



LJMU Research Online

Smiles, WJ, Parr, EB, Coffey, VG, Lacham-Kaplan, O, Hawley, JA and Camera, DM

Protein coingestion with alcohol following strenuous exercise attenuates alcohol-induced intramyocellular apoptosis and inhibition of autophagy

<http://researchonline.ljmu.ac.uk/7428/>

Article

Citation (please note it is advisable to refer to the publisher's version if you intend to cite from this work)

Smiles, WJ, Parr, EB, Coffey, VG, Lacham-Kaplan, O, Hawley, JA and Camera, DM (2016) Protein coingestion with alcohol following strenuous exercise attenuates alcohol-induced intramyocellular apoptosis and inhibition of autophagy. American Journal of Physiology Endocrinology and

LJMU has developed **LJMU Research Online** for users to access the research output of the University more effectively. Copyright © and Moral Rights for the papers on this site are retained by the individual authors and/or other copyright owners. Users may download and/or print one copy of any article(s) in LJMU Research Online to facilitate their private study or for non-commercial research. You may not engage in further distribution of the material or use it for any profit-making activities or any commercial gain.

The version presented here may differ from the published version or from the version of the record. Please see the repository URL above for details on accessing the published version and note that access may require a subscription.

For more information please contact researchonline@ljmu.ac.uk

<http://researchonline.ljmu.ac.uk/>

1 Protein Coingestion with Alcohol Following Strenuous Exercise Attenuates Alcohol-Induced
2 Intramyocellular Apoptosis and Inhibition of Autophagy

3

4 William J. Smiles¹, Evelyn B. Parr¹, Vernon G. Coffey², Orly Lacham-Kaplan¹, John A.
5 Hawley^{1,3} and Donny M. Camera¹

6

7 ¹Mary MacKillop Institute for Health Research, Centre for Exercise and Nutrition,
8 Australian Catholic University, Melbourne, Victoria, Australia; ²Bond Institute of Health and
9 Sport and Faculty of Health Sciences and Medicine, Bond University, Queensland, Australia;
10 ³Research Institute for Sport and Exercise Sciences, Liverpool John Moores University,
11 Liverpool, United Kingdom

12

13 **Running Title:** Alcohol, exercise and muscle homeostasis

14

15 **Author Contributions:** WJS, EBP, VGC, JAH and DMC designed the research. WJS, EBP,
16 VGC, and DMC conducted the research. WJS, OLK and DMC analysed the data and/or
17 performed statistical analysis. WJS, VGC, OLK, JAH and DMC wrote the paper. WJS, OLK,
18 JAH and DMC had primary responsibility for its final content.

19

20

21 Author for correspondence:

22 Donny Camera

23 Mary MacKillop Institute for Health Research

24 Centre for Exercise and Nutrition Australian Catholic University

25 Fitzroy VIC 3165

26 Australia

27 Email: donny.camera@acu.edu.au

28 Phone: +61 3 9953 3527

29 Fax: +61 3 9953 3095

30

31

32

33

34 **ABSTRACT**

35 Alcohol ingestion decreases post-exercise rates of muscle protein synthesis, but the
36 mechanism(s) (e.g., increased protein breakdown) underlying this observation are unknown.
37 Autophagy is an intracellular “recycling” system required for homeostatic substrate and
38 organelle turnover; its dysregulation may provoke apoptosis and lead to muscle atrophy. We
39 investigated the acute effects of alcohol ingestion on autophagic cell signaling responses to a
40 bout of concurrent (combined resistance- and endurance-based) exercise. In a randomized
41 cross-over design, 8 physically active males completed three experimental trials of concurrent
42 exercise with either post-exercise ingestion of alcohol and carbohydrate (12±2 standard
43 drinks; ALC-CHO), energy-matched alcohol and protein (ALC-PRO), or protein (PRO) only.
44 Muscle biopsies were taken at rest and 2 and 8 h post-exercise. Select autophagy-related gene
45 (Atg) proteins decreased compared to rest with ALC-CHO (P<0.05), but not ALC-PRO.
46 There were parallel increases (P<0.05) in p62 and PINK1, commensurate with a reduction in
47 BNIP3 content, indicating a diminished capacity for mitochondria-specific autophagy
48 (mitophagy) when alcohol and carbohydrate were coingested. DNA fragmentation increased
49 in both alcohol conditions (P<0.05); however, nuclear AIF accumulation preceded this
50 apoptotic response with ALC-CHO only (P<0.05). In contrast, increases in the nuclear
51 content of p53, TFEB and PGC-1 α in ALC-PRO were accompanied by markers of
52 mitochondrial biogenesis at the transcriptional (Tfam, SCO2, NRF-1) and translational
53 (COXIV, ATPAF1, VDAC1) level (P<0.05). We conclude that alcohol ingestion following
54 exercise triggers apoptosis, whereas the anabolic properties of protein coingestion may
55 stimulate mitochondrial biogenesis to protect cellular homeostasis.

56

57 **Keywords:** Alcohol, exercise, autophagy, apoptosis, protein

58 **INTRODUCTION**

59 Exercise sensitizes skeletal muscle to exogenous nutrient availability, such that substrate
60 availability interacts with cellular processes regulating protein turnover responses that
61 ultimately form the basis for exercise adaptation (20). While the carbohydrate and protein
62 requirements to promote adaptation to divergent exercise stimuli (i.e., endurance- and
63 resistance-based) are well-characterized (8, 44), far less is known regarding the impact of
64 alcohol ingestion on exercise response-adaptation processes. Given the widely reported
65 alcoholic drinking practices of professional and recreational athletes (9), and the capacity for
66 “binge” alcohol consumption to impair recovery-performance and muscle protein synthesis
67 (MPS) rates following strenuous exercise (5, 41), elucidating the mechanisms that underpin
68 these detrimental effects of exercised skeletal muscle exposed to alcohol is warranted.

69

70 Previous studies in rodents report that large quantities of alcohol dysregulate the
71 stimulation of MPS by growth factors and amino acids, largely due to attenuated signal
72 transduction of the mammalian target of rapamycin (mTOR) complex 1 pathway (30, 31), a
73 principal modulator of cellular growth. Similarly, alcohol was found to reduce the maximal
74 MPS response to high-force mechanical overload in rodents (56, 57). Recently, we showed
75 that alcohol ingested following a single bout of strenuous exercise attenuated the maximal
76 exercise- and nutrition-induced stimulation of the myofibrillar (contractile) fraction of protein
77 synthesis, despite exogenous protein availability (41). While the capacity for alcohol to
78 impair MPS is recognized, its impact upon intramuscular protein degradative pathways is less
79 clear. Acute alcohol exposure had no effect on proteasome-dependent proteolysis in rodents
80 (62) and we found no synergistic effect of alcohol toward the transcription of key
81 proteasome-related ubiquitin ligases following exercise (41). Whether autophagy, the
82 constitutive turnover of cellular components such as long-lived organelles (e.g.,

83 mitochondria) that protects homeostasis and is required for skeletal muscle integrity (24),
84 promotes skeletal muscle catabolism in response to alcohol ingested following an acute
85 exercise bout, is unknown.

86

87 Alcohol metabolism produces highly-toxic intermediates that may accelerate cellular
88 metabolism and exacerbate tissue breakdown responses to vigorous contractile activity (33).
89 Furthermore, autophagy is sensitive to the cellular energetic balance and “recycles” nutrient
90 substrate and organelles to maintain cellular viability (52). Thus, a post-exercise autophagic
91 response to alcohol may represent a compensatory removal of harmful protein aggregates that
92 can trigger cell death processes (apoptosis) (1). Accordingly, the primary aim of this study
93 was to investigate the activation of autophagic cell signaling responses to binge alcohol
94 consumption during recovery from a single bout of combined resistance and endurance
95 (“concurrent”) exercise. Due to the anabolic properties of amino acids and their capacity to
96 inhibit autophagic flux (39), we hypothesized that the largest autophagic response would
97 occur when alcohol and carbohydrate were coingested compared to protein following
98 exercise. We also hypothesized that alcohol would elicit an increased mitophagic
99 (mitochondria-selective autophagy) cell signaling response, since the catabolism of alcohol
100 metabolites predominates in the mitochondria and is a source of reactive oxygen species
101 (ROS) production, which may stimulate mitophagy and provoke muscle protein breakdown
102 responses (47, 61).

103

104

105

106

107

108 **MATERIALS AND METHODS**

109 Subjects

110 Eight young, healthy, physically active male subjects [age 21.4 ± 4.8 yr, body mass (BM)
111 79.3 ± 11.9 kg, were recruited from a previous study (41). Full subject characteristics,
112 preliminary testing procedures (VO_{2peak} and maximal muscular strength), diet and exercise
113 control, and complete details of the experimental design and experimental trials have been
114 reported (41). Due to the legal drinking age in Australia, no minors (<18 yr old) were
115 involved in the study. Subjects were advised of any possible risks associated with the study
116 prior to providing written informed consent. The study was approved by the Human Research
117 Ethics Committee of RMIT University (43/11) and was carried out in accordance with the
118 standards set by the latest revision of the Declaration of Helsinki.

119

120 Experimental Design

121 The study employed a randomized, counter-balanced crossover design in which each subject
122 completed, on three separate occasions, trials consisting of a bout of concurrent exercise
123 (described subsequently) with either post-exercise ingestion of alcohol and carbohydrate
124 (ALC-CHO), alcohol and protein (ALC-PRO) or protein only (PRO), in which carbohydrate
125 and protein beverages were consumed twice; before and after the alcohol drinking protocol.
126 Each experimental trial was separated by a two week recovery period, during which time
127 subjects resumed their habitual pattern of physical activity.

128

129 Experimental Trials

130 Subjects reported to the laboratory at ~0700 h on the morning of an experimental trial
131 following a 10 h overnight fast and a resting muscle biopsy was taken from the vastus
132 lateralis under local anaesthesia (1% lidocaine) using a 5 mm Bergstrom needle modified for

133 suction. Subjects then commenced the concurrent exercise bout which consisted of heavy
134 resistance exercise (8×5 knee extension repetitions at 80% of 1 repetition maximum),
135 followed by 30 min of cycling at $\sim 70\%$ of $VO_{2\text{peak}}$, and then a high-intensity (10×30 s,
136 110% PPO) interval cycling bout. Immediately post-exercise and following 4 h of recovery,
137 subjects ingested 500 mL of a carbohydrate (CHO: 25 g maltodextrin) or an isoenergetic
138 protein (PRO: 25 g whey) beverage. Consistent with recommendations for CHO feeding and
139 glycogen resynthesis following training (8), an additional CHO-based meal ($1.5 \text{ g}\cdot\text{kg}^{-1}$ BM)
140 was provided immediately after the first post-exercise (2 h) muscle biopsy with a final muscle
141 biopsy obtained 8 h post-exercise. Muscle biopsies were taken from separate incision sites,
142 cleared of visible adipose and/or connective tissue, snap-frozen in liquid nitrogen and stored
143 at -80°C for subsequent analysis. Subjects commenced drinking alcohol 1 h post-exercise
144 and drinks were consumed in 6 equal volumes of 1 part vodka (~ 60 mL) to 4 parts orange
145 juice (~ 240 mL, $1.8 \text{ g}\cdot\text{kg}^{-1}$ BM) across a 3 h period (12 ± 2 standard drinks consumed),
146 in which alcoholic beverages were consumed within 5 min every 30 min. For PRO trials,
147 subjects still consumed orange juice with a matched volume of water (Fig. 1).

148

149 Analytical Procedures

150 Skeletal Muscle Sample Preparation

151 For generation of whole muscle lysates, ~ 40 mg of skeletal muscle was homogenized in
152 buffer containing 50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1%
153 Triton X-100, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM DTT, $10 \mu\text{g}/\text{mL}$ trypsin
154 inhibitor, $2 \mu\text{g}/\text{mL}$ aprotinin, 1 mM benzamidine and 1 mM PMSF. Samples were spun at
155 $16,000 \text{ g}$ for 30 min at 4°C and the supernatant was collected for Western blot analysis.
156 Nuclear and cytoplasmic extracts were prepared using an NE-PER fractionation kit according
157 to the manufacturer's instructions (Thermo Scientific, Rockford, IL). In brief, ~ 30 mg of

158 skeletal muscle was homogenized on ice, by hand, using a glass Dounce homogenizer in
159 CER-I (cytoplasmic extraction reagent-I) buffer supplemented with protease and phosphatase
160 inhibitors. Homogenized samples were vortexed and incubated on ice for 10 min, after which
161 the CER-II buffer was added. CER-II-containing samples were vortexed and subsequently
162 spun at 16,000 g for 5 min at 4 °C with the supernatants containing cytoplasmic proteins
163 removed and the remaining nuclei-containing pellet resuspended in nuclear extraction reagent
164 buffer (supplemented with inhibitors). Nuclear lysates were subsequently incubated on ice
165 and vortexed for 15 s every 10 min for a total of 40 min, with a final 10 min centrifugation
166 (16,000 g at 4 °C) for collection of nuclear supernatants.

167

168 Western Blotting

169 After determination of protein concentration using a BCA protein assay (Pierce, Rockford,
170 USA), all lysates (50 µg and 10 µg of protein for whole muscle and fractionated lysates,
171 respectively) were resuspended in Laemmli sample buffer and loaded into 4–20% Mini-
172 PROTEAN TGX Stain-Free™ Gels (Bio-Rad, California, USA). Following electrophoresis,
173 gels were activated according to the manufacturer's instructions (Chemidoc, Bio-Rad,
174 Gladesville, Australia) and transferred to polyvinylidene fluoride (PVDF) membranes as
175 performed previously (55). After transfer, a Stain-Free image of the PVDF membranes for
176 total protein normalization was obtained before membranes were rinsed briefly in distilled
177 water and blocked for 1 h with 5% non-fat milk, washed 3 times (5 min each wash) with 10
178 mM Tris·HCl, 100 mM NaCl and 0.02% Tween 20 (TBST) and incubated with a primary
179 antibody diluted in TBST (1:1,000) overnight at 4 °C on a shaker. Membranes were
180 incubated for 1 h the next day with a secondary antibody diluted in TBST (1:2,000) and
181 proteins were detected via enhanced chemiluminescence (Amersham Biosciences,
182 Buckinghamshire, UK; Pierce Biotechnology) and quantified by densitometry (Chemidoc,

183 BioRad, Gladesville, Australia). All sample time points for each subject were run singularly
184 on the same gel. Antibodies against phospho-ULK1 Ser³¹⁷ and Ser⁷⁵⁷ (no. 12753 and no.
185 6888, respectively), Atg4b (no. 5299), Atg5 (no. 2630), cAtg12 (no. 4180), Beclin-1 (no.
186 3738), LC3b (no. 2775), p62 (no. 5114), NEDD4 (no. 2740), PINK1 (no. 6946), Parkin (no.
187 2132), BNIP3 (no. 44060), AIF (no. 5318), PARP1 (no. 9542), caspase-3 (no. 9662), TFEB
188 (no. 4240), phospho-AMPK^{Thr172} (no. 2531), AMPK α (no. 2532), phospho-p53^{Ser15} (no.
189 9284), p53 (no. 2527), COXIV (no. 4850), Mitofusin-2 (no. 11925), H2B (no. 8135) and
190 GAPDH (no. 2118) were purchased from Cell Signaling Technology (Danvers, MA).
191 Antibodies directed against PGC-1 α (ab54481), ATPAF1 (ab101518), VDAC1 (ab14734)
192 and an additional LC3b antibody (ab48394) were purchased from Abcam (Melbourne,
193 Australia). For all proteins, volume density of each target band was normalized to the total
194 protein loaded into each lane (Fig. 2A) using Stain-FreeTM technology (Bio-Rad, California,
195 USA) (18, 55). Purity of the cytoplasmic and nuclear fractions was determined by
196 immunoblotting for the glycolytic enzyme GAPDH and the nuclear histone 2B, respectively
197 (Fig. 2B). Due to limited tissue size, analysis of fractionated samples was restricted for some
198 time points: For the majority of proteins measured, ALC-CHO 2 h (n=7), ALC-PRO 2 h
199 (n=6) and 8 h (n=7), and PRO 2 h (n=6) and 8 h (n=7) time points were restricted. There was
200 n=5 for some time points of Parkin (ALC-PRO 2 h, PRO 8 h), PARP1 (ALC-PRO 2 h, PRO
201 2 h), phospho-AMPK^{Thr172} and ATPAF1 (both PRO 8 h). Due to difficulties in quantification
202 (non-specific binding), cytoplasmic p53 analysis was also based on n=5.

203

204 RNA Extraction, Quantification, Reverse Transcription and Real-Time PCR

205 Skeletal muscle tissue RNA extraction was performed using a TRIzol-based kit according to
206 the manufacturer's protocol (Invitrogen, Melbourne, Australia, Cat. No. 12183-018A).
207 Briefly, ~20 mg of frozen skeletal muscle tissue was homogenized in TRIzol with chloroform

208 added to form an aqueous RNA phase. This RNA phase was then eluted through a spin
209 cartridge with extracted RNA quantified using a NanoDrop 2000 Spectrophotometer (Thermo
210 Scientific, Scoresby, Australia) according to the manufacturer's protocol. Reverse
211 transcription and real-time Polymerase Chain Reaction (RT-PCR) was performed as
212 previously described (55). Quantification of mRNA in duplicate was performed using a
213 CFX96 Touch™ Real-Time PCR Detection System (Bio Rad, California, USA). TaqMan-
214 FAM-labelled primer/probes for Atg12 (Hs01047860), BECN1 (Hs00186838), LC3b
215 (Hs00797944), SQSTM1/p62 (Hs01061917), Atg4b (Hs00367088), BNIP3 (Hs00969291),
216 SCO2 (Hs04187025), SESN-2 (Hs00230241), PUMA (Hs00248075), Bax (Hs00180269),
217 PGC-1 α (Hs01016719), Tfam (Hs00273372) and NRF-1 (Hs01031046) were used. GAPDH
218 (Hs999999905) has been validated as an exercise housekeeping gene (23) and was used to
219 normalize threshold cycle (CT) values. GAPDH values were stably expressed between
220 conditions (data not shown). The relative amount of mRNA was calculated using the relative
221 quantification ($\Delta\Delta$ CT) method (35).

222

223 Cell Death ELISA

224 Detection of DNA damage and cell death (apoptosis) was performed using a cell death
225 detection ELISA (Roche Diagnostics, Mannheim, Germany). The cell death ELISA
226 quantitatively determines apoptotic DNA fragmentation by measuring the cytoplasmic
227 histone-associated mono- and oligonucleosomes. Briefly, even concentrations (120 μ g) of
228 nuclei-free cytoplasmic lysates were loaded as an antigen source to an anti-histone
229 monoclonal antibody fixated to the walls of microplate modules. Lysates were loaded prior to
230 the addition of an anti-DNA secondary antibody conjugated to peroxidase. The amount of
231 peroxidase retained in the immunocomplex was determined photometrically by incubating
232 with 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) as a substrate on a plate shaker

233 (300 rpm) for ~15 min at room temperature (20 °C). Absorbance was measured at a
234 wavelength of 405 nm using a SpectraMax Paradigm plate reader (Molecular Devices,
235 Sunnyvale, CA, USA). Samples were measured in duplicate on the same plate and
236 absorbance values were normalized to µg of protein loaded in the assay per sample. Due to
237 limited cytoplasmic lysate availability sample analysis was restricted for ALC-CHO 2 h
238 (n=7), ALC PRO 2 h (n=6), ALC PRO 8 h (n=7), PRO 2 h (n=5) and PRO 8 h (n=6).

239

240 GSH/GSSG Ratio

241 Detection of oxidative stress was performed using a GSH/GSSG Ratio Detection Assay Kit
242 (Abcam, Melbourne, Australia). Cytoplasmic lysates were first deproteinized by adding
243 trichloroacetic acid (TCA) to the samples in a 1:10 dilution. Samples were then incubated on
244 ice (15 min) and briefly centrifuged (5 min, 12,000 g at 4 °C) with the supernatant removed
245 and neutralized of excess TCA using a neutralizing solution (Abcam, Melbourne, Australia).
246 The assay was performed using standards for reduced (GSH) and oxidized (GSSG)
247 glutathione. Samples (n=4) were incubated for ~45 min at room temperature (20 °C) in a
248 Thiol Green Stock solution diluted in assay buffer (GAM) and a 25X GSSG Probe diluted in
249 GAM solution for the detection of reduced and oxidized glutathione, respectively.
250 Fluorescence was measured at an excitation/emission wavelength of 490/520 nm using a
251 SpectraMax Paradigm plate reader (Molecular Devices, Sunnyvale, CA, USA). Samples were
252 measured in duplicate on the same plate and fluorescence values were normalized to µg of
253 protein loaded in the assay per sample.

254

255 Statistical Analysis

256 Data were analyzed using two-way repeated measures analysis of variance (ANOVA) with
257 Student-Newman-Keuls post-hoc analysis (time × treatment) performed when an overall

258 statistically significant difference in group means of a particular comparison was found
259 (SigmaPlot for Windows; Version 12.5). Significance was set to $P < 0.05$ and all data are
260 presented as mean \pm standard deviation (SD). When tests for normality and/or equal variance
261 failed, data were log-transformed and statistical inferences were made based on these data.

262

263 **RESULTS**

264 Autophagy Regulatory Proteins

265 Autophagy involves the formation of vesicles termed autophagosomes that sequester and
266 deliver cellular constituents to lysosomes for degradation in a process regulated by
267 autophagy-related gene (Atg) proteins (38). AMP-activated protein kinase (AMPK)-targeted
268 phosphorylation of Atg1 or unc-51-like kinase 1 (ULK1) at Ser³¹⁷ that activates autophagy
269 was unchanged with any exercise-nutrient stimulus (data not shown). In contrast, ULK1^{Ser757}
270 phosphorylation, a site targeted by mTOR that inhibits autophagy induction (28), increased
271 ($P < 0.05$) with PRO above rest and each alcohol treatment (data not shown). Whole muscle
272 abundance of Atg4b, Atg5, the conjugated form of Atg5 (cAtg12) and Beclin-1 all decreased
273 compared to rest at 8 h post-exercise in ALC-CHO only ($P < 0.05$; Fig. 3A-D), while Atg5 and
274 cAtg12 values at 8 h post-exercise were greater in ALC-PRO than ALC-CHO ($P < 0.05$). All
275 Atg proteins except Beclin-1 were higher than ALC-CHO in PRO at 8 h post-exercise
276 ($P < 0.05$). An indirect marker of autophagosome formation is an increase in the lipidated
277 membrane-bound form of light chain 3b (LC3b-II) and a decrease in p62 (59). There were no
278 differences in the non-lipidated LC3b-I isoform with any exercise-nutrient condition (Fig.
279 3E), although LC3b-II decreased below rest at 2 h ($P < 0.05$) and remained attenuated by 8 h
280 post-exercise in ALC-CHO ($P = 0.052$; Fig. 3F). LC3b-II similarly decreased below rest at 8 h
281 with PRO ($P < 0.05$). p62 increased above rest at 8 h post-exercise in ALC-CHO ($P < 0.01$; Fig.
282 3G), while similar increases also occurred between 2-8 h of exercise recovery in ALC-PRO

283 (P<0.05). These findings altogether suggest that alcohol coingested with carbohydrate, but
284 not protein, inhibited autophagy. To gain insight into whether this inhibition culminated from
285 elevated Atg degradation by the proteasome, we measured the ubiquitin ligase neural
286 precursor cell expressed developmentally downregulated 4 (NEDD4), which has been shown
287 to target Beclin-1 for proteasomal degradation (45). However, NEDD4 declined below rest at
288 8 h post-exercise with ALC-CHO only (P<0.05; Fig. 3H), suggesting that the alcohol-
289 induced reduction in Atgs was probably not attributable to their proteasomal breakdown.

290

291 Autophagy Regulatory Genes

292 There were no changes in the mRNA transcripts of Atg12, BECN1, LC3b and SQSTM1/p62.
293 However, Atg4b increased (P<0.05) above rest at 8 h following exercise in both alcohol
294 treatments (data not shown).

295

296 Mitochondria-Specific Autophagy (Mitophagy)

297 PTEN-induced putative protein kinase-1 (PINK1) and Parkin regulate canonical mitophagy.
298 There were large increases in PINK1 above rest for ALC-CHO and PRO at 8 h post-exercise
299 (P<0.01; Fig. 4A). However, cytoplasmic Parkin only increased at 8 h with PRO above ALC-
300 CHO (P<0.05; Fig. 4B). Another mitophagy-specific protein, Bcl-2/adenovirus E1B 19 kDa-
301 interacting protein-3 (BNIP3), decreased below rest at 8 h post-exercise in ALC-CHO
302 (P<0.05; Fig. 4C). BNIP3 was higher than both ALC-CHO and PRO treatments with ALC-
303 PRO at 8 h post-exercise, an effect paralleled by similar temporal increases in its gene
304 expression (P<0.05; Fig. 4D). Given the previously mentioned reduction in “general”
305 autophagy, these findings suggest that mitophagy was also inhibited by alcohol/carbohydrate
306 coingestion post-exercise.

307

308 DNA Fragmentation and Apoptotic Signaling

309 To determine whether attenuated autophagic/mitophagic responses to ALC-CHO reflected
310 activation of apoptotic (cell death) events, we measured fragmented DNA in cytoplasmic
311 lysates as a marker of cellular apoptosis. DNA fragmentation increased significantly by
312 ~185% above rest at 8 h post-exercise with ALC-CHO only ($P<0.05$; Fig. 5A). Detection of
313 DNA fragmentation at 8 h in both alcohol treatments was greater than PRO ($P<0.05$).
314 Apoptosis can be executed by caspase-dependent and -independent pathways, the latter
315 involving poly (ADP-ribose) polymerase 1 (PARP1) triggering the mitochondria-to-nuclear
316 translocation of apoptosis inducing factor (AIF) (64). Nuclear abundance of AIF doubled
317 above rest during early (2 h) exercise recovery in ALC-CHO ($P<0.05$; Fig. 5B), but declined
318 sharply thereafter by 8 h ($P<0.01$). Within the cytoplasm, AIF levels with ALC-PRO and
319 PRO were greater than ALC-CHO at 8 h post-exercise ($P<0.05$; Fig. 5C). Full-length (~116
320 kDa) nuclear PARP1 decreased below resting levels by 8 h in ALC-CHO only ($P<0.05$; Fig.
321 5D). As a result, PARP1 was greater than ALC-CHO with ALC-PRO and PRO at this 8 h
322 time point ($P<0.05$). Immunoreactive ~89 kDa bands of PARP1, reduced proteolytic targets
323 of caspase-3, were undetectable in nuclear lysates. Furthermore, we could not detect bands
324 for the cleaved, active (~17 kDa) caspase-3 in whole muscle homogenates. Notably, pro-
325 caspase-3 levels were greater than ALC-CHO in response to ALC-PRO and PRO at 8 h post-
326 exercise ($P<0.05$; Fig. 5E). These findings suggest that alcohol-induced DNA fragmentation
327 was elicited by caspase-independent pathways.

328

329 Nuclear and Cytoplasmic TFEB, PGC-1 α , AMPK and p53

330 The apoptogenic effects of alcohol led us to investigate changes in the nuclear and
331 cytoplasmic levels of several proteins highly-sensitive to changes in cellular energy
332 availability. Nuclear and cytoplasmic levels of transcription factor EB (TFEB), a

333 transcriptional regulator of autophagy, decreased below rest in ALC-CHO at 8 h post-
334 exercise ($P < 0.05$; Fig. 6A, B). Nuclear TFEB at 8 h in ALC-PRO was greater than the ALC-
335 CHO ($P < 0.001$) and PRO ($P < 0.01$) conditions. Following 2 h of exercise recovery in both
336 alcohol treatments, nuclear PPAR γ -coactivator-1 α (PGC-1 α) doubled above rest ($P < 0.05$;
337 Fig. 6C). However, its nuclear abundance returned to basal levels by 8 h for ALC-CHO only
338 ($P < 0.01$). Cytoplasmic PGC-1 α in ALC-PRO increased above rest and ALC-CHO at 8 h
339 ($P < 0.05$; Fig. 6D). While there were no differences in nuclear AMPK α at any time point
340 (data not shown), cytoplasmic phospho-AMPK α^{Thr172} increased above rest at 8 h in ALC-
341 CHO only ($P < 0.05$; Fig. 6E). The apoptogenic p53 increased in the nucleus above rest in
342 ALC-PRO and PRO at 8 h post-exercise ($P < 0.05$; Fig. 6F). Within cytoplasmic fractions, p53
343 with each alcohol treatment at 8 h increased above rest and PRO ($P < 0.05$; Fig. 6G), of which
344 the largest effect occurred for ALC-CHO ($P < 0.01$). In addition, whole muscle p53^{Ser15}
345 phosphorylation was highest in ALC-CHO after exercise, increasing above rest at 8 h
346 ($P < 0.001$) and above ALC-PRO and PRO treatments ($P < 0.05$; Fig. 6H).

347

348 p53- and PGC-1 α -Target Genes and Oxidative Stress

349 We next chose to specifically focus on select p53/PGC-1 α transcriptional targets that are
350 involved in cell fate (i.e., survival or death) responses to changes in metabolic homeostasis
351 (50). There were differential responses of p53-target genes to post-exercise alcohol ingestion.
352 SCO2 (synthesis of cytochrome c oxidase 2) expressing a protein regulating assembly of the
353 respiratory chain cytochrome c oxidase (COX) complex, increased above rest with ALC-PRO
354 at 8 h post-exercise ($P < 0.05$; Fig. 7A). SESN-2 is an endogenous antioxidant (6) and was also
355 elevated at this time point with ALC-CHO compared to 2 h ($P < 0.05$; Fig. 7B). PUMA is a
356 pro-apoptotic gene involved in mitochondrial ROS generation (10, 34) and increased above
357 rest in all treatments ($P < 0.05$; Fig. 7C), whereby the largest effect occurred for ALC-CHO

358 (P<0.001). In contrast, there were no changes for the pro-apoptotic Bax following any
359 exercise-nutrient stimulus (data not shown). These changes in SESN-2 and PUMA suggested
360 alcohol may have induced an increase in ROS production. We therefore determined in
361 cytoplasmic lysates the ratio of reduced to oxidized glutathione (GSH/GSSG), a major
362 endogenous antioxidant. However, there were no significant changes in the GSH/GSSG ratio
363 with any exercise-nutrient stimulus (Fig. 7D). PGC-1 α can affect its own transcription (22)
364 and there were large increases in PGC-1 α gene expression during exercise recovery in all
365 treatments with the largest effect prevailing 2 h post-exercise (P<0.001; Fig. 7E).
366 Mitochondrial biogenesis-related PGC-1 α -target genes similarly revealed divergent responses
367 to the experimental conditions. Tfam (mitochondrial transcription factor A) increased above
368 rest at 8 h post-exercise in both alcohol treatments (P<0.05; Fig. 7F), whereby ALC-PRO
369 demonstrated the largest effect (P<0.01). NRF-1 (nuclear respiratory factor-1) increased
370 above rest at 8 h after exercise in response to ALC-PRO alone (P<0.05; Fig. 7G).

371

372 Mitochondrial Proteins

373 Suspecting that the transcriptional induction of SCO2, Tfam and NRF-1 reflected a
374 mitochondrial biogenesis response to post-exercise alcohol/protein ingestion, we measured
375 the abundance of several key proteins regulating mitochondrial function. At 8 h post-exercise
376 with ALC-PRO, there were increases above rest in the cytoplasmic content of COX subunit
377 IV; these changes were greater than ALC-CHO and PRO (P<0.01; Fig. 8A). Similarly, the
378 respiratory chain F₁-ATP synthase complex assembly factor 1 (ATPAF1) increased above
379 rest at 8 h post-exercise in ALC-PRO only (P<0.05; Fig. 8B) and this change was greater
380 than ALC-CHO (P<0.001). ATPAF1 was also elevated in PRO above ALC-CHO between 2-
381 8 h of exercise recovery and above ALC-PRO at 2 h only (P<0.05). Voltage-dependent anion
382 channel-1 (VDAC1), an abundant mitochondrial regulator of substrate trafficking, was higher

383 than ALC-CHO and PRO treatments at 8 h post-exercise in response to ALC-PRO ($P < 0.01$;
384 Fig. 8C). Whole muscle levels of the mitochondrial membrane fusion protein Mitofusin-2
385 decreased below rest at 8 h in ALC-CHO ($P < 0.05$; Fig. 8D). Mitofusin-2 levels in ALC-PRO
386 and PRO were greater than ALC-CHO at 8 h post-exercise ($P < 0.05$). Altogether these data
387 suggest that high-protein availability with alcohol stimulates mitochondrial biogenesis.

388

389 **DISCUSSION**

390 This is the first study to characterize the pro-apoptotic effects of acute binge alcohol
391 consumption in human skeletal muscle following exercise. The main finding was that alcohol
392 coingested with carbohydrate (i.e., before and after the alcohol drinking protocol) following a
393 single bout of strenuous concurrent exercise represses autophagy and triggers
394 intramyocellular apoptosis as indicated by DNA fragmentation. In contrast, energy-matched
395 protein consumption attenuated alcohol-induced apoptotic responses following exercise and
396 was accompanied by increases in markers of mitochondrial biogenesis. This apoptotic
397 response may have been the result of alcohol imposing an additional metabolic stress to a
398 cellular environment already disrupted by prior exercise. However, the anabolic properties of
399 protein that increase mitochondrial protein synthesis may lower the magnitude of apoptotic
400 events when coingested with alcohol.

401

402 **Post-exercise ingestion of alcohol and carbohydrate dysregulates autophagy**

403 We previously demonstrated that alcohol consumption following a strenuous bout of exercise
404 repressed maximal rates of myofibrillar protein synthesis in human skeletal muscle compared
405 to consuming protein-only beverages during recovery (41). The observation that acute
406 alcohol exposure failed to augment proteasome-dependent protein breakdown (62) led us to
407 investigate whether aberrant activation of autophagy, an alternate lysosome-dependent

408 degradative pathway sensitive to cellular bioenergetics (52), may play a role in the post-
409 exercise alcohol-induced repression of myofibrillar protein synthesis by degrading
410 intracellular substrate and inducing a net negative protein balance. In contrast to one of our
411 original hypotheses, we found that by 8 h following exercise (4 h after ingesting the final
412 alcoholic beverage), several Atgs implicated in the biogenesis of autophagic vesicles
413 (autophagosomes) along with the nuclear and cytoplasmic content of TFEB, a transcriptional
414 regulator of autophagy (51), were consistently attenuated (below resting values) with alcohol
415 and carbohydrate ingestion, but not when alcohol was consumed with protein. Corresponding
416 gene expression (except Atg4b) was largely unaffected at any time point, while NEDD4, an
417 ubiquitin ligase that can “tag” Atgs such as Beclin-1 for proteasomal degradation (45),
418 followed the same alcohol/carbohydrate-only pattern of post-exercise decline. These findings
419 were surprising as substantial increases in autophagy alongside a reduction in proteasomal
420 activity have previously been observed in skeletal muscle of alcoholic cirrhosis patients (60),
421 suggesting that chronic alcohol exposure ultimately elevates skeletal muscle autophagy for
422 constitutive removal of damaged cellular compartments.

423

424 Alcohol metabolism is energetically expensive and produces highly-toxic intermediates
425 such as acetaldehyde and acetate (33), the latter of which has been shown to be taken up by
426 skeletal muscle following its release from the splanchnic region in response to an acute
427 ethanol infusion (26). Alcohol metabolism provokes mitochondrial production of ROS that
428 can signal for the mitophagic removal of these organelles (4). Canonical mitophagy involves
429 PINK1 accumulating on the surface of damaged/depolarized mitochondria where it recruits
430 and activates the ubiquitin ligase Parkin (27, 63). Ubiquitinated mitochondrial proteins are
431 subsequently delivered to autophagosomes by the bridging protein p62, which in turn
432 undergoes degradation itself (17). Alternatively, BNIP3 is a hypoxia-sensitive mitophagy

433 receptor containing an LC3-interacting motif that facilitates direct autophagosomal
434 engulfment of mitochondria (19). We found that coingestion of alcohol and carbohydrate
435 after exercise elicited a dysregulated mitophagic response as evidenced by parallel increases
436 in PINK1 and p62 and a reduction in BNIP3. Given that alcohol led to the consistent
437 reduction of Atgs, the molecular “machinery” required for autophagic digestion of
438 mitochondria, our data suggest that post-exercise alcohol intoxication prevented the disposal
439 of potentially damaged mitochondria. Notably, when the intensity of a given stressor (i.e.,
440 alcohol) overwhelms the protective capabilities of mitophagy, intrinsic cell death (apoptotic)
441 pathways can be activated (36). Our findings of alcohol ingestion disrupting autophagy along
442 with an apparent, alcohol-induced downregulation of proteasome-dependent proteolysis, led
443 us to investigate whether induction of apoptotic processes were responsible for this
444 degradation of signaling proteins.

445

446 **Post-exercise ingestion of alcohol triggers intramyocellular apoptosis**

447 Alcohol with carbohydrate and also alcohol with protein coingestion triggered apoptotic
448 DNA fragmentation, as revealed by the detection of post-exercise mono- and
449 oligonucleosomes in cytoplasmic (nuclei-free) lysates. However, the largest apoptotic
450 response prevailed for alcohol/carbohydrate and was preceded by AIF nuclear accumulation.
451 AIF normally resides in the mitochondrial intermembrane space (58), but upon mitochondrial
452 outer membrane permeabilization (MOMP), AIF rapidly redistributes to the nucleus where it
453 has been shown to elicit large-scale DNA fragmentation (14). Hyperactivity of PARP1, a
454 DNA repair enzyme, signals to AIF to facilitate its nuclear import as an alternative to the
455 canonical cytochrome c/caspase-dependent pathway of mitochondrial apoptosis (29, 64).
456 Whether PARP1 was responsible for initially signaling to AIF is unclear, as it followed a
457 similar pattern to AIF, decreasing sharply during late (8 h) exercise recovery with

458 alcohol/carbohydrate. In addition, DNA fragmentation was not detected until 8 h post-
459 exercise and was paralleled by increases in the cytoplasmic abundance of p53, a well-
460 characterized effector of apoptosis. p53 promotes apoptosis by transcription-dependent and –
461 independent mechanisms, the latter involving MOMP, cytochrome c release and caspase
462 activation (11, 16, 37). Despite increased cytoplasmic p53, precursor caspase-3 levels were
463 lowest after exercise in alcohol/carbohydrate and we were unable to detect its cleaved, active
464 fragment nor evidence for nuclear cleavage of PARP1, a major caspase-3 substrate (32);
465 these effects seemingly contrast denervation-induced apoptotic signaling in rodent skeletal
466 muscle (53, 54). Thus, our findings suggest that AIF nuclear import is the initial, caspase-
467 independent driver of post-exercise alcohol-induced apoptosis. Degradation of nuclear
468 PARP1 would also limit DNA repair capacity and its decay may have perpetuated the p53-
469 independent apoptotic response. Another consideration is that the apoptotic response we
470 observed could also affect the myonuclei of myogenic cells (i.e., satellite cells) that are
471 activated in response to vigorous exercise, thereby impairing regenerative recovery,
472 particularly from the high-mechanical demands imposed by resistance exercise contraction
473 (43).

474

475 Aberrant mitophagy signaling observed in the alcohol/carbohydrate condition raises the
476 possibility that the metabolic burden of alcohol added to an intracellular environment already
477 disrupted by contractile stress (i.e., substrate depletion, altered redox state, Ca²⁺ fluctuations
478 etc.) overwhelmed mitochondrial metabolism. In this regard, the potential ATP depletion and
479 ROS generated as a result of mitochondrial alcohol metabolism may have triggered MOMP,
480 release of AIF and initiation of apoptosis, which are well-characterized features of alcohol-
481 induced liver injury (1). In support of this hypothesis, phosphorylation of cytoplasmic
482 AMPK α ^{Thr172} and downstream (whole muscle) p53^{Ser15} that reflects a response to diminished

483 energy availability (25) peaked with alcohol/carbohydrate ingestion. This increased p53
484 activity could account for the largest alcohol/carbohydrate-induced mRNA expression of
485 PUMA and SESN-2, p53-inducible genes involved in mitochondrial ROS generation (10, 34)
486 and scavenging (6), respectively. As a DNA damage-sensitive antioxidant, SESN-2 may have
487 been upregulated to quench excessive ROS and combat further tissue damage. However, we
488 found no differences in the ratio of reduced (GSH) to oxidized (GSSG) glutathione, decreases
489 of which reflect oxidative stress (46). Direct mitochondrial measurements of ROS such as
490 hydrogen peroxide may be a more accurate reflection of oxidative stress in human skeletal
491 muscle (48). Nevertheless, changes in redox state arising from alcohol metabolism could
492 have posttranslationally modified (i.e., oxidatively degraded) intracellular signaling proteins,
493 including precursor caspase-3, which prevents its apoptotic activity (65). Indeed, limited ATP
494 availability facilitates AIF-driven apoptosis (15, 40). Future time course studies are required
495 to ascertain whether ATP depletion and/or mitochondrial ROS differentially activate
496 apoptotic signal transduction following exercise and alcoholic intoxication.

497

498 **Alcohol and protein coingestion may stimulate mitochondrial biogenesis**

499 The capacity for an increase in exogenous protein availability to prevent cellular damage
500 (e.g., preservation of Atgs) and attenuate the magnitude of alcohol-induced apoptotic DNA
501 fragmentation was associated with an apparent activation of mitochondrial biogenesis. PGC-
502 1 α is the transcriptional “master regulator” of mitochondrial anabolism and increased rapidly
503 in the nucleus following exercise when alcohol was consumed, deteriorating thereafter when
504 carbohydrate was coingested. Alcohol coingested with protein otherwise facilitated nuclear
505 retention of PGC-1 α and promoted its cytoplasmic accumulation along with raising nuclear
506 levels of TFEB, which in addition to regulating autophagosome and lysosome abundance, is a
507 coactivator of PGC-1 α and governs mitochondrial turnover (i.e., undulations of synthesis and

508 breakdown) (49). Hence, because autophagy (including mitophagy) was neither up- nor
509 downregulated with alcohol and protein coingestion, the TFEB response presumably
510 favoured mitochondrial biogenesis. Indeed, PGC-1 α -inducible genes (NRF-1, Tfam) and the
511 p53 transcriptional target SCO2 that promote mitochondrial anabolism (21), increased to the
512 greatest extent when alcohol and protein were coingested. Although large PGC-1 α mRNA
513 responses to exercise followed for all treatments, its preferential accumulation at the protein
514 level with alcohol/protein and resultant transcriptional response (e.g., NRF-1 upregulation)
515 suggests that this effect was compensatory to counter the effects of alcohol exposure, since
516 measureable increases in PGC-1 α protein and rates of mitochondrial protein synthesis
517 typically occur 18-24 h after acute exercise (3, 7, 42). In support of this postulate,
518 mitochondrial proteins required for ATP production (COXIV, ATPAF1) and ATP trafficking
519 (VDAC1) all increased following exercise to the greatest extent with alcohol and protein
520 coingestion, whereas Mitofusin-2 levels, an outer mitochondrial membrane fusion protein,
521 were unchanged, suggesting that these nascent mitochondrial proteins may have been import-
522 incorporated into existing organelles.

523

524 Alcohol-induced ROS production is particularly damaging to mitochondrial DNA
525 (mtDNA) due to its close proximity to the respiratory chain, and alcohol exposure has been
526 shown to prevent mtDNA-encoded translation of proteins encoding for subunits of
527 respiratory complexes (12). Thus, it is tempting to speculate that alcohol and protein-induced
528 mitochondrial biogenesis represented a homeostatic matching of ATP synthesis with ROS
529 formation to relieve mtDNA and cellular damage, thereby lowering the overall apoptotic
530 response (i.e., reducing oxidative damage to cellular proteins etc.). The reason(s) for
531 exogenous protein and not carbohydrate eliciting this protective response against alcohol
532 despite matched energy content is unknown, but could be related to branched-chain amino

533 acids (BCAAs), of which whey protein is enriched, harbouring an intrinsic capacity to induce
534 skeletal muscle mitochondrial biogenesis when mitochondrial function is impaired (13). Our
535 previous finding of attenuated myofibrillar protein synthesis with alcohol/protein coingestion
536 may have culminated from a concomitant stimulation of mitochondrial protein synthesis, in
537 turn restricting available amino acid substrate for maximal stimulation of the myofibrillar
538 fraction (41). Of note is that we previously reported lower blood alcohol levels from 6-8 h of
539 exercise recovery with alcohol/protein versus alcohol/carbohydrate (41). Although the
540 mechanism(s) of this protein-induced reduction in blood alcohol is unknown, these findings
541 suggest that protein availability reduced skeletal muscle uptake of alcohol and its metabolites
542 (e.g., acetate) thus, in part, relieving mitochondria from the burden of alcohol catabolism and
543 inevitable ROS formation. It is also unclear whether protein availability before or after
544 alcohol ingestion, or the combined effects of consuming two beverages (50 g total protein), is
545 the catalyst for attenuating alcohol-inflicted cellular damage. Nonetheless, timing and
546 distribution of exogenous protein used in the current study otherwise aligns with optimal
547 anabolic feeding strategies that elevate plasma aminoacidemia to levels likely necessary to
548 counter post-exercise alcohol-induced intramuscular toxicity (2, 41). Future studies
549 investigating rates of mitochondrial protein synthesis as well as mitochondrial respiration
550 following combined exercise- and alcohol-induced intracellular stress are required to confirm
551 this thesis.

552

553 **CONCLUSION**

554 We provide novel information showing that alcohol ingestion instigates cellular apoptosis
555 following strenuous exercise. Alcohol with exogenous protein availability appeared to
556 engender skeletal muscle with an abrupt, mitochondrial anabolic response that may have
557 combated the stress imposed by alcohol metabolism (Figure 9), an effect likely mediated by

558 the intrinsic anabolic properties of BCAAs enriched in the whey protein beverages. While the
559 effects of consuming alcohol alone after exercise are currently unknown, the post-exercise
560 feeding patterns of carbohydrate and protein employed in the present investigation are
561 consistent with recommendations for optimal nutrition following exercise-training (8, 44). As
562 such, our data reveal several potential mechanisms unravelling how binge drinking practices
563 could compromise sports- and exercise-training recovery-adaptation (5).

564

565 **Acknowledgements**

566 This study was funded by an ACU Collaborative Research Network Grant to JAH
567 (2013000443). The authors would like to thank Dr. Andrew Garnham for his technical
568 assistance during experimental trials and the subjects for their time and effort.

569

570 **Disclosures**

571 All authors report no conflict of interest.

572

573

574 **REFERENCES**

- 575 1. **Adachi M, and Ishii H.** Role of mitochondria in alcoholic liver injury. *Free Radic*
576 *Biol Med* 32: 487-491, 2002.
- 577 2. **Areta JL, Burke LM, Ross ML, Camera DM, West DW, Broad EM, Jeacocke**
578 **NA, Moore DR, Stellingwerff T, Phillips SM, Hawley JA, and Coffey VG.** Timing and
579 distribution of protein ingestion during prolonged recovery from resistance exercise alters
580 myofibrillar protein synthesis. *J Physiol* 591: 2319-2331, 2013.
- 581 3. **Baar K, Wende AR, Jones TE, Marison M, Nolte LA, Chen M, Kelly DP, and**
582 **Holloszy JO.** Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional
583 coactivator PGC-1. *FASEB J* 16: 1879-1886, 2002.
- 584 4. **Bailey SM, Pietsch EC, and Cunningham CC.** Ethanol stimulates the production of
585 reactive oxygen species at mitochondrial complexes I and III. *Free Radic Biol Med* 27: 891-
586 900, 1999.
- 587 5. **Barnes MJ, Mundel T, and Stannard SR.** Post-exercise alcohol ingestion
588 exacerbates eccentric-exercise induced losses in performance. *Eur J Appl Physiol* 108: 1009-
589 1014, 2010.

- 590 6. **Budanov AV, Sablina AA, Feinstein E, Koonin EV, and Chumakov PM.**
 591 Regeneration of peroxiredoxins by p53-regulated sestrins, homologs of bacterial AhpD.
 592 Science 304: 596-600, 2004.
- 593 7. **Burd NA, Andrews RJ, West DW, Little JP, Cochran AJ, Hector AJ, Cashaback**
 594 **JG, Gibala MJ, Potvin JR, Baker SK, and Phillips SM.** Muscle time under tension during
 595 resistance exercise stimulates differential muscle protein sub-fractional synthetic responses in
 596 men. J Physiol 590: 351-362, 2012.
- 597 8. **Burke LM, Hawley JA, Wong SH, and Jeukendrup AE.** Carbohydrates for
 598 training and competition. J Sports Sci 29 Suppl 1: S17-27, 2011.
- 599 9. **Burke LM, and Read RS.** A study of dietary patterns of elite Australian football
 600 players. Can J Sport Sci 13: 15-19, 1988.
- 601 10. **Chipuk JE, Bouchier-Hayes L, Kuwana T, Newmeyer DD, and Green DR.**
 602 PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. Science 309: 1732-
 603 1735, 2005.
- 604 11. **Chipuk JE, Kuwana T, Bouchier-Hayes L, Droin NM, Newmeyer DD, Schuler**
 605 **M, and Green DR.** Direct activation of Bax by p53 mediates mitochondrial membrane
 606 permeabilization and apoptosis. Science 303: 1010-1014, 2004.
- 607 12. **Cunningham CC, Coleman WB, and Spach PI.** The effects of chronic ethanol
 608 consumption on hepatic mitochondrial energy metabolism. Alcohol Alcohol 25: 127-136,
 609 1990.
- 610 13. **D'Antona G, Ragni M, Cardile A, Tedesco L, Dossena M, Bruttini F, Caliaro F,**
 611 **Corsetti G, Bottinelli R, Carruba MO, Valerio A, and Nisoli E.** Branched-chain amino
 612 acid supplementation promotes survival and supports cardiac and skeletal muscle
 613 mitochondrial biogenesis in middle-aged mice. Cell Metab 12: 362-372, 2010.
- 614 14. **Daugas E, Nochy D, Ravagnan L, Loeffler M, Susin SA, Zamzami N, and**
 615 **Kroemer G.** Apoptosis-inducing factor (AIF): a ubiquitous mitochondrial oxidoreductase
 616 involved in apoptosis. FEBS Lett 476: 118-123, 2000.
- 617 15. **Daugas E, Susin SA, Zamzami N, Ferri KF, Irinopoulou T, Larochette N,**
 618 **Prevost MC, Leber B, Andrews D, Penninger J, and Kroemer G.** Mitochondrio-nuclear
 619 translocation of AIF in apoptosis and necrosis. FASEB J 14: 729-739, 2000.
- 620 16. **Dumont P, Leu JI, Della Pietra AC, 3rd, George DL, and Murphy M.** The codon
 621 72 polymorphic variants of p53 have markedly different apoptotic potential. Nat Genet 33:
 622 357-365, 2003.
- 623 17. **Geisler S, Holmstrom KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, and**
 624 **Springer W.** PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and
 625 p62/SQSTM1. Nat Cell Biol 12: 119-131, 2010.
- 626 18. **Gurtler A, Kunz N, Gomolka M, Hornhardt S, Friedl AA, McDonald K, Kohn**
 627 **JE, and Posch A.** Stain-Free technology as a normalization tool in Western blot analysis.
 628 Anal Biochem 433: 105-111, 2013.
- 629 19. **Hanna RA, Quinsay MN, Orogo AM, Giang K, Rikka S, and Gustafsson AB.**
 630 Microtubule-associated protein 1 light chain 3 (LC3) interacts with Bnip3 protein to
 631 selectively remove endoplasmic reticulum and mitochondria via autophagy. J Biol Chem 287:
 632 19094-19104, 2012.
- 633 20. **Hawley JA, Burke LM, Phillips SM, and Spriet LL.** Nutritional modulation of
 634 training-induced skeletal muscle adaptations. J Appl Physiol 110: 834-845, 2011.
- 635 21. **Hood DA.** Invited Review: contractile activity-induced mitochondrial biogenesis in
 636 skeletal muscle. J Appl Physiol (1985) 90: 1137-1157, 2001.
- 637 22. **Jager S, Handschin C, St-Pierre J, and Spiegelman BM.** AMP-activated protein
 638 kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. Proc
 639 Natl Acad Sci U S A 104: 12017-12022, 2007.

- 640 23. **Jemiolo B, and Trappe S.** Single muscle fiber gene expression in human skeletal
641 muscle: validation of internal control with exercise. *Biochem Biophys Res Commun* 320:
642 1043-1050, 2004.
- 643 24. **Jokl EJ, and Blanco G.** Disrupted autophagy undermines skeletal muscle adaptation
644 and integrity. *Mamm Genome* 2016.
- 645 25. **Jones RG, Plas DR, Kubek S, Buzzai M, Mu J, Xu Y, Birnbaum MJ, and**
646 **Thompson CB.** AMP-activated protein kinase induces a p53-dependent metabolic
647 checkpoint. *Mol Cell* 18: 283-293, 2005.
- 648 26. **Jorfeldt L, and Juhlin-Dannfelt A.** The influence of ethanol on splanchnic and
649 skeletal muscle metabolism in man. *Metabolism* 27: 97-106, 1978.
- 650 27. **Kane LA, Lazarou M, Fogel AI, Li Y, Yamano K, Sarraf SA, Banerjee S, and**
651 **Youle RJ.** PINK1 phosphorylates ubiquitin to activate Parkin E3 ubiquitin ligase activity. *J*
652 *Cell Biol* 205: 143-153, 2014.
- 653 28. **Kim J, Kundu M, Viollet B, and Guan KL.** AMPK and mTOR regulate autophagy
654 through direct phosphorylation of Ulk1. *Nat Cell Biol* 13: 132-141, 2011.
- 655 29. **Kolthur-Seetharam U, Dantzer F, McBurney MW, de Murcia G, and Sassone-**
656 **Corsi P.** Control of AIF-mediated cell death by the functional interplay of SIRT1 and PARP-
657 1 in response to DNA damage. *Cell Cycle* 5: 873-877, 2006.
- 658 30. **Kumar V, Frost RA, and Lang CH.** Alcohol impairs insulin and IGF-I stimulation
659 of S6K1 but not 4E-BP1 in skeletal muscle. *Am J Physiol Endocrinol Metab* 283: E917-928,
660 2002.
- 661 31. **Lang CH, Frost RA, Deshpande N, Kumar V, Vary TC, Jefferson LS, and**
662 **Kimball SR.** Alcohol impairs leucine-mediated phosphorylation of 4E-BP1, S6K1, eIF4G,
663 and mTOR in skeletal muscle. *Am J Physiol Endocrinol Metab* 285: E1205-1215, 2003.
- 664 32. **Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, and Earnshaw WC.**
665 Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature*
666 371: 346-347, 1994.
- 667 33. **Lieber CS.** Metabolism of alcohol. *Clinics in liver disease* 9: 1-35, 2005.
- 668 34. **Liu Z, Lu H, Shi H, Du Y, Yu J, Gu S, Chen X, Liu KJ, and Hu CA.** PUMA
669 overexpression induces reactive oxygen species generation and proteasome-mediated
670 stathmin degradation in colorectal cancer cells. *Cancer Res* 65: 1647-1654, 2005.
- 671 35. **Livak KJ, and Schmittgen TD.** Analysis of relative gene expression data using real-
672 time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.
- 673 36. **Marino G, Niso-Santano M, Baehrecke EH, and Kroemer G.** Self-consumption:
674 the interplay of autophagy and apoptosis. *Nat Rev Mol Cell Biol* 15: 81-94, 2014.
- 675 37. **Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P, and Moll**
676 **UM.** p53 has a direct apoptogenic role at the mitochondria. *Mol Cell* 11: 577-590, 2003.
- 677 38. **Mizushima N, Yoshimori T, and Ohsumi Y.** The role of Atg proteins in
678 autophagosome formation. *Annu Rev Cell Dev Biol* 27: 107-132, 2011.
- 679 39. **Nicklin P, Bergman P, Zhang B, Triantafellow E, Wang H, Nyfeler B, Yang H,**
680 **Hild M, Kung C, Wilson C, Myer VE, MacKeigan JP, Porter JA, Wang YK, Cantley**
681 **LC, Finan PM, and Murphy LO.** Bidirectional transport of amino acids regulates mTOR
682 and autophagy. *Cell* 136: 521-534, 2009.
- 683 40. **Nicotera P, Leist M, Fava E, Berliocchi L, and Volbracht C.** Energy requirement
684 for caspase activation and neuronal cell death. *Brain Pathol* 10: 276-282, 2000.
- 685 41. **Parr EB, Camera DM, Areta JL, Burke LM, Phillips SM, Hawley JA, and**
686 **Coffey VG.** Alcohol ingestion impairs maximal post-exercise rates of myofibrillar protein
687 synthesis following a single bout of concurrent training. *PLoS One* 9: e88384, 2014.

- 688 42. **Perry CG, Lally J, Holloway GP, Heigenhauser GJ, Bonen A, and Spriet LL.**
 689 Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial
 690 proteins during training in human skeletal muscle. *J Physiol* 588: 4795-4810, 2010.
- 691 43. **Petrella JK, Kim JS, Mayhew DL, Cross JM, and Bamman MM.** Potent myofiber
 692 hypertrophy during resistance training in humans is associated with satellite cell-mediated
 693 myonuclear addition: a cluster analysis. *J Appl Physiol* (1985) 104: 1736-1742, 2008.
- 694 44. **Phillips SM, and Van Loon LJ.** Dietary protein for athletes: from requirements to
 695 optimum adaptation. *J Sports Sci* 29 Suppl 1: S29-38, 2011.
- 696 45. **Platta HW, Abrahamsen H, Thoresen SB, and Stenmark H.** Nedd4-dependent
 697 lysine-11-linked polyubiquitination of the tumour suppressor Beclin 1. *Biochem J* 441: 399-
 698 406, 2012.
- 699 46. **Powers SK, Duarte J, Kavazis AN, and Talbert EE.** Reactive oxygen species are
 700 signalling molecules for skeletal muscle adaptation. *Exp Physiol* 95: 1-9, 2010.
- 701 47. **Qiao S, Dennis M, Song X, Vadysirisack DD, Salunke D, Nash Z, Yang Z, Liesa
 702 M, Yoshioka J, Matsuzawa S, Shirihai OS, Lee RT, Reed JC, and Ellisen LW.** A
 703 REDD1/TXNIP pro-oxidant complex regulates ATG4B activity to control stress-induced
 704 autophagy and sustain exercise capacity. *Nat Commun* 6: 7014, 2015.
- 705 48. **Sahlin K, Shabalina IG, Mattsson CM, Bakkman L, Fernstrom M,
 706 Rozhdestvenskaya Z, Enqvist JK, Nedergaard J, Ekblom B, and Tonkonogi M.**
 707 Ultraendurance exercise increases the production of reactive oxygen species in isolated
 708 mitochondria from human skeletal muscle. *J Appl Physiol* (1985) 108: 780-787, 2010.
- 709 49. **Scott I, Webster BR, Chan CK, Okonkwo JU, Han K, and Sack MN.** GCN5-like
 710 protein 1 (GCN5L1) controls mitochondrial content through coordinated regulation of
 711 mitochondrial biogenesis and mitophagy. *J Biol Chem* 289: 2864-2872, 2014.
- 712 50. **Sen N, Satija YK, and Das S.** PGC-1alpha, a key modulator of p53, promotes cell
 713 survival upon metabolic stress. *Mol Cell* 44: 621-634, 2011.
- 714 51. **Settembre C, Di Malta C, Polito VA, Garcia Arencibia M, Vetrini F, Erdin S,
 715 Erdin SU, Huynh T, Medina D, Colella P, Sardiello M, Rubinsztein DC, and Ballabio A.**
 716 TFEB links autophagy to lysosomal biogenesis. *Science* 332: 1429-1433, 2011.
- 717 52. **Singh R, and Cuervo AM.** Autophagy in the cellular energetic balance. *Cell Metab*
 718 13: 495-504, 2011.
- 719 53. **Siu PM, and Alway SE.** Deficiency of the Bax gene attenuates denervation-induced
 720 apoptosis. *Apoptosis* 11: 967-981, 2006.
- 721 54. **Siu PM, and Alway SE.** Mitochondria-associated apoptotic signalling in denervated
 722 rat skeletal muscle. *J Physiol* 565: 309-323, 2005.
- 723 55. **Smiles WJ, Areta JL, Coffey VG, Phillips SM, Moore DR, Stellingwerff T, Burke
 724 LM, Hawley JA, and Camera DM.** Modulation of Autophagy Signaling with Resistance
 725 Exercise and Protein Ingestion Following Short-Term Energy Deficit. *Am J Physiol Regul*
 726 *Integr Comp Physiol* ajpgu 00413 02014, 2015.
- 727 56. **Steiner JL, and Lang CH.** Alcohol impairs skeletal muscle protein synthesis and
 728 mTOR signaling in a time-dependent manner following electrically stimulated muscle
 729 contraction. *J Appl Physiol* (1985) jap 00180 02014, 2014.
- 730 57. **Steiner JL, and Lang CH.** Alcohol intoxication following muscle contraction in
 731 mice decreases muscle protein synthesis but not mTOR signal transduction. *Alcohol Clin Exp*
 732 *Res* 39: 1-10, 2015.
- 733 58. **Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion
 734 J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R,
 735 Siderovski DP, Penninger JM, and Kroemer G.** Molecular characterization of
 736 mitochondrial apoptosis-inducing factor. *Nature* 397: 441-446, 1999.

- 737 59. **Tanida I, and Waguri S.** Measurement of autophagy in cells and tissues. *Methods*
738 *Mol Biol* 648: 193-214, 2010.
- 739 60. **Thapaliya S, Runkana A, McMullen MR, Nagy LE, McDonald C, Naga Prasad**
740 **SV, and Dasarathy S.** Alcohol-induced autophagy contributes to loss in skeletal muscle
741 mass. *Autophagy* 10: 677-690, 2014.
- 742 61. **Vainshtein A, Desjardins EM, Armani A, Sandri M, and Hood DA.** PGC-1alpha
743 modulates denervation-induced mitophagy in skeletal muscle. *Skelet Muscle* 5: 9, 2015.
- 744 62. **Vary TC, Frost RA, and Lang CH.** Acute alcohol intoxication increases atrogin-1
745 and MuRF1 mRNA without increasing proteolysis in skeletal muscle. *Am J Physiol Regul*
746 *Integr Comp Physiol* 294: R1777-1789, 2008.
- 747 63. **Vives-Bauza C, Zhou C, Huang Y, Cui M, de Vries RL, Kim J, May J, Tocilescu**
748 **MA, Liu W, Ko HS, Magrane J, Moore DJ, Dawson VL, Grailhe R, Dawson TM, Li C,**
749 **Tieu K, and Przedborski S.** PINK1-dependent recruitment of Parkin to mitochondria in
750 mitophagy. *Proc Natl Acad Sci U S A* 107: 378-383, 2010.
- 751 64. **Yu SW, Wang H, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, Poirier GG,**
752 **Dawson TM, and Dawson VL.** Mediation of poly(ADP-ribose) polymerase-1-dependent
753 cell death by apoptosis-inducing factor. *Science* 297: 259-263, 2002.
- 754 65. **Zeigler MM, Doseff AI, Galloway MF, Opalek JM, Nowicki PT, Zweier JL, Sen**
755 **CK, and Marsh CB.** Presentation of nitric oxide regulates monocyte survival through effects
756 on caspase-9 and caspase-3 activation. *J Biol Chem* 278: 12894-12902, 2003.

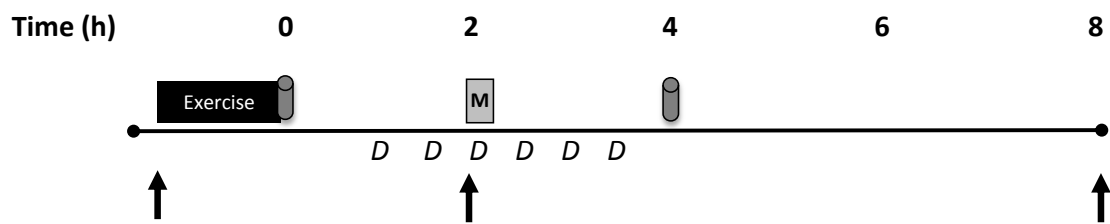
757

758

759

760

761



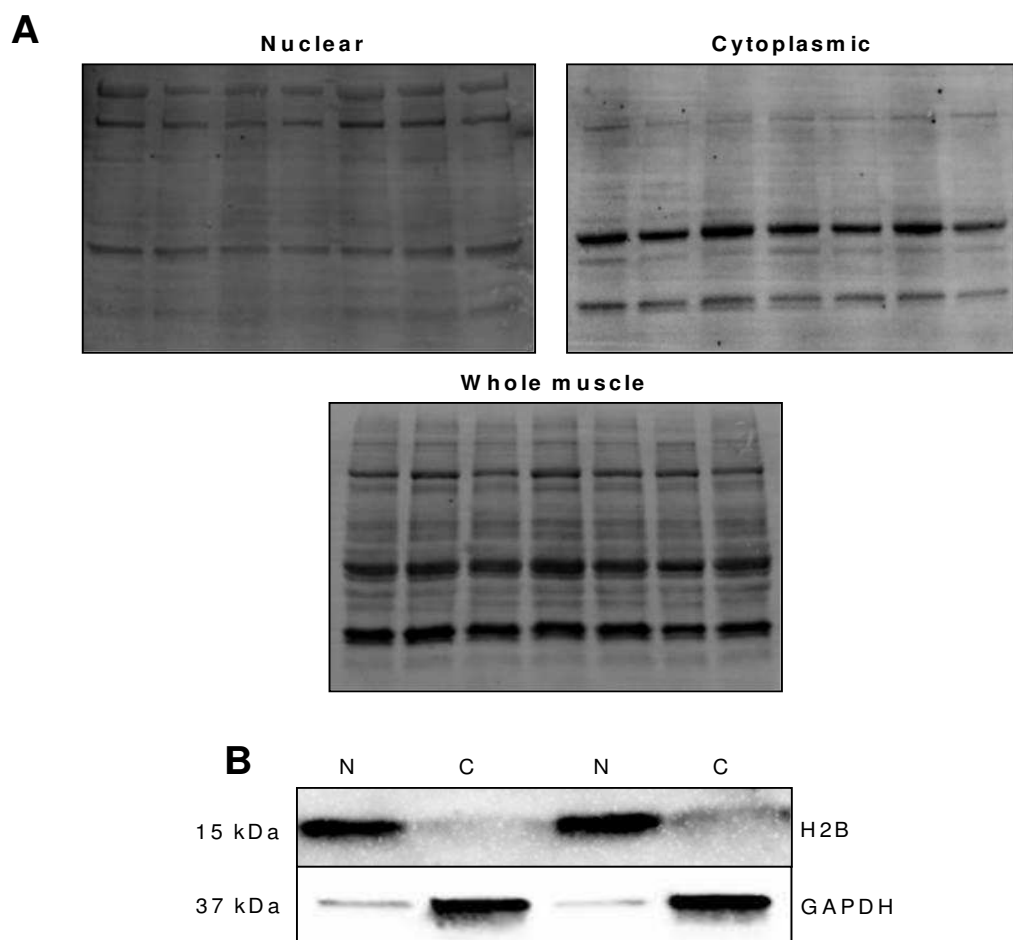
Key: = 25 g Protein OR CHO = muscle biopsy *D* = alcohol/placebo drink = CHO meal

762

763

764 **Figure 1.** Schematic overview of experimental trials.

765

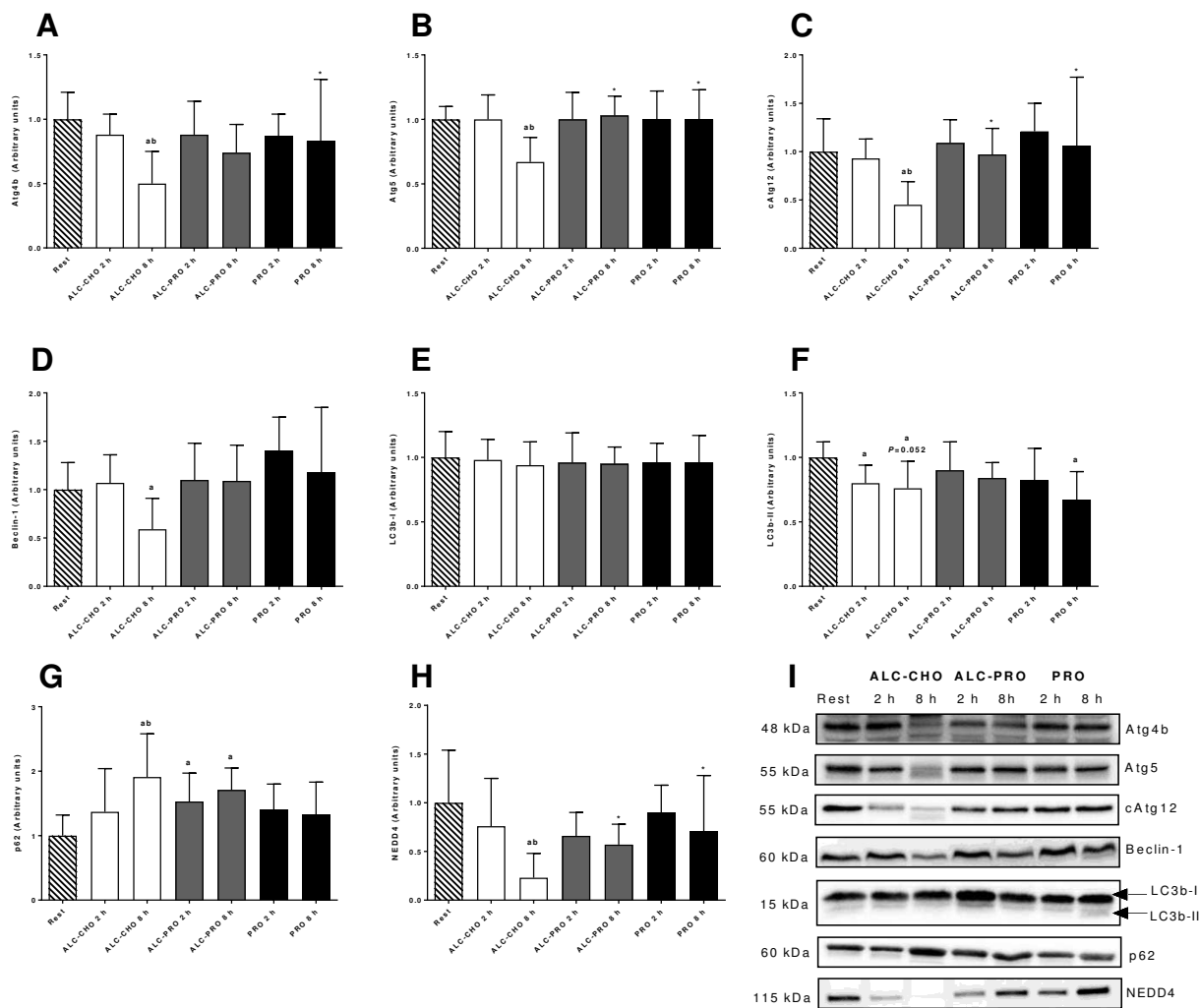


766

767 **Figure 2.** Representative Stain-Free images used for total protein normalization (A). Purity of

768 nuclear (N) and cytoplasmic (C) fractions were determined by immunoblotting for histone 2B

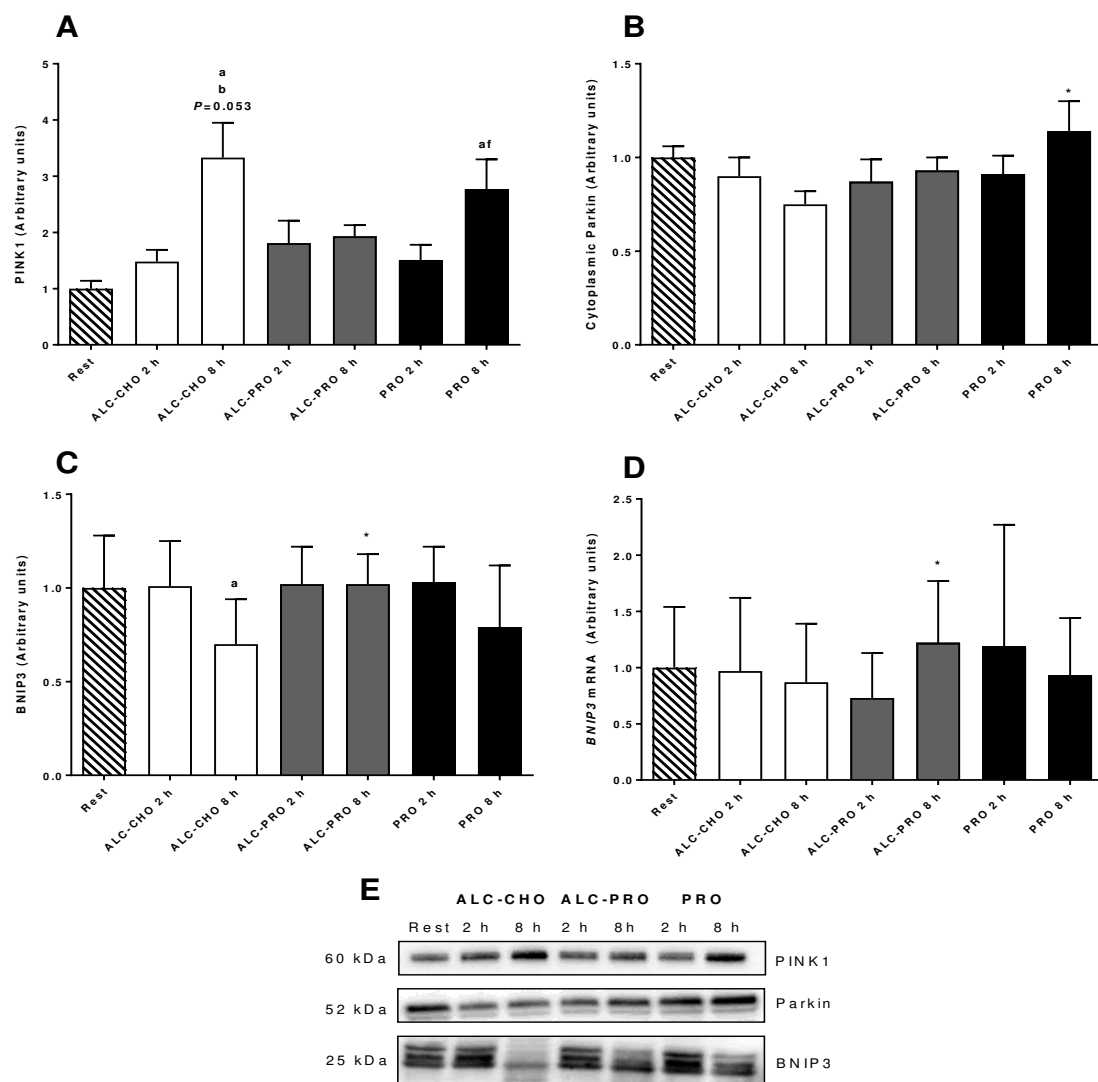
769 (H2B) and GAPDH, respectively (B).



770

771 **Figure 3.** Whole muscle abundance of autophagy regulatory proteins Atg4b (A), Atg5 (B),
 772 cAtg12 (C), Beclin-1 (D), LC3b-I (E), LC3b-II (F), p62 (G), and the ubiquitin ligase NEDD4
 773 (n=7 for ALC-CHO time points and ALC-PRO 2 h only; H) at rest and following a single
 774 bout of concurrent exercise with ingestion of either alcohol and carbohydrate (ALC-CHO),
 775 alcohol and protein (ALC-PRO), or protein only (PRO). I, representative images for all
 776 proteins. Data were analysed using a 2-way ANOVA with repeated measures and Student-
 777 Newman-Keuls post-hoc analysis. Values are presented (mean \pm SD) as a fold change
 778 relative to resting values. Significantly different ($P < 0.05$) vs. (a) rest, (b) ALC-CHO 2 h, and
 779 (*) 8 h between treatments.

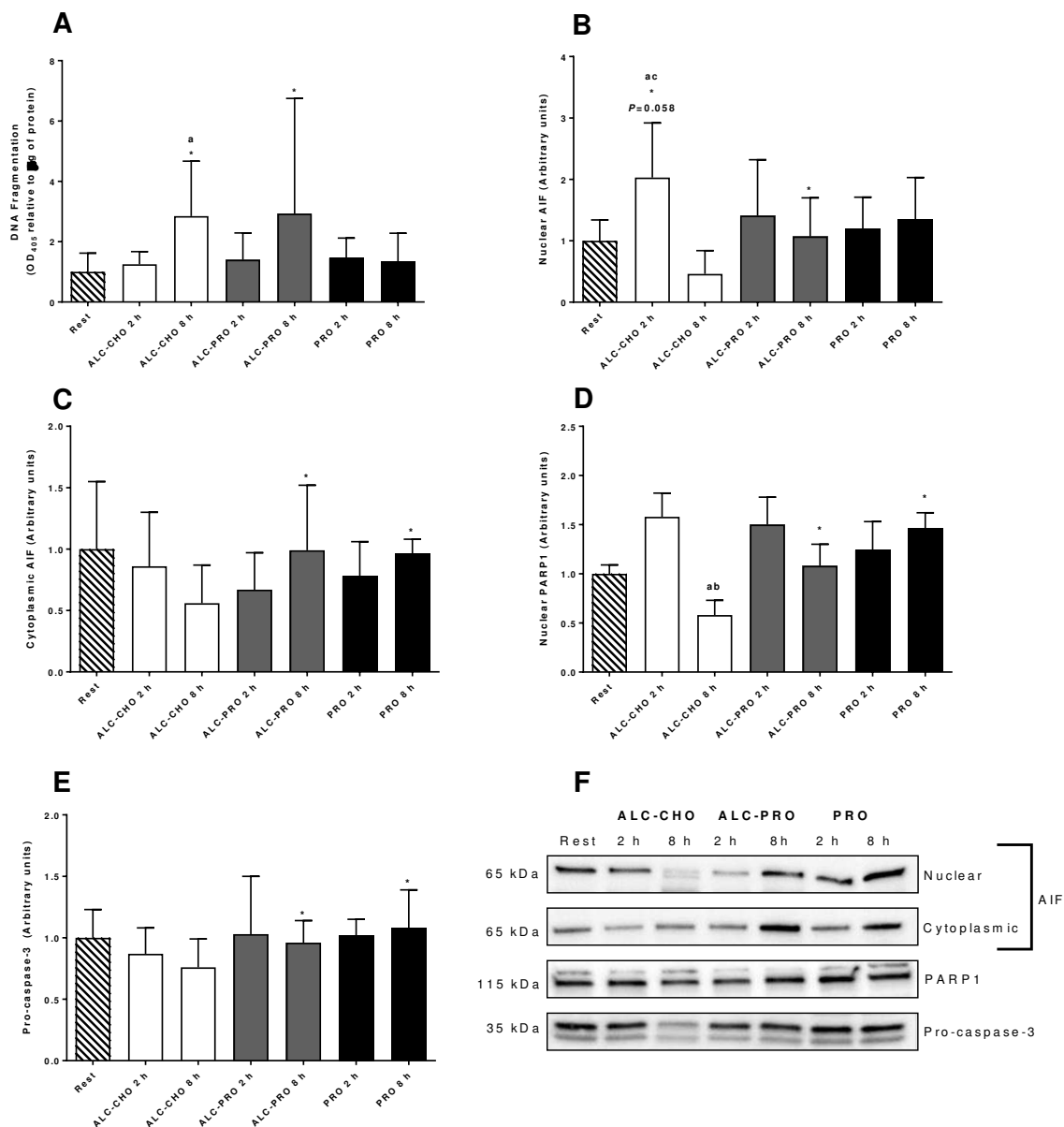
780



781

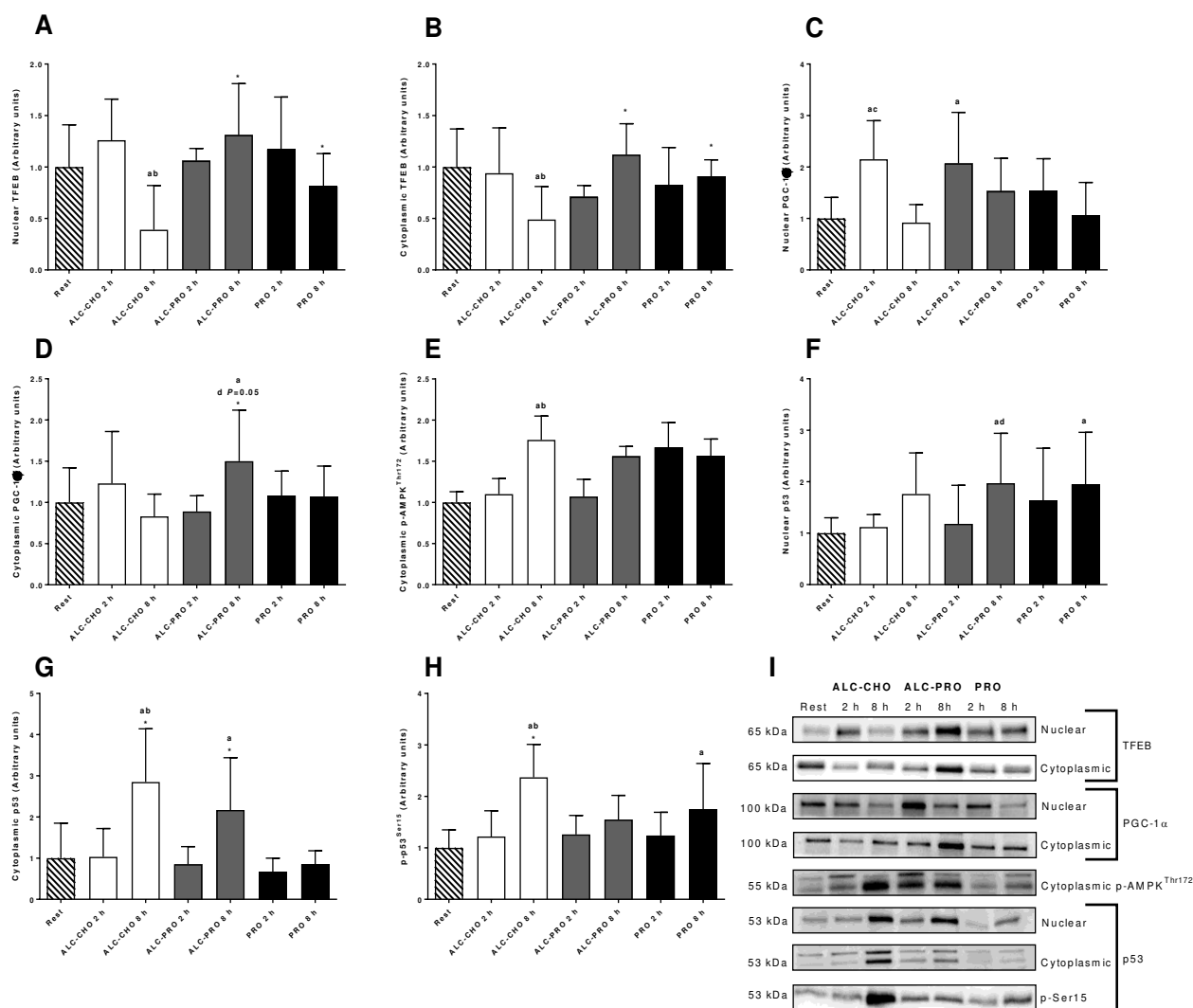
782 **Figure 4.** Mitophagy-related proteins PINK1 (A), Parkin (B), BNIP3 (n=7 for ALC-CHO 8 h
 783 and ALC-PRO 2 h only; C), and BNIP3 gene expression (n=7; D) at rest and following a
 784 single bout of concurrent exercise with ingestion of either alcohol and carbohydrate (ALC-
 785 CHO), alcohol and protein (ALC-PRO), or protein only (PRO). E, representative images for
 786 all proteins. Data were analysed using a 2-way ANOVA with repeated measures and Student-
 787 Newman-Keuls post-hoc analysis. Values are presented (mean \pm SD) as a fold change
 788 relative to resting values. Significantly different ($P < 0.05$) vs. (a) rest, (b) ALC-CHO 2 h, (f)
 789 PRO 2 h, and (*) 8 h between treatments.

790



791
 792 **Figure 5.** Apoptotic DNA fragmentation (A), nuclear (B) and cytoplasmic AIF (C), nuclear
 793 PARP1 (D), and whole muscle pro-caspase-3 (n=7 for ALC-CHO time points and ALC-PRO
 794 2 h only; E) at rest and following a single bout of concurrent exercise with ingestion of either
 795 alcohol and carbohydrate (ALC-CHO), alcohol and protein (ALC-PRO), or protein only
 796 (PRO). F, representative images for all proteins. Data were analysed using a 2-way ANOVA
 797 with repeated measures and Student-Newman-Keuls post-hoc analysis. Values are presented
 798 (mean ± SD) as a fold change relative to resting values. Significantly different (P<0.05) vs.
 799 (a) rest, (b, c) ALC-CHO 2 and 8 h, and (*) 8 h between treatments.

800



801

802 **Figure 6.** Nuclear (A) and cytoplasmic TFEB (B), nuclear (C) and cytoplasmic PGC-1α (D),803 cytoplasmic phospho-AMPK^{Thr172} (E), nuclear (F) and cytoplasmic p53 (G), and whole804 muscle phospho-p53^{Ser15} (H) at rest and following a single bout of concurrent exercise with

805 ingestion of either alcohol and carbohydrate (ALC-CHO), alcohol and protein (ALC-PRO),

806 or protein only (PRO). I, representative images for all proteins. Data were analysed using a 2-

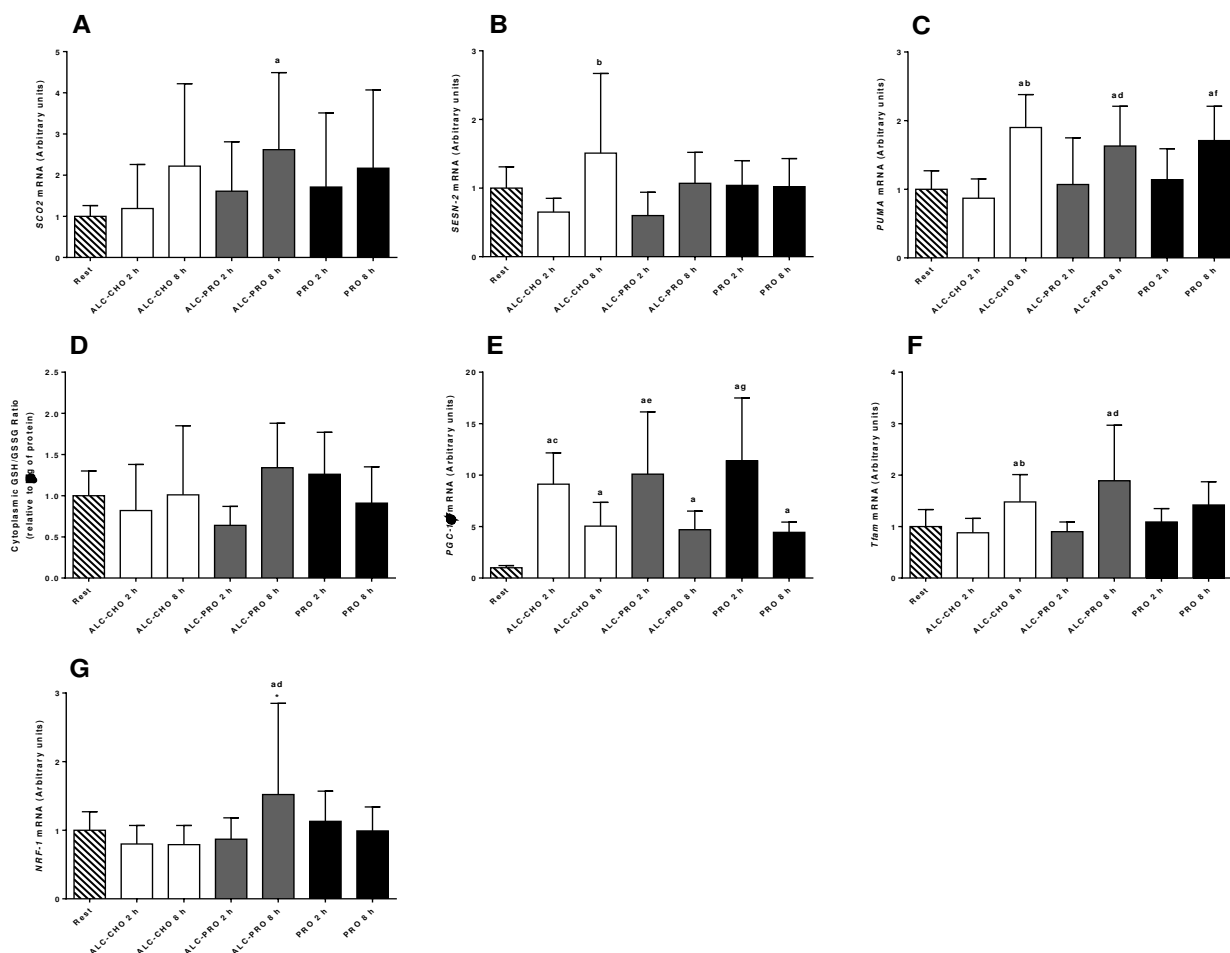
807 way ANOVA with repeated measures and Student-Newman-Keuls post-hoc analysis. Values

808 are presented (mean ± SD) as a fold change relative to resting values. Significantly different

809 (P<0.05) vs. (a) rest, (b, c) ALC-CHO 2 h and 8 h, (d) ALC-PRO 2 h, and (*) 8 h between

810 treatments.

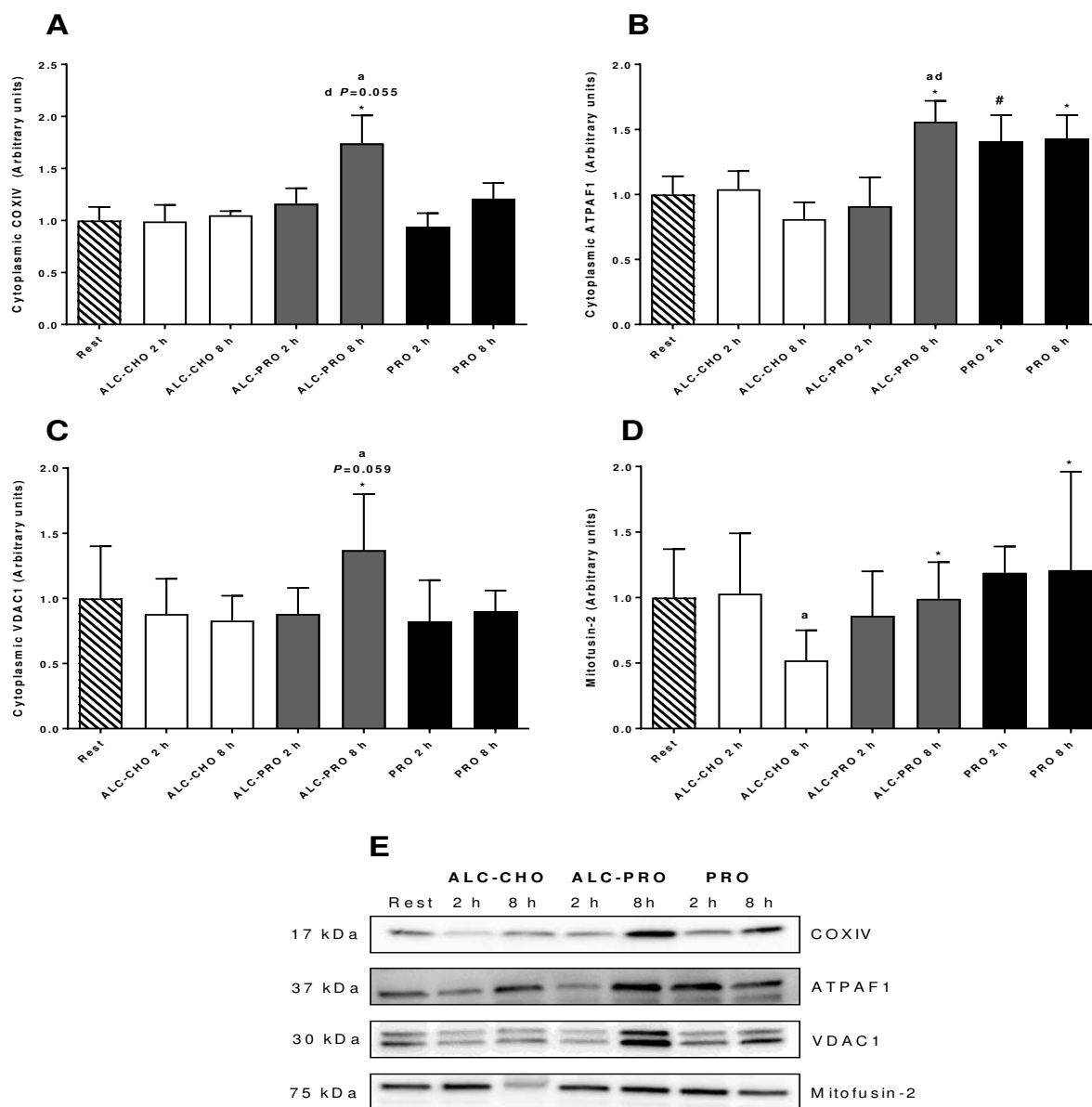
811



812

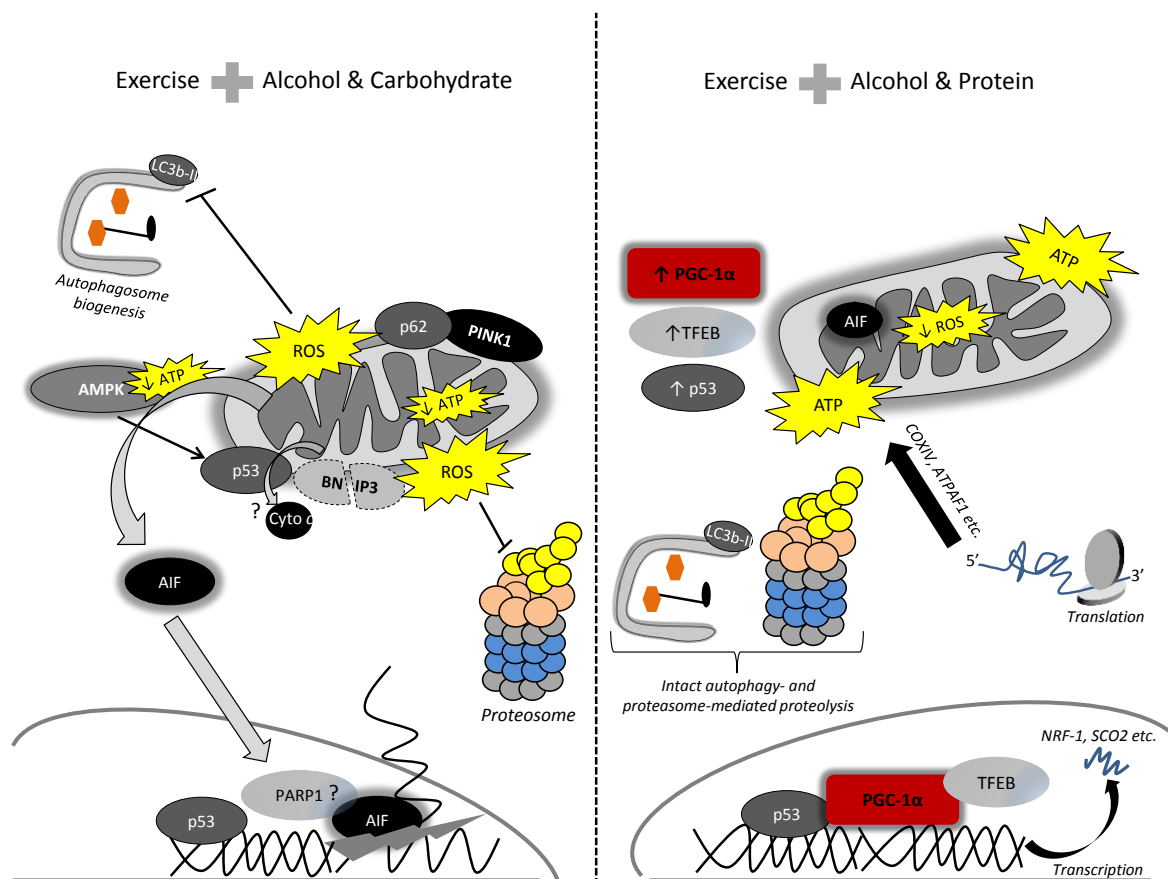
813 **Figure 7.** mRNA expression of SCO2 (A), SESN-2 (B) and PUMA (C), the cytoplasmic
 814 reduced (GSH) to oxidized (GSSG) glutathione ratio (n=4; D), and PGC-1α (E), Tfam (F)
 815 and NRF-1 (G) mRNA at rest and following a single bout of concurrent exercise with
 816 ingestion of either alcohol and carbohydrate (ALC-CHO), alcohol and protein (ALC-PRO),
 817 or protein only (PRO). Data were analysed using a 2-way ANOVA with repeated measures
 818 and Student-Newman-Keuls post-hoc analysis. Values are expressed relative to GAPDH and
 819 presented (mean ± SD; mRNA n=7) as a fold change relative to resting values. Significantly
 820 different (P<0.05) vs. (a) rest, (b, d, f) 2 h within treatments, (c, e, g) 8 h within treatments,
 821 and (*) 8 h between treatments.

822



823
 824 **Figure 8.** Cytoplasmic levels of mitochondrial proteins COXIV (A), ATPAF1 (B), VDAC1
 825 (C), and whole muscle Mitofusin-2 (n=7 for ALC-CHO time points and ALC-PRO 2 h only;
 826 D) at rest and following a single bout of concurrent exercise with ingestion of either alcohol
 827 and carbohydrate (ALC-CHO), alcohol and protein (ALC-PRO), or protein only (PRO). E,
 828 representative images for all proteins. Data were analysed using a 2-way ANOVA with
 829 repeated measures and Student-Newman-Keuls post-hoc analysis. Values are presented
 830 (mean ± SD) as a fold change relative to resting values. Significantly different (P<0.05) vs.
 831 (a) rest, (d) ALC-PRO 2 h, and (#) 2 h and (*) 8 h between treatments.

832



833

834

835 **Figure 9. Hypothetical model of the effects of alcohol coingested with carbohydrate**
 836 **versus protein following exercise-training**

837 Following strenuous exercise, binge alcohol consumption coingested with carbohydrate
 838 overwhelms mitochondrial respiration, leading to a reduction in cellular ATP levels and
 839 overproduction of reactive oxygen species (ROS) that causes oxidation of proteins implicated
 840 in cellular turnover (i.e., autophagy- and proteasome-mediated proteolysis). Consequently,
 841 failed clearance of damaged mitochondria, as indicated by a reduction in BNIP3 and increase
 842 in PINK1 and p62, and persistent ROS emissions could augment mitochondrial membrane
 843 permeability and trigger release of apoptogenic factors; notably, AIF (which may be recruited
 844 to the nucleus by PARP1) and cytochrome c, yet the latter requires confirmation. AIF nuclear
 845 translocation triggers apoptotic DNA fragmentation. In contrast, protein availability,

846 potentially owing to its enrichment of branched-chain amino acids, stimulates an abrupt,
847 mitochondrial anabolic response regulated by PGC-1 α , p53 and TFEB. Ultimately, the
848 transcription of mRNA and translation of proteins integral to mitochondrial biogenesis is a
849 homeostatic countermeasure (i.e., by increasing cellular ATP availability, attenuating ROS
850 formation and preserving constitutive degradative pathways) to the metabolic burden
851 imposed by alcohol introduced to an already disrupted, by prior exercise, cellular
852 environment.

853

854

855