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1 **The Effect of Acute Oral Phosphatidic Acid Ingestion on Myofibrillar Protein Synthesis**
2 **and Intracellular Signaling in Older Males.**

3

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26

27 **ABSTRACT**

28 **Background:** Age-related muscle loss (sarcopenia) may be driven by a diminished
29 myofibrillar protein synthesis (MyoPS) response to anabolic stimuli (i.e. exercise and
30 nutrition). Oral phosphatidic acid (PA) ingestion has been reported to stimulate resting
31 muscle protein synthesis in rodents, and enhance resistance training-induced muscle
32 remodelling in young humans. **Purpose:** This study examined the effects of acute oral PA
33 ingestion on resting and exercise-induced MyoPS rates in older individuals. **Methods:**
34 Sixteen older males performed a bout of unilateral leg resistance exercise followed by oral
35 ingestion of 750mg of soy-derived PA or a rice-flour placebo (PL) over 60 min post-exercise.
36 A primed-continuous infusion of L-[ring-¹³C₆]-phenylalanine with serial muscle biopsies was
37 used to determine MyoPS at rest and between 0-150 and 150-300 min post-exercise. **Results:**
38 Plasma [PA] concentrations were elevated above basal values from 180-300 min post-
39 exercise in PA only (P = 0.02). Exercise increased MyoPS rates above basal values between
40 150-300 min post-exercise in PL (P = 0.001), but not PA (P = 0.83). Phosphorylation of
41 p70S6K, rpS6, 4E-BP1 and Akt was elevated above basal levels in the exercised leg over
42 150-300 min post-exercise for PL only (P = 0.018, 0.007, 0.011 and 0.002, respectively), and
43 were significantly greater than PA (P < 0.01 for all proteins). The effects of oral PA ingestion
44 on proteolytic signaling markers was equivocal. **Conclusions:** Acute oral phosphatidic acid
45 ingestion appears to interfere with resistance exercise-induced intramuscular anabolic
46 signaling and MyoPS in older males and, therefore, may not be a viable treatment to
47 counteract sarcopenia. Clinicaltrials.gov registration no: NCT03446924

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51

52 INTRODUCTION

53 Age-related loss of skeletal muscle mass and strength, also termed sarcopenia [1], is
54 associated with premature mortality [2] and leads to myriad adverse health consequences
55 such as impaired functional capacity [3], increased risk of fractures [4], and metabolic disease
56 [5]. Whilst the incidence of age-related muscle wasting has been observed in people as young
57 as 45 y, its prevalence increases with advancing age [6]. The healthcare costs associated with
58 sarcopenia are extensive and, in the context of a rapidly expanding global ageing population,
59 are expected to increase considerably in the coming decades [7].

60 Skeletal muscle proteostasis is dependent on the equilibrium between muscle protein
61 synthesis (MPS) and muscle protein breakdown (MPB). One of the most effective strategies
62 to attenuate the progression of sarcopenia is resistance exercise (RE) training [8-10]. In
63 young individuals, RE robustly increases MPS by 2 to 3-fold [11], thereby enhancing overall
64 net protein balance for muscle hypertrophy [12, 13]. However, the acute MPS response to RE
65 is blunted in older compared with younger individuals [14, 15], which may explain the
66 impaired muscle remodelling response to prolonged training in older age [15, 16]. The age-
67 related blunting of the muscle anabolic response to RE may be underpinned by impairments
68 in ribosomal biogenesis and/or translational efficiency in the mechanistic target of rapamycin
69 complex 1 (mTORC1) signaling pathway [14, 15, 17]. Collectively, these data have
70 galvanized the search for strategies to abolish the impaired muscle anabolic response to RE
71 and maximize the therapeutic benefits of this non-pharmacological intervention in the old.

72 The lipid second messenger phosphatidic acid (PA) has been touted as an important
73 factor in the regulation and activation of mTORC1 for MPS. Specifically, mechanical
74 contraction increases the activity of diacylglycerol kinase ζ to convert diacylglycerol into PA,
75 which in turn, binds mTORC1 directly at the FKB12 rapamycin binding (FRB) domain [18,
76 19], increasing its activity and ultimately enhancing protein translational efficiency [20].

77 Exogenous PA administration in cell culture is converted to lysophosphatidic acid [21],
78 thereby inactivating the tuberous sclerosis complex (TSC1/2) through the extracellular
79 regulated kinase (ERK) pathway to bolster mTORC1-mediated signaling [22].
80 Mechanistically, acute oral PA ingestion was reported to stimulate mTORC1-mediated
81 signaling with a tendency to increase MPS in rodents [23]. Given that mechanical load-
82 induced PA and exogenous PA appear to converge on mTORC1 through distinct proximal
83 pathways, exogenous PA provision (via oral ingestion) may modulate the anabolic response
84 to RE in older individuals [24]. Indeed, studies in healthy young humans report that orally
85 ingested PA enhances strength and lean body mass gains when consumed daily during
86 prolonged RE training [25, 26]. However, there is a need for further *in vivo* human
87 investigation of the bioavailability and intramuscular mechanisms through which this
88 purported ‘nutraceutical’ compound acts before introduction to the older population [27].

89 Therefore, the primary aim of the present study was to establish the effects of acute
90 oral PA ingestion on rates of myofibrillar protein synthesis (MyoPS) and intramuscular
91 signaling at rest and in response to a bout of RE in older individuals. We hypothesised that
92 PA ingestion alone would not stimulate MyoPS, but would modulate RE-induced
93 intramuscular signaling and MyoPS rates in older individuals compared with a placebo.

94 95 **METHODS**

96 **Participants**

97 Sixteen older males were recruited for the present study (age 68.9 ± 2.8 yrs, range 65-75 yrs).
98 All participants were recreationally active and deemed healthy based on their responses to a
99 general health questionnaire. Participants suffering from uncontrolled hypertension or
100 generalised neuromuscular, metabolic or cardiovascular diseases were excluded from the
101 study. Furthermore, taking part in regular structured resistance exercise whilst partaking in

102 the current study, smoking, and consuming non-steroidal anti-inflammatory drugs or any
103 medication that might interfere with muscle metabolism, rendered the participant ineligible to
104 participate. Prior to obtaining written consent, participants were informed of the purpose and
105 methodology of the study. Ethical approval was obtained through the Solihull Research
106 Ethics Committee (15/WM/0228). The study conformed to the latest guidelines set by the
107 Declaration of Helsinki (7th edition). This trial is registered at Clinicaltrials.gov registration
108 no: NCT03446924

109

110 **Experimental design**

111 Following an initial screening, participants visited the laboratories of the School of Sport,
112 Exercise and Rehabilitation Sciences (SportExR) on two separate occasions. The study was
113 parallel-designed and single-blinded, with participants randomized to receive either the
114 placebo control (PL; n = 8) or phosphatidic acid treatment (PA, n = 8). During the initial
115 visit, anthropometric characteristics and isotonic leg strength were determined.

116 Approximately one week after this initial visit, participants underwent an experimental trial
117 to determine the muscle anabolic properties of supplemental PA. The experimental trial
118 consisted of a stable isotope amino acid infusion combined with serial muscle biopsies to
119 determine the MyoPS response to PA or PL consumption alone, or in combination with
120 resistance exercise.

121

122 **Preliminary Assessments**

123 **Body mass and height:** Participant body mass was recorded in loose clothing and without
124 shoes to the nearest 0.1kg using a digital balance scale. Height was determined to the nearest
125 0.1cm using a stadiometer.

126 **Body composition:** Following determination of body mass and height, participants underwent
127 a dual energy x-ray absorptiometry scan (DXA) (Discovery DXA Systems, Hologic Inc.,
128 Bedford, MA) to determine whole-body and regional bone mineral density, fat- and fat-free
129 mass. DXA scans were performed after a ~10 h overnight fast. Participants rested supine on
130 the scanner in loose clothing with their feet positioned at shoulder width apart and held in
131 place with micropore tape. Each scan took 7 min and was analysed by a trained DXA
132 operator.

133 **Isotonic leg strength:** Participant knee extensor one repetition maximum (1RM) strength was
134 estimated in the dominant leg using a leg extension machine (Cybex VR-3, Medway, MA,
135 USA) in order to determine the appropriate load of 75% 1RM for the subsequent
136 experimental trial. Briefly, prior to exercise commencement, a baseline blood pressure
137 measurement was obtained to ensure participants were normotensive (diastolic blood
138 pressure 60-90 mmHg, systolic blood pressure 120-140 mmHg) to reduce the risk of adverse
139 events during heavy-load, fatiguing RE. Following blood pressure assessment, participants
140 initiated a self-selected 1-set warm-up after which the exercising load was gradually
141 increased over subsequent sets until participants were unable to perform >10 repetitions. This
142 final load was used to estimate knee extensor 1RM strength via the Brzycki equation [28].
143 The increment in loading was based on subjective ratings of exercise intensity, which were
144 recorded immediately after each lifting attempt using a modified Borg category-ratio scale
145 (CR-10) [29]. Each exercise set was separated by 2 min of passive rest. Strength assessments
146 were led by a trained strength and conditioning specialist.

147 **Dietary and activity control:** Participants were asked to fast 10 hours overnight for both the
148 initial and experimental trial visit, but were provided with a standardised meal on the evening
149 prior to the experimental trial only. The meal contained ~787 kcal, comprised of ~19%
150 protein (~37.5 g), ~46% carbohydrate (~90.7 g) and 35% fat (30.8 g). Participants were asked

151 to abstain from alcohol and caffeine for 24 h prior to the experimental trial. Furthermore,
152 participants were asked to refrain from strenuous exercise for 48 h prior to the experimental
153 trial.

154

155 **Experimental Trial**

156 Following a 10-hour overnight fast, participants returned to the SportExR laboratory at 0630
157 h following a ~10 h overnight fast. Upon arrival, a 21G cannula was inserted in an
158 antecubital vein of both forearms. One cannula was used for frequent blood sampling, whilst
159 the other was used to administer a stable isotope amino acid infusion. After obtaining a
160 baseline blood sample, a primed continuous infusion of L-[ring-¹³C₆] phenylalanine was
161 initiated (prime: 2 $\mu\text{mol}\cdot\text{kg}^{-1}$; infusion: 0.05 $\mu\text{mol}\cdot\text{kg}^{-1}$, Cambridge Isotope Laboratories,
162 Andover, MA, USA). The contralateral arm was warmed to ~60°C using an electric heating
163 blanket to obtain arterialized blood samples at -180, -120, -60 and immediately prior to
164 exercise, and at 20, 40, 60, 90, 120, 180, 240 and 300 min of post-exercise recovery. A total
165 of 10 mL of arterialized blood was sampled at each time point and separated into
166 ethylenediaminetetraacetic (EDTA) and serum separator vacutainers (BD, Oxford, UK).
167 Blood samples were centrifuged for 10 min at 3000G and 4°C. Plasma and serum were
168 aliquoted and stored at -80°C until further analyses. After ~150 min of stable isotope
169 infusion, a muscle biopsy was obtained under local anaesthesia (1% lidocaine) from the
170 quadriceps *vastus lateralis* of the non-dominant, non-exercised leg using the Bergström
171 technique [30]. Muscle biopsy tissue was quickly rinsed in ice-cold saline and freed from any
172 visible blood, connective tissue and fat before being snap-frozen in liquid nitrogen and stored
173 at -80°C. Immediately after biopsy obtainment, participants completed a unilateral leg
174 extension RE bout of the dominant leg to elicit a rise, but not maximise, MyoPS [31].
175 Resistance exercise consisted of two warm-up sets of 12 repetitions at 50% of their

176 previously estimated 1RM followed by 6 sets of 12 repetitions at 75% of their estimated
177 1RM, to elicit a rating of ~8-9 on the Borg CR-10 scale. Exercise sets were interspersed by 2
178 min of passive rest during which participants remained seated on the machine. Participants
179 completed the exercise with a lifting-lowering cadence of ~1 sec without pause. Time-under-
180 tension and Borg CR-10 rating were recorded after each exercise set. Following RE
181 completion, participants consumed their respective PA or placebo control treatment over 1 h
182 post-RE (described in detail below) and remained in a supine position for the remainder of
183 the experimental trial. At 150 and 300 mins after starting treatment consumption, muscle
184 biopsies were obtained from the non-exercised and exercised legs. Muscle biopsies were
185 sampled ~2-3 cm from one another in a distal-to-proximal orientation. An overview of the
186 experimental trial is provided in Figure 1.

187

188 **Treatment Administration**

189 Immediately and 60 min after RE completion, participants consumed two gelatine capsules
190 (i.e. 4 capsules in total), each containing 375 mg of either a rice-flour placebo control (PL) or
191 phosphatidic acid-enriched soybean phospholipid supplement (PA; Mediator® 50P,
192 ChemiNutra, Austin, US). Treatment capsules were visually identical and ingested with
193 water. The phospholipid composition of PA was; 50-60% phosphatidic acid, 5-15%
194 phosphatidylcholine, 5-15% phosphatidylethanolamine, 1-5% phosphatidylinositol, 1-5%
195 lyso-phosphatidylcholine, 1-5% N-acyl phosphatidyl ethanolamine. Thus, the total 1500mg
196 of ingested supplement material provided ~750mg of PA over 1 h post-exercise. Supplements
197 were ingested post-RE completion to minimize any potential negative effects of RE on gut
198 absorption kinetics and minimize splanchnic extraction of PA [32] (i.e. absorption by
199 intestinal mucosa). Based on the data of Purpura et al. [33] and our own pilot work in
200 younger males, PA was ingested in a biphasic manner to ensure plasma PA concentrations

201 remained elevated for the majority of the RE recovery period. The PA supplement source and
202 total ingested dose was similar to that used by others demonstrating; i) robust increases in
203 p70S6K signaling in cultured cells [25], ii) elevated rates of MPS and mTORC1-mediated
204 signaling in rats when consumed acutely in a similar equivalent dose for humans [23], iii) an
205 increase in circulating PA and lysophosphatidic acid concentrations in a young male [33] and
206 iv) augmented lean body mass and strength increases when consumed during prolonged
207 resistance training in young males [25, 26], albeit using a different ingestion pattern to that
208 chosen here.

209

210 **Blood Analyses**

211 *Plasma amino acids, isotope enrichment and serum insulin*

212 Plasma [$^{13}\text{C}_6$] phenylalanine enrichment was determined by gas chromatography-mass
213 spectrometry (GCMS; model 5973; Hewlett Packard, Palo Alto, CA, USA) by monitoring
214 ions 234/240. Briefly, 300 μL of plasma was diluted 1:1 with acetic acid before being
215 purified through cation-exchange columns and dried down overnight under nitrogen. The
216 purified amino acids were then converted to their N-tert-butyltrimethylsilyl-N-
217 methyltrifluoroacetamide (MTBSTFA) derivative. Simultaneously, leucine and phenylalanine
218 concentrations were measured by GCMS using the internal standard method based on the
219 known volume of plasma and internal standard added and the known amino acid
220 concentration of the internal standard. The internal standards were U- $^{13}\text{C}_6$ leucine (ions
221 302/308) and U- $^{13}\text{C}_9$ - ^{15}N phenylalanine (ions 336/346) added in a ratio of 100 $\mu\text{L}\cdot\text{mL}^{-1}$ of
222 blood. Insulin concentrations were measured using commercially available enzyme-linked
223 immunosorbent assay kit (IBL International, Hamburg, Germany).

224

225 *Plasma phosphatidic acid concentration*

226 Plasma [PA] was determined in a subset of participants (PA: n = 4; PL: n = 4) using the
227 internal standard method, where 0.0013 mg of C17:0 PA (Avanti Polar Lipids, #830856) was
228 added to 500 mL of plasma. Plasma lipids were extracted according to Folch et al [34] using
229 a chloroform, methanol and acidified salt solution (2:1:0.8 v/v). Once extracted, lipids were
230 re-dissolved in chloroform:methanol (2:1 v/v) before being spotted onto an HPTLC silica
231 plate and separated into its component lipid fractions using a chloroform, petroleum ether,
232 methanol, acetic acid and boric acid solvent mix (40:30:20:10:1:8 v/v). The PA position was
233 identified using a TLC identification standard (Larodan AB, #37-0140 Phosphatidic Acid
234 (egg PC)) before being removed and esterified, worked up and separated into fatty acids by
235 gas chromatography (Agilent 7890 Gas Chromatograph with FID Detector, Agilent UK)
236 using a carbowax column (Agilent, CP-Wax52CB). Plasma [PA] concentrations are
237 expressed as $\mu\text{g}/\text{mg}$ plasma and fold-change from basal values, due to inherent background
238 variability in [PA] between participants.

239

240 **Muscle Tissue Analyses**

241 ***Myofibrillar protein enrichment***

242 The myofibrillar protein fraction and muscle free pool were extracted for the analysis of $^{13}\text{C}_6$
243 phenylalanine enrichment from ~30 mg of muscle tissue as previously described [35].

244 Briefly, muscle tissue samples were homogenised in a 2 mL microtube using clean sharp
245 scissors. Homogenised samples were placed on a shaker (IKA, Vibrax, Germany) for 10 min
246 at room temperature at 1500 rpm and subsequently centrifuged at 11000 g for 15 min at 4°C.

247 The supernatant containing the amino acid free pool (IC) was transferred to a clean microtube
248 and the myofibrillar pellet washed twice through centrifugation at 11000 g for 15 min at 4°C
249 with 500 μL homogenisation buffer. To separate the collagen fraction from the myofibrillar
250 pellet, the pellet was incubated in 750 μL of 0.3 M NaOH for 30 min at 30°C, giving the

251 sample a vortex mix at 15 and 30 min. The sample was then spun at 13000 rpm for 10 min at
252 4°C and the supernatant transferred to a 4 mL glass collection tube. A 750 µL volume of 0.3
253 M NaOH was then added to the pellet, centrifuged at 13000 rpm for 10 min at 4°C and the
254 supernatants combined. Myofibrillar proteins were then precipitated by adding 1 mL of 1 M
255 PCA, which was centrifuged at 3200 rpm for 20 min at 4°C. The pellet was washed twice
256 with 2 mL of 70% ethanol. The remaining myofibrillar pellet was hydrolysed at 110°C
257 overnight in 1 mL of 0.05 M HCl and 1 mL of activated Dowex 50W-X8 100-200 resin (Bio-
258 Rad laboratories INC, USA). Constituent amino acids of the myofibrillar fraction were
259 purified on cation-exchange columns by eluting with 2 M NH₄OH and evaporating to
260 dryness. Amino acids were then derivatised as their n-acetyl-n-propylester, and phenylalanine
261 labelling determined by gas chromatography-combustion-isotope mass spectrometry (Delta
262 Plus XP, Thermofisher Scientific, Hemel Hempstead, UK).

263

264 *Intramuscular signaling*

265 Western blot analyses were performed on the sarcoplasmic protein fraction obtained during
266 myofibrillar protein isolation (described above). Ubiquitin protein conjugates were analysed
267 using separate sarcoplasmic protein fractions with the addition of 2-chloroacetamide (Sigma-
268 Aldrich, St. Louis, MO) to a final concentration of 100 mM. Sarcoplasmic protein content
269 was determined by a DC protein assay before western blot aliquots of 2 µg protein per 1 µL
270 were prepared in 4x Laemmli sample buffer and sucrose lysis buffer and subsequently boiled
271 for 5 min. Equal amounts of protein (30 µg) were loaded onto 7.5–15 % gels and separated
272 by SDS-PAGE for 1 h. Following electrophoresis, proteins were transferred onto a biotrace
273 nitrocellulose membrane (Pall Laboratory, Portsmouth, U.K.) for 1 h at 100 V, or in regard to
274 the ubiquitinated proteins, S6 ribosomal protein (rps6), (LC3 a/b) and Caspase-3 onto a
275 polyvinylidene fluoride (PVDF) membrane for 2.5h at 60 V. Membranes were subsequently

276 blocked in 5 % milk for 1 h and washed 3 times for 5 min in TBST before being incubated
 277 overnight at 4°C in following primary antibodies: Muscle Ring Finger protein 1 (MuRF1:
 278 Santa Cruz Biotechnology, Dallas, Texas, U.S; sc-398608), mono- and polyubiquitinated
 279 conjugates (FK2: Enzo Life Sciences LTD, Exeter, U.K.) phospho-p70S6K1 Thr389 (#9205),
 280 total 70 kDa S6 protein kinase (p70S6K1; #9202), phospho-eukaryotic initiation factor 4E
 281 binding protein (4E-BP1) Thr37/46 (#9459), total 4E-BP1 (#9452), phospho-eukaryotic
 282 elongation factor 2 (eEF2) Thr56 (#2331), total eEF2 (#2332), phospho-protein kinase B
 283 (Akt) Ser473 (#3787), total Akt (#9272), phospho-AMP activated protein kinase α (AMPK α)
 284 Thr172 (#2535), total AMPK α (#5831), phospho-p44/42 MAPK (Erk 1/2)^{Thr202/Tyr204}
 285 (#4370), total p44/42 MAPK (#4695), phospho-rps6 Ser240/244, total rps6 (#2217), LC3a/b
 286 (#12741) and Caspase-3 (#9665) each purchased from Cell Signaling Technology (New
 287 England Biolabs (UK) Ltd, Hitchin, U.K.). Membranes were then washed 3 times for 5 min
 288 in TBST and incubated for 1 h in their respective secondary antibody and washed again 3
 289 times for 5 min in TBST. Protein quantification was achieved by incubating the membranes
 290 for 5 min in Immobilon Western chemiluminescent HRP substrate (Merck Millipore,
 291 Watford, UK) before being imaged using a G:BOX Chemi XT4 imager using GeneSys
 292 capture software (Syngene, Cambridge, U.K.). Bands were quantified using Image Studio
 293 Lite (Li-Cor, Lincoln, Nebraska, U.S.).

294

295 **Calculations**

296 MyoPS rates were calculated from ¹³C₆ phenylalanine incorporation by using the standard
 297 precursor-product model:

$$298 \text{FSR } (\% \cdot \text{h}^{-1}) = \Delta E_b / E_p \times 1 / t \times 100$$

299 Where ΔE_b is the difference in bound ¹³C₆ phenylalanine enrichment between two biopsy

300 samples, E_p is the mean plasma precursor enrichment and t is the time in min between muscle

301 biopsy samples. Basal MyoPS rates were calculated in the rested/fasted state using the pre-
302 infusion plasma $^{13}\text{C}_6$ phenylalanine enrichment as a proxy for basal muscle protein
303 enrichment, a technique that has previously been previously validated in tracer naïve older
304 individuals [36].

305

306 **Statistics**

307 Data analysis was performed using SPSS version 22 (IBM, Chicago, IL, USA).

308 Anthropometric and exercise parameters were analysed using a one-way ANOVA. MyoPS
309 rates, intracellular signaling, plasma PA concentrations and insulin were analysed using a
310 two-way, repeated measures ANOVA with one within (three levels; basal, early and late
311 MyoPS) and one between factor (two levels; group). A Tukey's HSD post hoc test was
312 performed whenever a significant F ratio was found to determine specific differences.

313 Significance was set at $p < 0.05$ for all analyses. All values are presented as means \pm SD or
314 SEM.

315

316 **RESULTS**

317 **Anthropometric and Exercise Variables**

318 There were no significant differences between PA and PL for anthropometric or strength
319 characteristics (Table 1). Similarly, RE variables including knee extension 1RM ($P = 0.43$),
320 total time-under-tension ($P = 0.61$), time-under-tension per set ($P = 0.43$), total volume ($P =$
321 0.46), volume per set ($P = 0.46$) and average load per set ($P = 0.43$) were not significantly
322 different between PLA and PA (Table 1).

323

324 **Plasma Amino Acid, Phosphatidic Acid and Serum Insulin Concentrations**

325 Plasma leucine and phenylalanine concentrations did not change from basal values during the
326 experimental trial and were similar between groups at all time-points (Figure 2A and B,
327 respectively). Serum insulin concentration decreased significantly below basal values by 90
328 min post-exercise cessation until 240 and 300 min post-exercise in PL ($P < 0.05$) and PA ($P <$
329 0.05), respectively, with no differences between groups (Figure 2C). Plasma $^{13}\text{C}_6$
330 phenylalanine enrichment significantly increased above basal values (-180 min) 60 min after
331 initiation of the stable isotope tracer infusion and remained elevated for the duration of the
332 trial in PL and PA ($P < 0.001$). Linear regression analysis revealed that the $^{13}\text{C}_6$
333 phenylalanine enrichment slopes in both groups were not significantly different from zero,
334 confirming the obtainment of an isotopic steady state (Figure 2D). Plasma PA concentration
335 was significantly elevated above basal values at 180 min post-exercise ($P = 0.02$) until 300
336 min post-exercise ($P = 0.01$) in PA only. There was no difference from basal plasma [PA]
337 values in PL (Figure 3A and B). Fold-change in plasma [PA] concentration was significantly
338 greater in PA compared with PL at 240 and 300 min post-exercise ($P = 0.026$ and 0.020 ,
339 respectively).

340

341 **Myofibrillar Protein Synthesis**

342 Basal-state, temporal MyoPS rates (0-150 min and 150-300 min) and aggregate MyoPS rates
343 (0-300 min) in rested and exercised legs are presented in Figure 4A and 4B. MyoPS rates in
344 the rested leg did not differ from basal values at any time-point, nor between groups. In the
345 exercised leg, MyoPS rates over 150-300 min were significantly greater than basal values (P
346 $= 0.001$) and 0-150 min ($P = 0.019$) in PL only, and were ~40% greater than basal values
347 over the aggregate 0-300 min in PL only ($P = 0.023$). In the exercised leg, there was a strong
348 trend for greater MyoPS rates over 150-300 min in PL compared with PA ($P = 0.051$).

349 Temporal and aggregate MyoPS rates did not differ from basal values in rested or exercised

350 legs in PA, although there was a tendency for a reduction in MyoPS from basal values in the
351 rested leg over 150-300 min in PA only ($P = 0.053$).

352

353 **Intramuscular Signaling**

354 *Anabolic Signaling*

355 Phosphorylation of p70S6K^{Thr389}, 4EBP1^{Thr37/46}, Akt^{Ser473} and rps6^{Ser240/244} in the PL exercised
356 leg were, respectively, elevated 3.6-, 1.7-, 2.4- and 2.2-fold compared with baseline values at
357 150 min ($P = 0.002$; $P = 0.019$, $P < 0.001$ and $P = 0.021$ respectively), and remained
358 significantly elevated 300 min post RE ($P = 0.003$; $P = 0.001$, $P = 0.025$ and $P = 0.007$
359 respectively). In the PL rested leg, AMPK α ^{Thr172} phosphorylation at 300 min was,
360 respectively, 1.7- and 1.9-fold greater than corresponding values at baseline ($P = 0.028$) and
361 150 min ($P = 0.002$). At 150 min, p70S6K^{Thr389}, Akt^{Ser473} and rps6^{Ser240/244} phosphorylation in
362 the PL exercised leg were greater than corresponding resting values at 150 min ($P = 0.002$, P
363 $= 0.002$ and $P = 0.004$ respectively). At 300 min, 4EBP1^{Thr37/46}, p70S6K^{Thr389} and Akt^{Ser473}
364 phosphorylation in the PL exercised leg were elevated compared with the corresponding
365 rested leg ($P = 0.002$, $P = 0.001$ and $P = 0.009$ respectively). In the PA group, p70S6K^{Thr389}
366 phosphorylation was significantly elevated from basal values 150 min upon exercise
367 completion ($P = 0.047$). Rps6^{Ser240/244} was 2.1-fold elevated in the PA exercised compared
368 with the rested leg at 150 min ($P = 0.037$), whilst AMPK α ^{Thr172} was 1.9-fold greater in the
369 exercised compared with rested leg at 300 min ($P = 0.027$). Between-group differences were
370 observed at 150 min in the rested leg for 4E-BP1^{Thr37/46} ($P = 0.022$) and in the exercised leg
371 for Akt^{Ser473} ($P < 0.001$), whilst between-group differences at 300 min were apparent in the
372 rested leg for AMPK α ^{Thr172} ($P = 0.001$) and in the exercised leg for p70S6K^{Thr389} ($P = 0.046$),
373 4EBP1^{Thr37/46} ($P = 0.005$) and rps6^{Ser240/244} ($P = 0.003$) phosphorylation. No changes were
374 found for MAPK^{Ser44/42} and eEF2^{Thr56} phosphorylation (Figure 5A-F).

375

376 *Proteolytic Signaling*

377 MuRF1 protein content (Figure 6A) was elevated 1.2-fold from baseline values in the PA
378 rested leg at 300 min ($P = 0.019$), and 1.4-fold in PL exercised leg at 300 min compared with
379 their corresponding resting value ($P = 0.002$). Between-group differences for Murf1 protein
380 content occurred in the rested leg at 300 min ($P = 0.019$). Total ubiquitinated protein
381 conjugates (Figure 6B) in the PL rested leg were, respectively, elevated 1.4- and 1.3-fold
382 above basal values at 150 ($P = 0.001$) and 300 min ($P = 0.005$), and were greater than
383 corresponding exercising values at 150 ($P = 0.008$) and 300 min ($P = 0.007$). Between-group
384 differences were apparent for the resting leg at 150 ($P < 0.001$) and 300 min ($P = 0.002$).
385 Quantification of a single distinct band at ~30 kDa (Figure 6C) revealed significantly
386 elevated total protein ubiquitination in the PL exercised leg at 150 min compared with basal
387 values ($P = 0.011$) and corresponding resting values ($P = 0.047$; Figure 6B). Ubiquitinated
388 proteins seemed to occur mainly above 100 kDa. LC3 II protein content (Figure 6D) was
389 elevated from baseline values 150 min post exercise in PL ($P = 0.027$). Higher LC3 II protein
390 contents were found in PL compared with PA at 150 ($P < 0.001$) and 300 min ($P = 0.015$) in
391 the exercising leg, and at 150 min in the resting leg ($P = 0.003$). Total Caspase-3 (Figure 6E)
392 protein decreased in the PL resting leg from 150 min to 300 min ($P = 0.022$).

393

394 **DISCUSSION**

395 At the forefront of strategies to combat sarcopenia is the development of novel
396 interventions to enhance MyoPS and overcome the ‘anabolic resistance’ of aged skeletal
397 muscle. In this regard, PA has been touted as a nutraceutical with muscle anabolic properties,
398 phosphorylating mTORC1-mediated signaling leading to enhanced protein translational
399 efficiency [24, 27]. Both endogenous PA production [37] and exogenous PA provision [22]

400 phosphorylate mTORC1 and associated distal signaling proteins (i.e. p70S6K and 4E-BP1) in
401 cell culture and animal models, albeit via different pathways. Herein, we aimed to investigate
402 these separate but synergistic effects of distinct PA-mediated signaling pathways on MyoPS
403 in older individuals through acute oral PA provision alone or combined with mechanical
404 loading. Following PA ingestion, plasma [PA] concentrations rose above basal values at 180
405 min post-exercise. Surprisingly, PA ingestion inhibited the RE-induced rise in intramuscular
406 anabolic signaling and MyoPS observed in PL over 5 h of recovery. No robust group
407 interactions were found, likely due to the relatively small sample size studied. Thus, our
408 findings suggest that, in an acute experimental setting, oral PA provision has an interference
409 effect on the muscle remodelling response to RE in older individuals.

410 Reconciling the present findings of a potential PA-induced interference effect on
411 MyoPS is difficult considering the established *in vitro* evidence that PA exerts anabolic
412 effects on mTORC1-mediated signaling [22, 25, 38]. Support for our findings can be gleaned
413 from the work in rodents [23], in which a whey protein-induced rise in MPS was attenuated
414 by co-ingestion of PA, at a dose equivalent to that provided herein. Despite dampened MPS
415 rates with PA co-ingestion, mTORC1-mediated signaling events were numerically greater
416 with combined whey protein *plus* PA. The authors suggested PA may have altered mTORC1-
417 mediated signaling, causing the peak MPS time to shift. The results of our study support
418 similar inhibitory effects of PA on MyoPS, particularly in the presence of a second anabolic
419 stimulus (i.e. RE). In contrast to the work of Mobley et al. [23], our findings demonstrate that
420 acute oral PA ingestion prevented any RE-induced rise in anabolic signaling, which most
421 likely impaired protein translational efficiency and prevented a similar MyoPS response to
422 that observed in PL [39]. Elucidating the mechanisms underlying the tendency towards
423 lower resting MyoPS rates over 150-300 min post-PA ingestion is more challenging. These
424 observations are in stark contrast to Mobley et al. [23], in which PA ingestion alone increased

425 anabolic signaling and potentially MPS. These discrepant findings could be attributed to the
426 different species studied (rodents vs. humans). Besides PA-related alterations in downstream
427 mTORC1-mediated signaling, acute oral PA supplementation did not alter the ERK
428 signaling pathway, which has been suggested to be an important mechanistic link between
429 exogenous PA provision and mTORC1-mediated signaling *in vivo* [40]. The PA-induced
430 activation of ERK, and thus mTORC1 signaling, hinges on the conversion of PA to
431 lysophosphatidic acid (LPA), which, in turn, depends on the presence of the converting
432 enzyme phospholipase A (PLA) [22]. Thus, the absence of between group differences in
433 MAPK signaling, in response to exogenous oral PA ingestion, suggests that this route of
434 administration may not have altered LPA production. Taken together, the present findings
435 demonstrate an impaired anabolic signaling response to RE when combined with acute PA
436 ingestion, reinforcing the lack of any MyoPS response.

437 In addition to MyoPS and anabolic signaling, changes in proteolysis and proteolytic
438 signaling events may play an important role in the overall net muscle protein balance to RE
439 [41]. Therefore, we were interested to see if acute oral PA ingestion modulated any potential
440 change in RE-induced proteolytic signaling. Earlier work conducted by Jaafar et al. [42]
441 revealed distinctive anti-proteolytic characteristics for PA. Specifically, overexpressing
442 myotubes with the PA precursor, phospholipase D1, protected against the atrophy-promoting
443 agent dexamethasone, whilst PA provision protected against the atrophying effects of TNF- α .
444 In our *in vivo* human ageing model, we found equivocal effects of oral PA ingestion on
445 intracellular proteolytic signaling. Whereas Caspase-3 did not reveal any significant between
446 group differences, 30kDa Ubiquitin-conjugates and LC3-II seemed to be higher in PL
447 compared with PA. However, it is important to acknowledge that others have previously
448 observed that alterations in the ubiquitin proteasome pathway are not easily reconciled with
449 observed effects on muscle protein turnover [43]. Furthermore, the proteolytic markers

450 measured in the present study only represent a small fraction of the vast complexity of the
451 signaling proteins that regulate proteolysis and offer only snapshots of this dynamic
452 processes. Without direct measurement of myofibrillar breakdown rates, which is technically
453 challenging, the effects of orally ingested PA on proteolysis remain inconclusive

454 The specific mechanisms through which PA interferes with RE-induced MyoPS and
455 potentially impairs basal-state MyoPS in older individuals are intriguing. One possibility is
456 that PA-derived production of diacylglycerol, catalysed by the enzyme phosphatidic acid
457 phosphatase, may impair insulin signaling and blunt MyoPS rates [44-46]. Indirect evidence
458 for lipid-induced impairment in MyoPS is apparent from our recent findings of a negative
459 association between fat mass and postprandial MyoPS rates in older individuals [47].
460 Furthermore, Stephens et al. [48] reported a suppression in postprandial MPS rates and
461 mTORC1-mediated signaling (i.e. anabolic resistance) when insulin resistance was induced
462 via lipid infusion in healthy young individuals. It is important to note that Stephens et al. [48]
463 infused lipid intravenously over 7 h, bypassing splanchnic absorption, resulting in a
464 circulating lipid milieu that was substantially greater and occurred more rapidly than our
465 acute oral PA ingestion protocol. Furthermore, intravenous lipid infusion resulted in
466 significant intramuscular lipid metabolite accumulation, which would directly explain
467 impairments in insulin signaling and MPS. In the present study, acute oral PA ingestion
468 significantly increased plasma [PA] ~2-3-fold from 180 min post-ingestion, which coincided
469 with the temporal impairment in MyoPS over 150-300 min post-exercise. Unfortunately, due
470 to limitations on biopsy tissue sampling, we can only speculate as to whether the increase in
471 plasma [PA] following acute oral PA ingestion altered intracellular lipid content and insulin
472 resistance. What is clear, is that events arising from the extracellular increase in [PA] were
473 sufficient to impair mTORC1-mediated signaling and MyoPS.

474 Although we have provided the first *in vivo* mechanistic investigation of orally
475 ingested PA in humans, there is still much to be understood about the muscle anabolic
476 properties of this purported nutraceutical. Centred around previous animal studies
477 establishing the muscle anabolic properties of acute PA consumption [23], we chose to
478 administer a similar equivalent oral dose based on the per species conversion calculations of
479 Reagan-Shaw et al [49], in an attempt to understand whether these responses would translate
480 to an *in vivo* human ageing model. The delayed rise in plasma [PA] following PA ingestion,
481 compared with case study values reported by others [33], might be due to the biphasic
482 ingestion protocol, where the second dose of PA was ingested 60 min after the first.
483 However, we acknowledge that the timing of PA ingestion around RE may influence any
484 potential anabolic effects of this compound. Indeed, enhancements in RE-induced strength
485 and lean body mass in young males were reported when supplemental PA was consumed
486 prior-to (450mg) and immediately after (300mg) RE training [25], although others failed to
487 replicate these findings [50]. In theory, replicating the PA ingestion pattern of Joy et al. [25]
488 would have shifted the increase in plasma [PA] above basal values to ~60 min earlier than
489 observed (i.e. 120 min post-exercise), which, we posit, is unlikely to have significantly
490 altered MyoPS responses. Nonetheless, given that plasma [PA] concentrations remained
491 elevated above basal levels at 5 h post-RE, we cannot rule out MyoPS responses >5 h post-
492 RE might have differed between PL and PA. In light of evidence of the importance of
493 contraction-induced elevations in intracellular [PA] for mTORC1 activation, and the reported
494 anabolic properties of prolonged PA supplementation [25, 51], our finding of an inhibitory
495 effect of acute PA ingestion could indicate that longer-term supplementation may be
496 necessary to increase in intracellular [PA] and muscle anabolism in older individuals. In
497 support of this notion, supplementation of lipid-based nutritional compounds, omega-3 poly-
498 unsaturated fatty acids and arachidonic acid, over 4-8 weeks has been reported to alter

499 intracellular lipid composition and modulate the acute anabolic signaling and ribosome
500 biogenesis response to RE in young individuals [52, 53]. Thus, an important next step is to
501 investigate whether longer-term PA supplementation alters intramuscular [PA] and enhances
502 the acute MyoPS response to RE in older individuals, in addition to better understanding of
503 how supplemental PA dosing and ingestion pattern can be altered to ‘optimize’ delivery.

504 In summary, this is the first *in vivo* study to characterise the effects of acute oral PA
505 ingestion on MyoPS and intramuscular signaling at rest and post-RE in older individuals.
506 Oral PA ingestion attenuated the RE-induced increase in MyoPS. The precise cause of the
507 apparent interference effect of oral PA ingestion on RE-induced muscle anabolism in older
508 individuals remains to be fully elucidated, but seems to be underpinned by impaired
509 intramuscular anabolic signaling. Based on our acute mechanistic findings, oral PA ingestion
510 does not appear to be an effective means of enhancing the muscle anabolic response to RE in
511 older individuals. However, longer-term supplementation studies are required to fully
512 understand whether this purported nutraceutical holds any anabolic potential for aged skeletal
513 muscle.

514

515 Disclosures

516 The authors have no conflicts of interest to declare.

517

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531

532 Authorship Statement

533 All authors gave their final approval of the version of the article to be published. BS, PJA and
534 LB designed the study. BS and LB organized and carried out the clinical experiments with
535 the assistance of JM. BS, YN, JM, ML, KS, PJA and LB performed all data analyses. BS, and
536 LB performed the statistical analysis of the data. BS, PJA and LB wrote the manuscript
537 together. BS and LB are the guarantors of this work and take responsibility for the integrity
538 and accuracy of the data analysis.

539

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TABLES

Table 1. Participant anthropometric, strength and resistance exercise characteristics.

	PL (n = 8)	PA (n = 8)
Age (yrs)	69.4 ± 3.6	68.4 ± 1.8
Age range (yrs)	65 – 75	66 – 70
Body mass (kg)	73.9 ± 7.4	73.7 ± 8.9
BMI (kg·m ⁻²)	23.1 ± 1.6	24.4 ± 2.1
Whole-body FM (kg)	16.4 ± 2.9	19.4 ± 4.0
Leg FM (kg)	5.2 ± 1.0	5.9 ± 1.5
Whole-body FFM (kg)	53.6 ± 5.7	50.6 ± 5.6
Leg FFM (kg)	17.1 ± 1.4	16.1 ± 1.9
Knee extension 1RM (kg)	87.4 ± 18.3	79.4 ± 20.7
Training volume (kg)	1918 ± 377	1725 ± 602
Volume per set (kg)	320 ± 63	288 ± 100
Time-under-tension total (sec)	183 ± 37	170 ± 66
Time-under-tension per set (sec)	31 ± 6	28 ± 11
Average load per set (kg)	26.9 ± 4.3	24.3 ± 8.2
Average Borg CR-10	8.4 ± 1.4	8.1 ± 1.5

Values are presented as means ± SD. BMI; body mass index, FM; fat-mass, FFM; fat-free mass, 1RM; one-repetition maximum, Borg CR-10; Borg category-ratio scale.

FIGURES

Figure 1: Schematic overview of the experimental trial. PA; 750mg of phosphatidic acid-enriched soybean phospholipid supplement, PL; 750mg of rice-flour placebo.

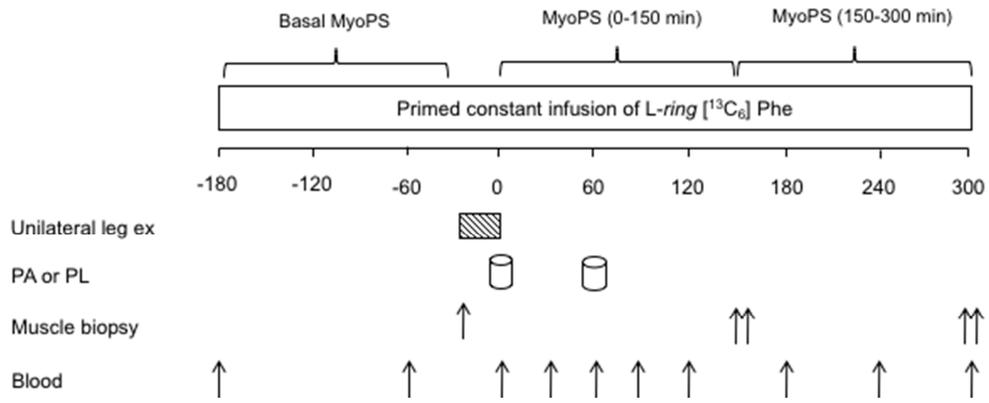
Figure 2: Plasma phenylalanine (A), leucine (B) and insulin concentration (C) and $^{13}\text{C}_6$ phenylalanine enrichment (D) in experimental trials. At $t = 0$ and 60 min, participants orally ingested 750mg of PA or PL with water. * indicates significantly different from basal fasting values (-180 min) for PL and PA. † indicates significantly different from basal fasting values for PA only. Values are means \pm SEM. Significance was set at $P < 0.05$.

Figure 3: Plasma PA concentrations expressed as absolute values (A) and fold-change from basal (B). At $t = 0$ and 60 min, participants orally ingested 750mg of PA or PL with water. Subscript *a* indicates significantly greater than basal values (0 min) for PA. Subscript *b* indicates significantly greater than values at 60 min for PA. * indicates significant between-group difference at the same time-point. Values are means \pm SEM. Significance was set at $P < 0.05$.

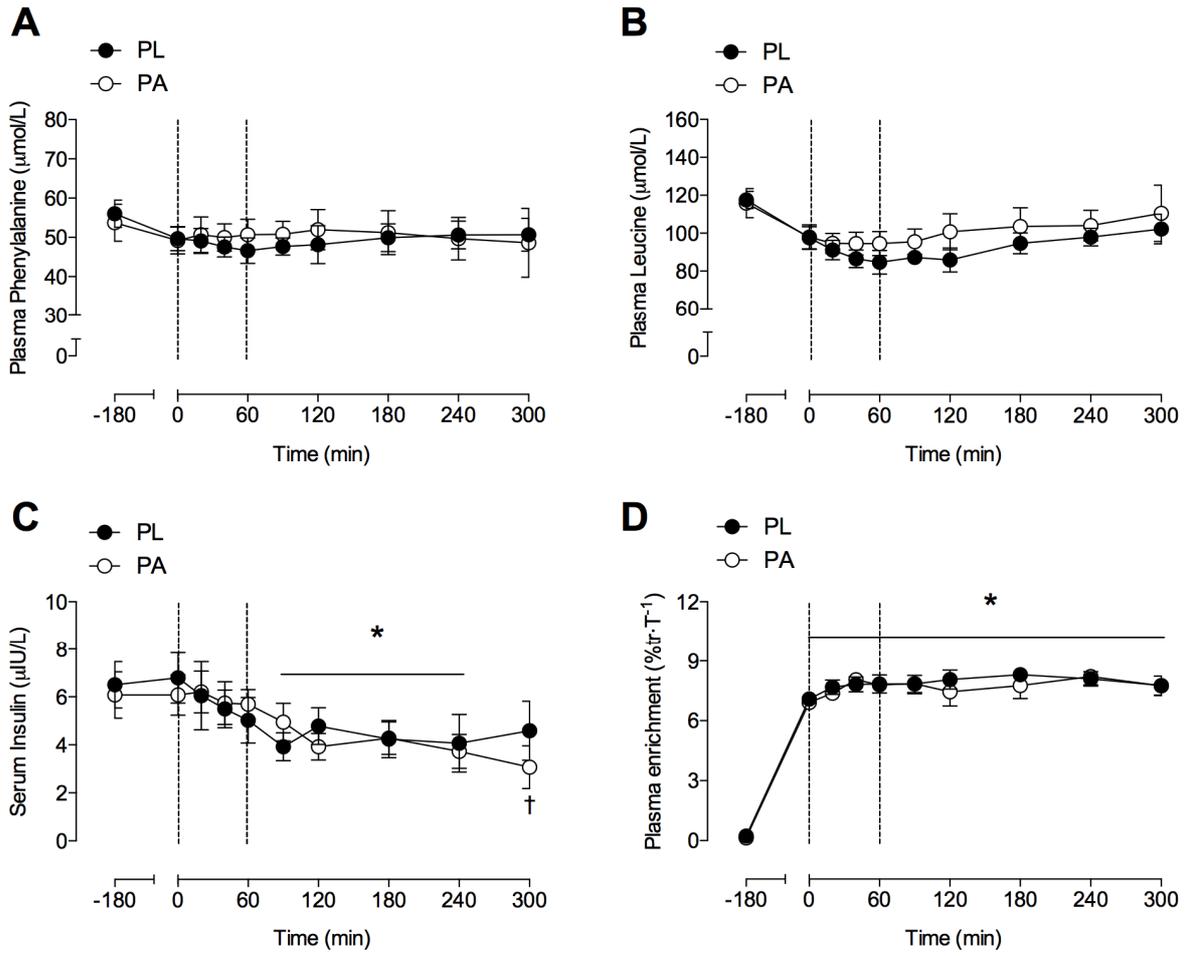
Figure 4: Myofibrillar fractional synthesis rate (FSR) during basal postabsorptive conditions (prior to exercise and/or treatment administration) and over the 0-150 and 150-300 or aggregate 0-300 min post-exercise recovery period in the resting leg (REST; A) and exercised leg (EX; B) after oral ingestion of PA or PL. * indicates significantly greater than basal state MyoPS rates in the same leg, † indicates significantly greater than MyoPS rates over 0-150 min in the same leg. Values are means \pm SEM. Significance was set at $P < 0.05$.

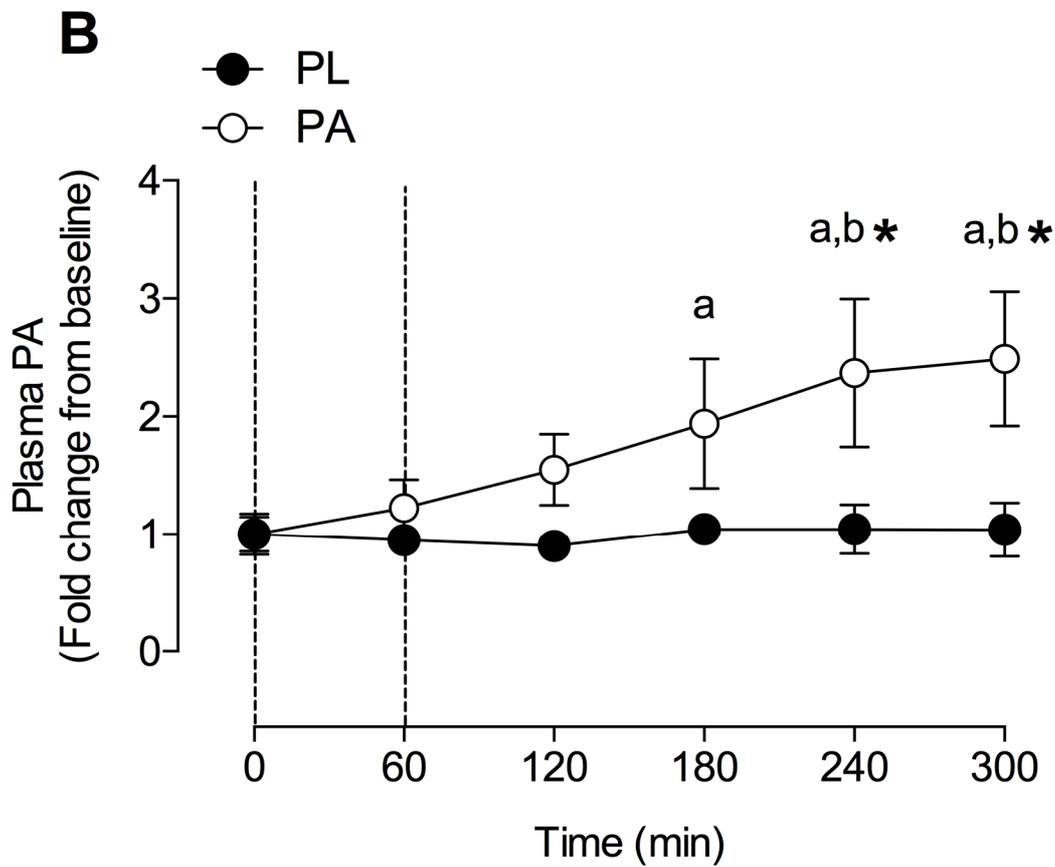
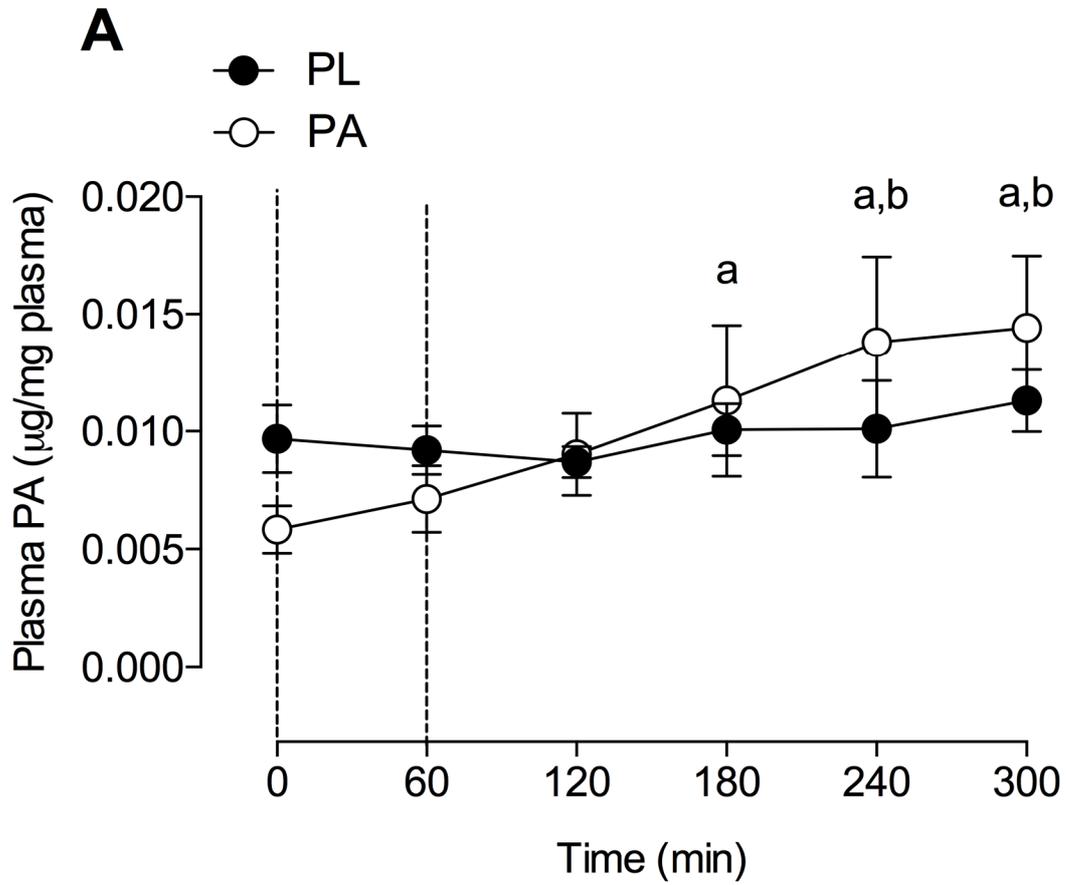
Figure 5: Intracellular signaling phosphorylation of p70S6K^{Thr389} (A), 4E-BP1^{Thr37/46} (B), Akt^{Ser473} (C), eEF2^{Thr56} (D), ERK1/2^{Thr202/Tyr204} (E) and AMPK^{Thr172} (F) expressed as fold-change from basal values at 150 and 300 min post-exercise in rested and exercised legs with PA and PL ingestion. * indicates significantly greater than basal value, ‡ indicates significant within-treatment difference from value at 150 min, † indicates significant different from rested leg at the same time-point, # indicates significant between-treatment difference at same time-point. Values are presented as means \pm SEM. Significance was set at $P < 0.05$.

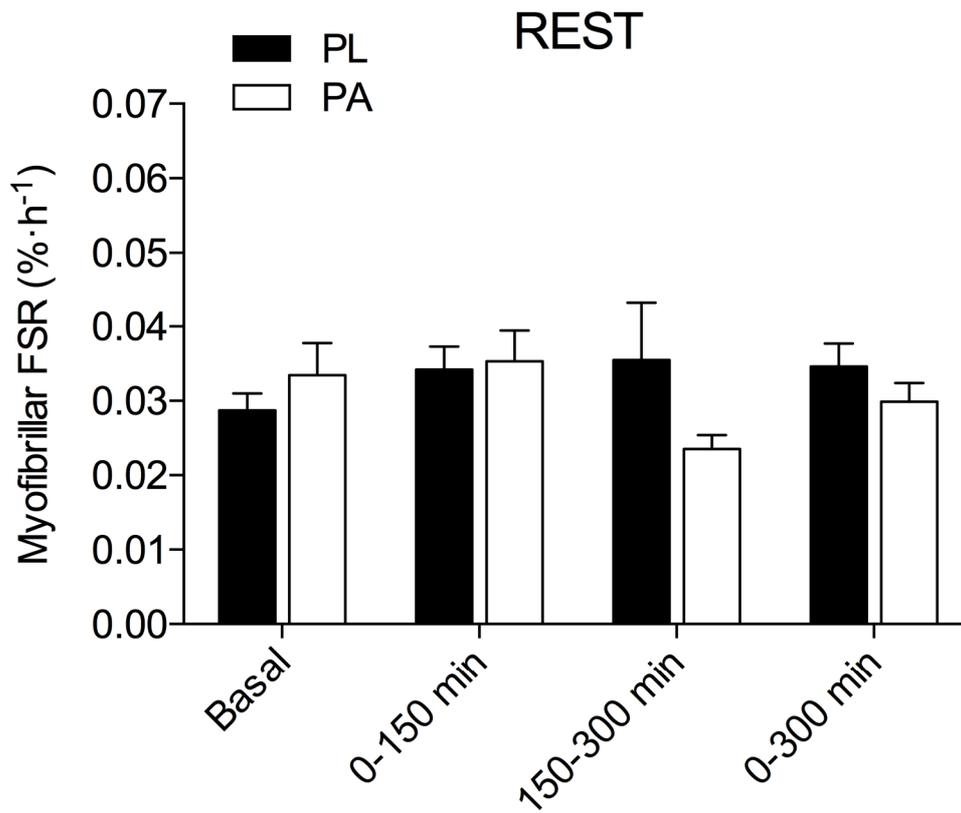
Figure 6: Intracellular proteolytic markers for the abundance of MuRF1 (A), total ubiquitin conjugates (B) ubiquitinated proteins at 30 kDa (C), LC3 (D) and Caspase-3 (E) expressed as fold-change from basal, normalized to Ponceau. * indicates significantly greater than basal value, † indicates significant different from rested leg at the same time-point, # indicates significant between-treatment difference at same time-point. Values are presented as means \pm SEM. Significance was set at $P < 0.05$.



ACCEPTED MANUSCRIPT





A**B**