations

355   in mitochondrial supercomplexes formation, morphology, and lipidome are unlikely to be

356   responsible for the improvement in muscle P/O induced by semaglutide.

357

358   ***Semaglutide-induced weight loss does not robustly influence the abundances of***

359   ***OXPHOS subunits.***

360   The improvement in OXPHOS efficiency may result from changes in the abundance of

361   respiratory subunits. Thus, we performed proteomic analyses on skeletal muscle mitochondria

362   with or without semaglutide treatment. Consistent with our previous experiments with dietary

363   intervention-induced weight loss (10), semaglutide treatment neither appeared to influence

364   muscle mitochondrial proteome by principal component analyses (Fig. 5B) nor were there a

365   single mitochondrial protein whose abundance was statistically significantly different (q<0.05)

366   (Fig. 5C-H). However, two subunits associated with complex III assembly were trending to be

367   elevated (Lyrm7: increased by 41%, Ttc19: increased by 38%, both p<0.05 without correcting

368   for multiple comparison) (Fig. 5F). It is unclear whether changes in these complex III subunits

369   are sufficient to explain the improvement in skeletal muscle OXPHOS efficiency with

370   semaglutide treatment.

371

Discussion

GLP-1 receptor agonists including semaglutide have revolutionized obesity-related health care by its efficacy to induce weight loss, as well as other comorbidities including diabetes, cardiovascular disease, and chronic kidney disease. Weight loss, including those induced by dietary intervention or those induced by GLP-1 receptor agonists, are known to induce a reduction in energy expenditure. The consequence of a perpetual reduction in resting and exercise-induced energy expenditure extends beyond the propensity for weight regain, as they may attenuate all secondary benefits of elevated energy expenditure such as glycemic control. With the emergence of GLP-1 receptor agonists and other weight loss drugs, it is important more than it has ever been to study the biology of weight-reduce state. In this study, we showed that semaglutide promotes an increase in skeletal muscle OXPHOS energy efficiency, in a similar fashion to our previous observation with weight loss induced by a dietary intervention. The mechanism by which semaglutide treatment improved OXPHOS efficiency remains to be fully elucidated, but we found two components of complex III, Lyrm7 and Ttc19, to become elevated after semaglutide treatment.

We found that semaglutide treatment improved skeletal muscle mitochondrial efficiency when analyzed in permeabilized muscle fiber bundles. JATP tended to increase after 1 week of treatment without a change in JO$_2$, and this effect became more pronounced after 3 weeks, with significantly attenuated JO$_2$. These changes resulted in approximately a 50% increase in the P/O ratio, indicating improved OXPHOS efficiency. These findings align with previous studies showing that weight loss, whether induced by diet or exercise, can improve mitochondrial efficiency in skeletal muscle (10, 23). Consistent with our previous study, the higher P/O ratio was driven by a reduction in JO$_2$ in response to the same ADP stimuli (10). We also observed a trend toward increased JATP with semaglutide treatment. Activation of GLP-1 receptor signaling enhances ATP synthesis in pancreatic β-cells (24, 25). Nevertheless, increased JATP was not

398   observed in our previous paper with dietary-intervention induced weight loss (10). Our previous

399   dietary-intervention study induced weight loss by switching HFD back to standard chow, while

400   the mice remained on HFD for our current study with semaglutide-induced weight loss. Thus,

401   these nuanced differences between dietary-intervention and semaglutide treatment may be due

402   to differences in their dietary compositions.

403

404   No significant improvements in mitochondrial efficiency were detected when measuring

405   OXPHOS efficiency in isolated mitochondria. This may suggest the critical role of the intact

406   cellular environment, which is preserved in permeabilized muscle fibers but disrupted during

407   mitochondrial isolation (26, 27). Permeabilized muscle fiber bundles (PmFB) maintain the

408   integrity of key cellular components, including mitochondrial structural arrangement, intracellular

409   organelles, and multi-enzyme systems essential for energy transfer (26, 28, 29). Intact

410   myofibrillar ATPases, including myosin ATPase, in PmFB potentially lead to this difference. We

411   used blebbistatin, which inhibits specifically myosin II ATPase, to prevent spontaneous

412   contraction during the measurement (17, 30). There is a possibility that the addition of

413   blebbistatin did not inhibit other myosin ATPases, such as myosin I ATPases, presented in

414   PmFB since we precisely dissected red gastrocnemius (type I; oxidative) for PmFB. Moreover, it

415   has been reported that increased skeletal muscle work efficiency during, as well as after, weight

416   loss has been linked to switch myosin isoforms from more glycolytic (IIa, IIb and IIx) to oxidative

417   (I) (4). Other candidates for intact myofibrillar ATPases in PmFB, including Ca^{2+}-activated

418   myosin ATPase and SERCA, can also involve in generating this different observation between

419   PmFB and isolated mitochondria as they are largely participated in ATP consumption. To avoid

420   this possibility, we added a chelating agent to our experimental buffer (see details in

421   Methodology). Different types of fiber or myofibrillar ATPases possibly result in distinct outcomes

422   (27, 31, 32). This likely explains why observed improvements were specific to PmFB

423   underscoring the importance of studying mitochondrial function in a more physiological relevant

424   context. These findings suggest the future studies should investigate how different fiber types

425   and mitochondria sources contribute to OXPHOS efficiency under weight loss conditions.

426

427   Semaglutide-induced improvement in OXPHOS efficiency did not appear to be related to

428   changes in the mitochondrial lipidome. Our previous study found shifts in some cardiolipin

429   species after diet-induced weight loss to potentially mediate increased OXPHOS efficiency (10).

430   However, we did not observe any changes in mitochondrial phospholipids with semaglutide

431   intervention. It remains possible that semaglutide influences other aspects of skeletal muscle

432   mitochondria to influence bioenergetics. One possibility is that GLP-1 receptor agonists

433   decrease mitochondrial electron leak. In leukocytes from patients with type 2 diabetes, GLP-1

434   receptor agonists reduced the reactive oxygen species production (33), potentially suggesting

435   that it might reduce mitochondrial electron leak to improve efficiency. However, electron leak

436   usually accounts for a very small portion of total electron flux (often less than 1% when

437   quantified) (34). Thus, it is difficult to imagine that a 2-fold increase in P/O is completely

438   accounted for by reduced electron leak. GLP-1 receptor agonists are also known to affect

439   mitochondrial dynamics in substantia nigra (35). Even though we did not observe a robust

440   change in mitochondrial morphology, there may be other subtle effects not detected by EM that

441   contribute to improved OXPHOS efficiency.

442

443   Semaglutide treatment resulted in a 12% and 23% reduction in body weight after 1 and 3 weeks

444   of treatment, respectively. Fat mass was reduced by 26% and 42% reduction over the same

445   time point. Additionally, lean mass decreased by approximately 10% at both time points. These

446   findings are consistent with previous clinical trials and animal studies showing that GLP-1

447   receptor agonists effectively promote weight loss (11, 36). Skeletal muscle is a significant

448   contributor to energy expenditure (3, 37). A decrease in skeletal muscle mass during weight loss

449   has been associated with a reduction in energy expenditure (3). In addition to this effect, energy

450   expenditure per unit of lean mass appears to be reduced, promoted by an increase in skeletal

451   muscle work efficiency (4, 5, 10).

452

453   In the current study, semaglutide did not significantly alter the skeletal muscle mitochondrial

454   proteome when corrected for multiple comparisons. However, we identified two proteins related

455   to complex III assembly—Lyrm7 and Ttc19—that trended to be greater (p<0.05 with t-tests).

456   Lrym7 is involved in the incorporation of the iron-sulfur cluster into Rieske (Fe-S) protein

457   (UQSRFC1) (38). Ttc19 is known to interact with UQCRC1 and UQCRFS1 (39), though

458   semaglutide did not influence the abundance of these subunits. Defects in Lyrm7 and Ttc19 are

459   linked to complex III deficiencies, nuclear type 8 (MC3DN8) and nuclear type 2 (MC3DN2),

460   respectively, which result in increased oxidative stress and inefficient electron transfer (40-42).

461   The association between weight loss and these proteins is unknown. Thus, semaglutide may

462   influence Lyrm7 and Ttc19, potentially influencing complex III to enhance electron transport

463   efficiency and reducing oxidative stress.

464

465   In summary, our study demonstrates that semaglutide treatment significantly improves

466   mitochondrial OXPHOS efficiency in skeletal muscle. Increased OXPHOS efficiency was only

467   observed when quantified in permeabilized fibers, suggesting that intact subcellular structures

468   may be necessary for this effect. In contrast to our previous findings with dietary intervention-

469   induced weight loss, we did not find semaglutide to influence skeletal muscle mitochondrial

470   proteome or lipidome. Alternatively, improved mitochondrial energy efficiency may be mediated

471   by post-translational modifications or by changes in mitochondria dynamics and ultrastructure.

472   While tools to study these changes in vivo are currently limited, future studies should explore

473   alternative molecular mechanisms that drive the improved OXPHOS efficiency with semaglutide

474   treatment. Greater understanding of these effects could help identify targets to mitigate risks for

475   weight regain.

476

483 **Figure Legends**

484 **Figure 1. Semaglutide induces weight loss in overweight mice.** (A) Schematic of the

485 experimental design. Twenty-week-old C57BL/6J mice were fed 42% HFD diet for 12 weeks

486 before semaglutide (3 nmol/kg of body weight/day) intervention. Semaglutide was

487 subcutaneously injected daily for 1 or 3 weeks. Vehicle groups received an equal volume of

488 PBS. (B) Body weight was recorded every week. (C) Percent change in body weight after

489 semaglutide treatment was calculated from the baseline of 12-wk HFD. (D) Fat mass was

490 determined by NMR before and after treatment. (E) Percentage change in fat mass was

491 analyzed by NMR. (F) Lean mass was determined by NMR before and after treatment. (G)

492 Percentage change in lean mass was calculated by NMR. (H) After the completion of

493 semaglutide treatment, gastrocnemius muscles were harvested, and wet weight was measured.

494 Data are represented as mean± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ significant

495 difference vs. corresponding controls. ## $p<0.01$, ### $p<0.001$ significant difference vs. vehicle-

496 3wk. N=8 and 9 for vehicle-1wk and vehicle-3wk, respectively, and N=11 for both semaglutide 1-

497 wk and 3-wk.

498

499 **Figure 2. Semaglutide treatment increases skeletal muscle OXPHOS energy efficiency.** A

500 small piece of red gastrocnemius was collected and permeabilized in the buffer with saponin (30

501 µg/ml). (A and B) The rate of ATP production (JATP) was determined by fluorometer. (C and D)

502 Mitochondrial O_2 consumption (JO$_2$) was analyzed by high-resolution respirometry. (E and F)

503 OXPHOS efficiency was determined as P/O ratio. (G and H) Abundance of OXPHOS subunits

504 in whole lysate of gastrocnemius muscle was determined by Western blot. Data are represented

505 as mean± SEM. * $p<0.05$ significant difference vs. corresponding controls. N=10 and 13 for

506 vehicle-1wk and vehicle-3wk and N=10 and 14 for semaglutide 1-wk and 3-wk, respectively.

507

508 **Figure 3. Semaglutide-induced increase in mitochondrial OXPHOS efficiency is NOT**

509 **observed when quantified in isolated mitochondria from skeletal muscle.** Mitochondria

510 were isolated from whole gastrocnemius muscle. (A and B) The rate of ATP production (JATP)

511 was determined by fluorometer. (C and D) Mitochondrial O_2 ($J O_2$) consumption was analyzed by

512 high-resolution respirometry. (E and F) OXPHOS efficiency was determined as P/O ratio. (G and

513 H) Abundance of OXPHOS subunits in isolated mitochondria from gastrocnemius muscle was

514 determined by Western blot. Data are represented as mean± SEM. N=12 and 13 for vehicle-

515 1wk and vehicle-3wk, respectively, and N=15 for both semaglutide 1-wk and 3-wk.

516

517 **Figure 4. Semaglutide treatment does not alter skeletal muscle mitochondrial**

518 **supercomplexes formation.** (A-F) Mitochondrial supercomplexes formations were analyzed by

519 Native PAGE and quantified. (G) Representative mitochondrial morphology analyzed by

520 electron microscopy. All data are from 3-wk semaglutide treatment. N=6 for both vehicle and

521 semaglutide for Native PAGE.

522

523 **Figure 5. Semaglutide-induced weight loss does not robustly influence the abundances**

524 **of OXPHOS subunits.** (A) Relative abundance of mitochondrial lipids. (B) Principal component

525 analysis of mitochondrial proteomic data. (C) Volcano plot of differentially abundant

526 mitochondria proteins between vehicle (Veh) and semaglutide (Sema) groups. (D-H) Heatmap

527 of abundance of OXPHOS subunits determined by mass spectrometry. All data are from 3-wk

528 semaglutide treatment. N=6 for both vehicle and semaglutide.

529

530   **Figure S1. Skeletal muscle mitochondrial lipidome.**

531   Mitochondrial lipidome was analyzed in isolated mitochondrial from gastrocnemius muscle of 3

532   weeks vehicle and semaglutide groups. (A) Heatmap of abundance of each mitochondrial

533   phospholipidome (B) Total phospholipids from vehicle and semaglutide group (C) Absolute

534   abundance of mitochondrial lipids (D – K) Absolute abundance of individual phospholipids

535   species (D) Phosphocholine (PC), (E) Phosphatidylethanolamine (PE), (F) Phosphatidylinositol

536   (PI), (G) Phosphatidylserine (PS), (H) Cardiolipin (CL), (I) Phosphatidylglycerol (PG), (J)

537   Lysophosphatidylcholine (LPC), and (K) Lysophosphatidylethanolamine (LPE). All data are from

538   3-wk semaglutide treatment. N=6 for both vehicle and semaglutide. ** $p<0.01$ significant

539   difference vs. Veh.

540

541   **Figure S2. Skeletal muscle mitochondrial supercomplexes.**

542   (A) High molecular weight (HMW) supercomplexs (SCs) or V_n, (B) Supercomplex (SC) I+III$_2$+IV

543   and SC I+III$_2$ or V_n,(C) V_n,(D) SC III$_2$+IV or V_n, (E) III$_2$ or IV$_2$, (F) IV, and (G) II. All data are from

544   1-wk semaglutide treatment. N=5-6 for both vehicle and semaglutide.

545

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705
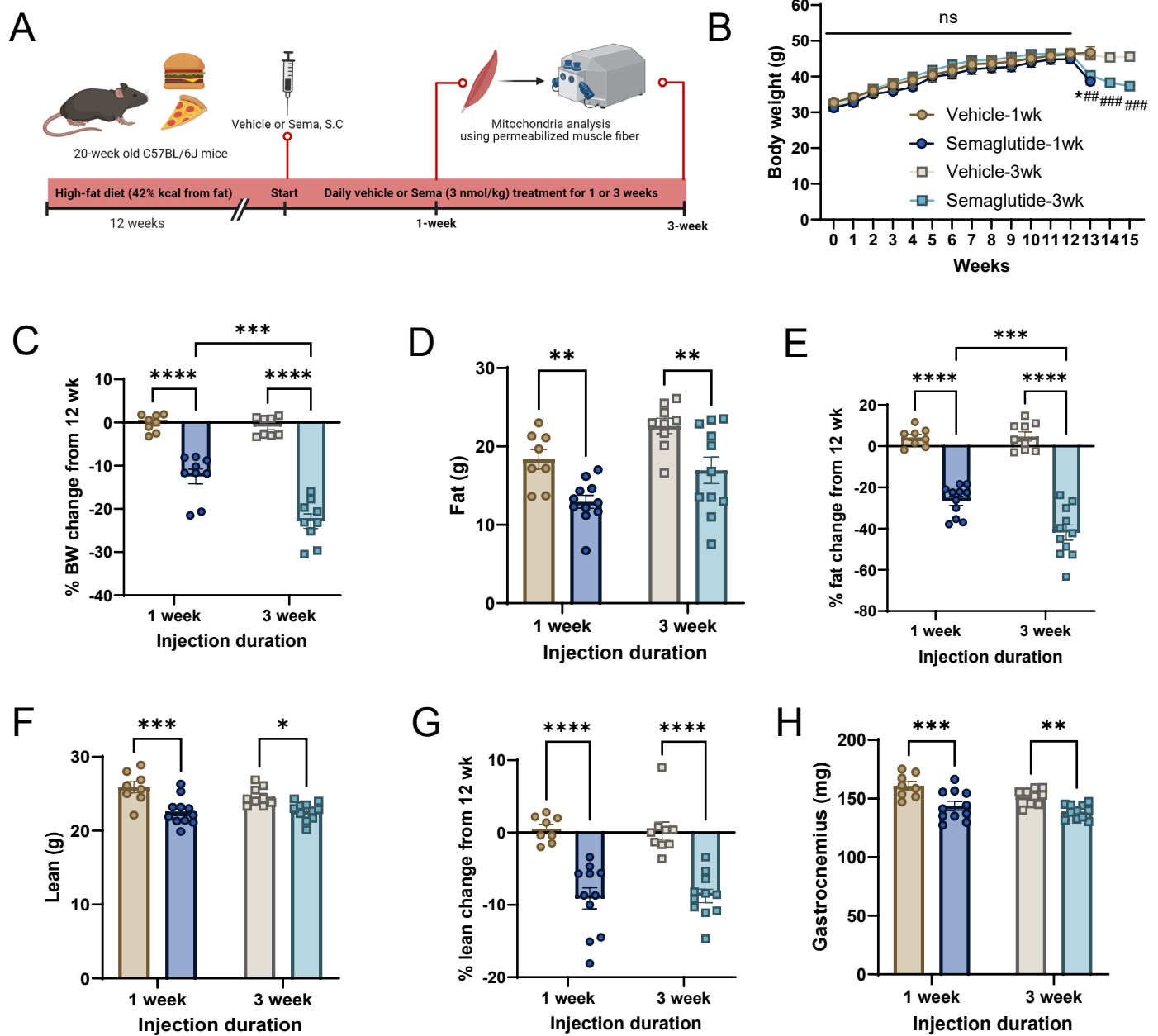
Figure 1.

Figure 2

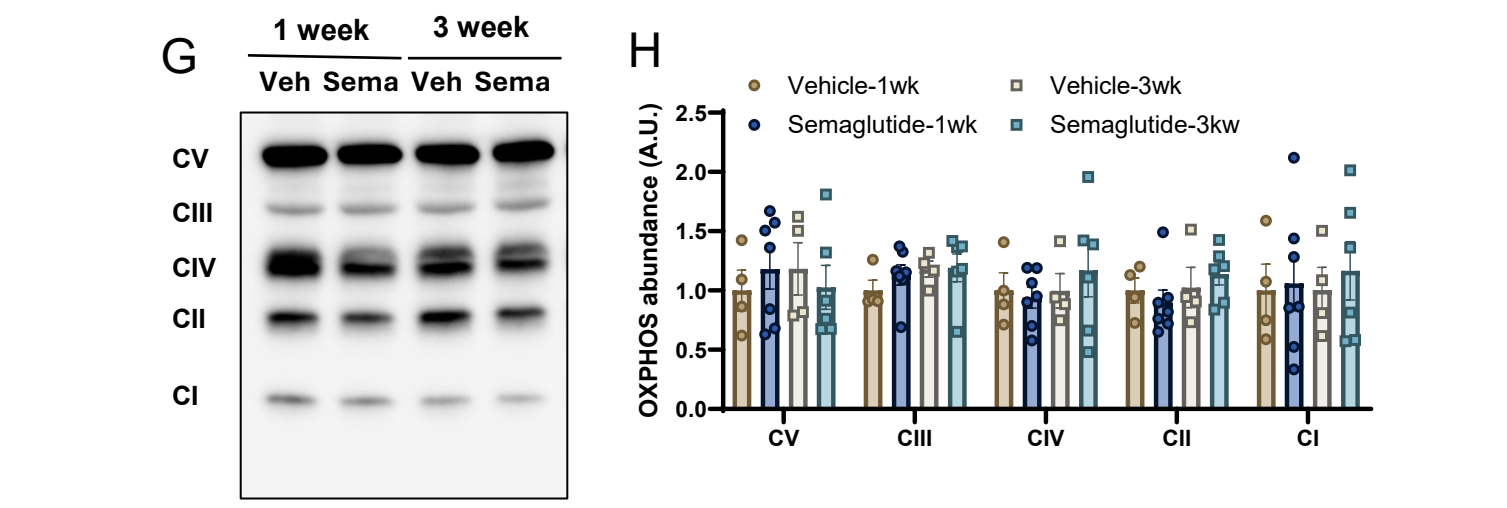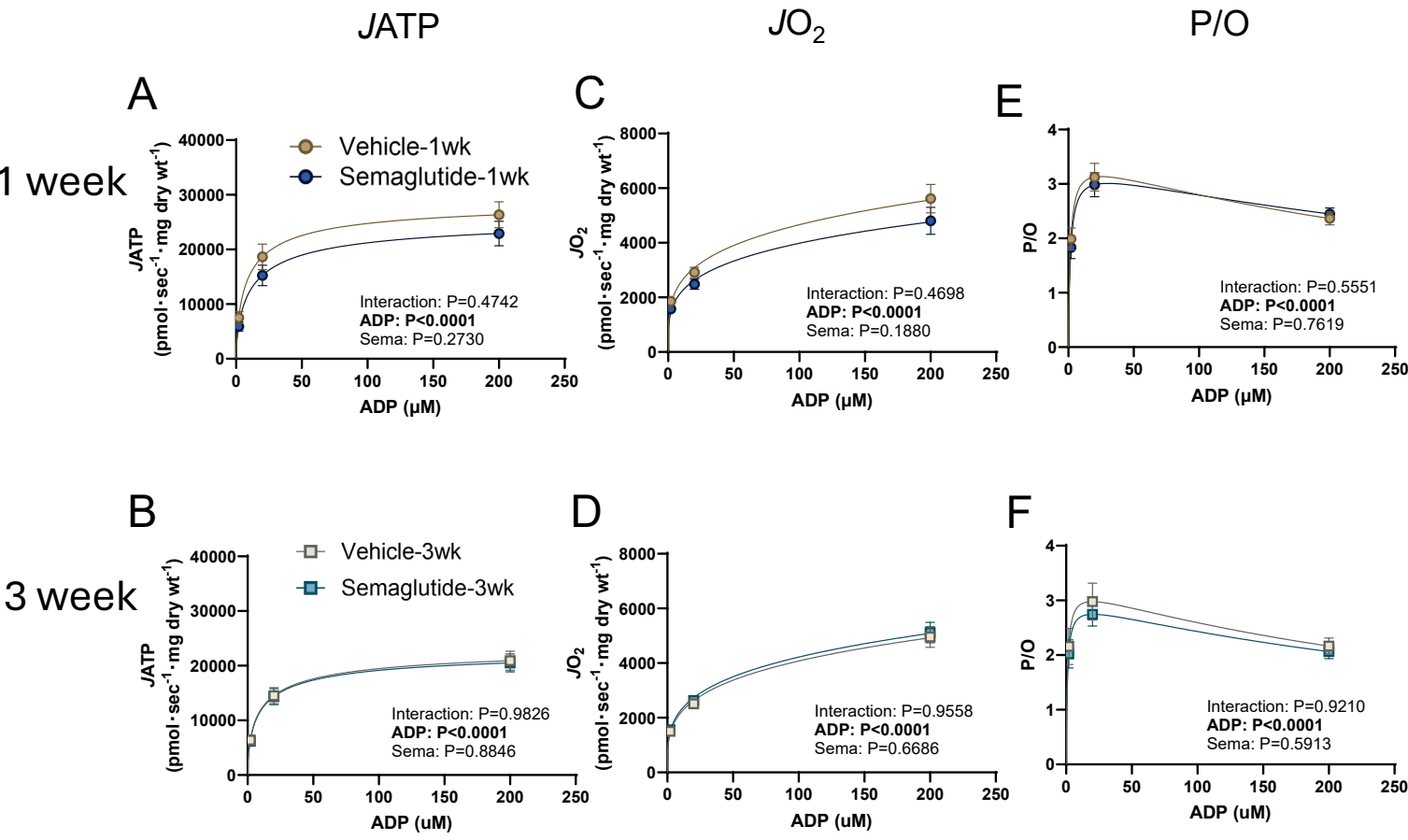## Permeabilized skeletal muscle fiber bundles
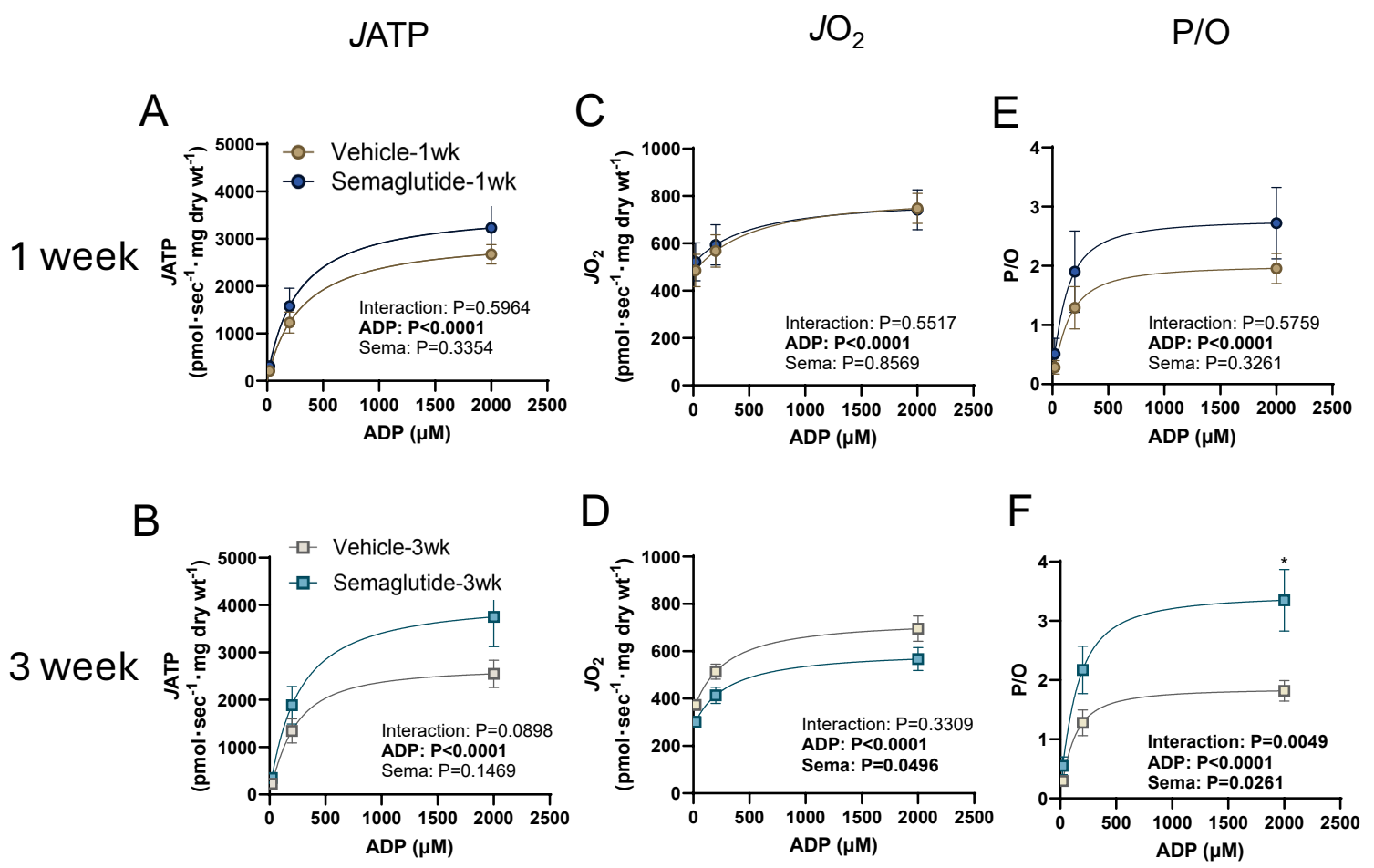
Skeletal muscle tissue

Figure 3

Figure 4

A

(kDa)

Veh Sema

1236 ►
1048 ►

720 ►

480 ►

242 ►

146 ►

HMW SCs or Vn
SC I+III$_2$+IV,
SC I+III$_2$, or V$_n$
V$_n$
III$_2$ or IV$_2$
IV
II

B HMW SCs or V$_n$

C HMW SCs or V$_n$

D V$_n$

E SC III$_2$+IV or V$_n$

F III$_2$ or IV$_2$

G Vehicle   Semaglutide

1 µm   1 µm

Figure 5

Figure S1.

Figure S2