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In Vivo Potentiation of Muscle Torque is Enhanced in Female Mice through Estradiol-Estrogen Receptor Signaling

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1 ***In Vivo* Potentiation of Muscle Torque is Enhanced in Female Mice Through Estradiol-**
2 **Estrogen Receptor Signaling**
3
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14
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27 **ABSTRACT**

28 Estradiol affects several properties of skeletal muscle in females including strength. Here, we
29 developed an approach to measure *in vivo* post-tetanic twitch potentiation (PTP) of the anterior
30 crural muscles of anesthetized mice and tested the hypothesis that 17β-estradiol (E_2) enhances
31 PTP through estrogen receptor (ER) signaling. Peak torques of potentiated twitches were ~40-
32 60% greater than those of unpotentiated twitches and such PTP was greater in ovary-intact mice,
33 or ovariectomized (Ovx) mice treated with E_2, compared to Ovx mice ($p \leq 0.047$). PTP did not
34 differ between mice with and without ERα ablated in skeletal muscle fibers ($p=0.347$). Treatment
35 of ovary-intact and Ovx mice with ERβ antagonist and agonist (PHTPP and DPN, respectively)
36 did not affect PTP ($p \geq 0.258$). Treatment with G1, an agonist of the G protein-coupled estrogen
37 receptor (GPER), significantly increased PTP in Ovx mice from 41 ± 10 to 66 ± 21% (mean ±
38 SD; $p=0.034$). Collectively, these data indicate that E_2 signals through GPER, and not ERα or
39 ERβ, in skeletal muscle of female mice to augment an *in vivo* parameter of strength, namely
40 PTP.

41

42 **NEW & NOTEWORTHY** A novel *in vivo* approach was developed to measure potentiation of
43 skeletal muscle torque in female mice and highlight another parameter of strength that is
44 impacted by estradiol. The enhancement of PTP by estradiol is mediated distinctively through
45 the G-protein estrogen receptor, GPER.

46 Dynapenia, defined as a loss of muscle strength with age unrelated to a neuromuscular
47 disease or muscle atrophy, is highly associated with physical disability, falls, and mortality (1).
48 Strategies to combat dynapenia will be enhanced as cellular and molecular mechanisms
49 underlying the condition are continually unveiled. It will also be crucial to determine if and how
50 such mechanisms differ by sex. For example, evidence continues to accumulate showing that the
51 major female sex hormone, estradiol (E_2), contributes to skeletal muscle (dys)function in females
52 (2, 3). Thus, estrogenic pharmacological strategies, such as those used for improving bone health
53 in post-menopausal women, may present sex-specific strategies to mitigate dynapenia as well.

54 E_2 is the main estrogen in females of reproductive age and primarily exerts its biological
55 action by binding to estrogen receptors (ERs). Ligand bound ERs can then act genomically as
56 transcription factors or non-genomically to elicit rapid cellular actions. While ERα and ERβ
57 subtypes are considered the classical nuclear ERs, a G protein-coupled estrogen receptor (GPER)
58 has more recently been recognized for its quick extranuclear actions (4). Notably, all three ER
59 subtypes are expressed in skeletal muscle (5) with ERα being the most abundant. Elimination or
60 pharmacological blockade of ERα is detrimental to muscle contractility (6), myogenesis and
61 regeneration following an injury (7, 8), and has shown negative consequences in muscle
62 metabolism, insulin sensitivity (9), and mitochondrial function (10) in some but not all studies on
63 mice (11). Evidence for the involvement of ERβ in skeletal muscle myogenesis and regeneration
64 also exists (12, 13).

65 Specific to skeletal muscle strength in females, when E_2 is deficient force generation is
66 diminished (6, 14-17). The molecular force generator in muscle is myosin and myosin-based
67 mechanisms underlying E_2-related decrements force are supported by studies conducted on
68 rodents (14, 18-20) and are consistent with results from human studies (21, 22). For example,

69 specific force and force per myosin crossbridge were greater in single fibers from biopsies of
70 post-menopausal twins on estrogenic hormone therapy compared to fibers from sisters not on
71 hormone therapy (21). A specific myosin modification that affects force production in striated
72 muscle is phosphorylation of the regulatory light chain (RLC) (23) as RLC phosphorylation
73 (pRLC) facilitates myosin binding with actin (24). A physiological measurement in skeletal
74 muscle that is highly sensitive to pRLC is potentiation of twitch force following a tetanic
75 contraction, i.e., post-tetanic twitch potentiation (PTP) (25). Phosphorylation of RLC is primarily
76 mediated by myosin light chain kinase. The direct link between PTP and pRLC is substantiated
77 by results showing that mice lacking myosin light chain kinase in skeletal muscle have
78 attenuated PTP due to reduced pRLC (26).

79 E_2 has been shown to affect kinase signaling in C2C12 cells (7, 27, 28), isolated rodent
80 muscles (29), mouse muscles *in situ* (30), and in human muscle (31). Thus, it follows that greater
81 kinase signaling mediated by E_2 to phosphorylate RLC would result in enhanced PTP.
82 Supporting this concept, Lai and coworkers (32) demonstrated that pRLC and PTP were
83 attenuated in isolated extensor digitorum longus (EDL) and soleus muscles from ovariectomized
84 (Ovx) mice compared to muscles from ovary intact mice. Directly implicating E_2 in affecting
85 pRLC and PTP are data showing that incubation of EDL muscles from Ovx mice in a buffer
86 containing E_2 restored both pRLC and PTP (32). Further, *in vitro* pharmacological blockade of
87 specific ERs in muscles or depleting specific ERs by siRNA in C2C12 cells indicated that E_2
88 signaled through ERβ and GPER, but not ERα, to mediate the phosphorylation of RLC (32).
89 Despite the evidence that E_2–ER signaling facilitates enhanced force production *in vitro*, there is
90 a lack of evidence showing that E_2 affects PTP *in vivo*. The primary objectives of this study were
91 to establish an *in vivo* PTP protocol in mice and test the hypothesis that PTP is enhanced in

92 skeletal muscle by E_2-ER signaling, and to specifically identify which ER subtype mediates the
93 PTP enhancement.
94

95 **MATERIALS AND METHODS**

96 **Ethical Approval: Animals and procedures common across experiments**

97 The Institutional Animal Care and Use Committee at the University of Minnesota, which
98 operates under the national guidelines set by the Association for Assessment and Accreditation
99 of Laboratory Animal Care, approved all protocols. Investigators understand the ethical
100 principles and ensure that the work complies with the animal ethics of American Physiological
101 Society guidelines.

102 Female C57BL/6J mice aged 3-4 mo (n = 41) were obtained from Jackson Laboratories
103 (Bar Harbor, ME). Female skeletal muscle specific estrogen receptor α knockout mice (ERαKO;
104 n= 7) and their wildtype littermates (ERαWT; n= 8) on a C57BL/6 were bred onsite and used at
105 4-5 mo of age (6). Mice were housed in groups of 4-5 and had access to phytoestrogen-free
106 rodent chow (Harlan-Teklad #2019; Indianapolis, IN) and water *ad libitum*. The housing room
107 was specific pathogen free with a 14:10 h light:dark cycle and controlled temperature and
108 humidity.

109 Mice not born and raised in the animal facility at the University of Minnesota were
110 acclimated to their new housing for at least 1 wk before a surgery was conducted to implant a
111 stimulating nerve cuff on the common peroneal nerve of the left leg. A subset of the nerve cuff-
112 implanted C57BL/6J mice also had electromyography (EMG) recording electrodes implanted on
113 the left tibialis anterior muscle. The EMG electrode surgery was done no less than 3 wk after
114 implanting the stimulating nerve cuff and PTP measurements were done no less than 3 wk after

the second surgery. For mice undergoing only the nerve cuff surgery, 6 wk later mice were randomly assigned either to maintain regular 4-5 d ovarian hormone cycles with intact ovaries (Intact) or to surgically remove both ovaries to induce ovarian hormone deficiency (Ovx). Mice instrumented with stimulating electrodes only were reused in up to three experiments (specifically, Experiments 1, 3 and 4 as described below) with at least a 1-mo washout between experiments.

Mice were euthanized after completion of their final *in vivo* PTP measurements. At the time of euthanasia, mice were first anesthetized by an intraperitoneal injection of pentobarbital sodium (100 mg/kg body mass; Diamondback Drugs, Scottsdale, AZ) and tissues were excised. Uteri were dissected and weighed; mean (SD) uterine mass for Intact and Ovx mice were 112.7 (19.6) and 18.0 (2.9) mg, respectively. Based on our experience, uterine mass of <30 mg in Ovx mice reflects successful ovariectomy surgery. Mice were euthanized by an overdose of pentobarbital sodium (200 mg/kg body mass).

Surgical Procedures

Each mouse received extended-release buprenorphine subcutaneously (1.0 mg/kg body mass) as an analgesic immediately prior to any surgical procedure. Surgical procedures were done under anesthesia using an induction chamber containing isoflurane and then maintained using inhalation of 2-3% isoflurane in oxygen at a flow rate of 100-200ml/min. Depth of anesthesia was assessed and maintained by monitoring respiratory rate and toe pinch withdrawal reflex. This anesthetic regimen was also used when isometric dorsiflexion torque and EMG measurements were made with the exception that 1.5% isoflurane was used.

137 Stimulating nerve cuffs were surgically implanted on the common peroneal nerve as
138 described previously (33). In brief, an incision was made through the biceps femoris muscle of
139 the left hindlimb and a nerve cuff made of platinum iridium wire (Medwire-Sigmund Cohn
140 10Ir9/49T, Mt. Vernon, NY) and silastic tubing was placed around the common peroneal nerve.
141 For inducing muscle contraction, ends of the nerve cuff were connected to a stimulator and
142 stimulus isolation unit (Models S48 and SIU5, respectively, Grass Technologies, West Warwick,
143 RI).

144 M-wave amplitude was measured as described previously (33-35). Briefly, deinsulated
145 ends of two platinum iridium wires, offset by ~2 mm, were routed underneath the superficial
146 fascial sheath of the tibialis anterior muscle. The electrode wire spacing theoretically permitted
147 sampling of EMG activity from the full thickness of the tibialis anterior muscle beneath the
148 electrodes. The wires were secured to adjacent tissue by suture and the proximal ends of the
149 wires were run subcutaneously and externalized in the dorsal cervical region. When EMG
150 measurements were conducted, the proximal wire ends were connected to an EMG amplifier
151 (Model P55, Grass Technologies).

152 Approximately one-half of the mice with nerve cuffs underwent a second surgery to
153 remove ovaries. For this, bilateral ovariectomy was performed through two small dorsal incisions
154 between the iliac crest and the lower ribs (18).

155

156 **Experimental design and methods**

157 **Establishing an *in vivo* post-tetanic potentiation (PTP) protocol:** The *in vivo* PTP
158 protocol for mouse anterior crural muscles (tibialis anterior, EDL, and extensor hallucis longus
159 muscles) was modeled after *in vitro* protocols for mouse EDL muscle (32, 36). In preliminary

160 experiments, testing conditions were optimized including the stimulation frequency and duration
161 of the potentiating tetanic contraction, as well as timing of the twitches before and after the
162 tetanic contraction in order to obtain the highest PTP values. The final protocol involved one
163 baseline twitch contraction elicited by a 0.1-ms pulse. Thirty seconds later, muscles were
164 stimulated using 0.1-ms pulses at 100 Hz and 7 V for 1 s to produce a prolonged, isometric
165 tetanic contraction. Two post-tetanic twitches were elicited at 2 and 30 s after the tetanic
166 contraction (Fig. 1). Immediately before each twitch contraction a 100-ms resting torque baseline
167 was determined. The average of the resting torque baseline was calculated from 3000 data points
168 (i.e., all sampling was done at 30,000 Hz) and was then subtracted from the subsequent twitch
169 contraction torque values. This was done to adjust for any minor baseline changes before to after
170 the tetanus as well as between twitches. PTP was calculated as the percent increase in isometric
171 dorsiflexion torque from the baseline twitch to the greater of the two post-tetanic twitches.
172 Initial PTP experiments were conducted on Intact mice to demonstrate that twitch torque
173 was potentiated *in vivo* (n=23; Table 1). A subset of those mice were subsequently
174 ovariectomized and retested ~6 wk later to demonstrate PTP still could be measured (n=14).
175 Further validation was established as some Ovx mice were acutely treated with vehicle and/or E_2
176 in preliminary experiments (n=10 each; Table 1). 17β-estradiol (E_2, 80056-424; VWR
177 Calbiochem, Radnor, PA; 0.15 mg E_2 per kg body weight) was dissolved in saline plus ethanol
178 (0.3%); 0.3% ethanol in saline was the vehicle. The dose of E_2 was based on previous use of
179 pellets that released 0.18 mg of E_2 over 60 d (3 μg/d) and resulted in physiological levels of
180 serum E_2 for female mice (14, 37). To determine optimal timing of E_2 delivery, Ovx mice
181 anesthetized with isoflurane were placed on a heating pad for 5 – 10 min to cause vasodilation,
182 one of the two lateral tail veins was located, and E_2 was injected *via* an insulin syringe. PTP was

183 then measured 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h and 3 h after injection. The optimal time point selected
184 was based on the minimal time tested after injection that elicited the greatest PTP and this time
185 point was 1 h. As such, data from Ovx+ mice in Table 1 as well as for mice in Experiments 1, 3,
186 and 4 involved a pre-treatment PTP measurement, delivery of the estrogenic agent or a vehicle,
187 maintenance of anesthesia for 1 h, and then measurement of post-treatment PTP.

188 To determine that *in vivo* PTP occurs independent of changes in fiber excitability, Intact
189 mice implanted with both stimulating and EMG electrodes had M-waves recorded and PTP
190 measured simultaneously (n = 9). Analysis of the electrically-evoked myoelectric signal, i.e.,
191 measurement of M-wave amplitude, was done by calculating root-mean-square of the M-wave as
192 previously described (33, 35). Change in M-wave amplitude (in mV) was calculated as percent
193 increase from baseline to that following tetanic stimulation using the same post-tetanic twitch as
194 was used for calculating PTP.

195 **Determining effects of estrogenic interventions on *in vivo* PTP:** *Experiment #1:* To
196 determine the effect of acute E_2 treatment on PTP *in vivo*, PTP was measured in mice (Intact or
197 Ovx) before and after treatment with either vehicle (ethanol) or 17β-E_2 *via* tail vein injection (n =
198 6-11 per treatment, per group).

199 *Experiment #2:* Once demonstrated that E_2 accentuates PTP *in vivo*, we sought to
200 determine if E_2 signals through an ER to enhance force. Thus, we first measured PTP in female
201 mice lacking skeletal muscle ERα, and compared data from those ERαKO mice (n = 8) to
202 control littermates, ERαWT mice (n = 7).

203 *Experiment #3:* To determine if E_2-estrogen receptor β (ERβ) signaling contributes to
204 PTP *in vivo*, the anterior crural muscles were stimulated immediately before and 1 h after
205 treatment with ERβ specific agonists/antagonists. Treatments included either vehicle (ethanol),

206 the ERβ agonist diarylpropionitrile (DPN, H5915; Sigma, St. Louis, MO; 5 nmol dissolved in
207 ethanol), or the ERβ antagonist 4-[2-Phenyl-5,7-bis (trifluoromethyl) pyrazolo [1,5-a]-pyrimidin-
208 3-yl] phenol (PHTPP, 2662; Tocris Bioscience, Minneapolis, MN; 33 nmol dissolved in ethanol)
209 each *via* tail vein injection. Treatment dosages for DPN and PHTPP were chosen according to
210 Meyers and coworkers (38) and Compton and coworkers, respectively (39). Only Ovx mice were
211 used in the agonist experiments and only Intact mice were used in the antagonist experiments (n
212 = 4-10 per treatment, per group).
213 *Experiment #4:* To determine if E_2-GPER signaling contributes to PTP *in vivo*, vehicle
214 (Dimethyl Sulfoxide [DMSO]), GPER agonist *rel*-1-[4-(6-bromo-1,3-benzodioxol-5-yl)-
215 3aR,4S,5,9bS-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone (G1, 10008933; Cayman
216 Chemical, Ann Arbor, MI; 2.4 nmol dissolved in DMSO), or GPER antagonist
217 (3aS*,4R*,9bR*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinolone
218 (G15, 41004003; Sandia Biotech, Albuquerque, NM; 27 nmol dissolved in DMSO) was tested.
219 Treatment dosages for G1 and G15 were chosen according to Dennis and coworkers (40). Ovx
220 mice were used in the agonist experiments and only Intact mice were used in the antagonist
221 experiments (n = 7-10 per treatment, per group).
222
223 **Statistical analyses**
224 Paired *t-tests* were used to assess differences between unpotentiated and potentiated
225 twitch torques and M-wave amplitudes, and between pre- and post-treatments. Student *t-tests*
226 were used to assess differences between Intact vs Ovx mice, Ovx+Veh vs Ovx+E_2 mice, and
227 ERαWT vs ERαKO mice. Prior to performing the *t-tests*, assumptions of parametric tests, i.e.,
228 normality and homoscedasticity, were checked. An α level of 0.05 was used for all analyses.

229 Values are presented as means ± SD. Statistical testing was conducted using IBM SPSS Statistics
230 ver. 24 (Armonk, NY) or SigmaStat version 12.5 (Systat Software, San Jose, CA).
231

232 **RESULTS**

233 ***In vivo* PTP**

234 PTP of anterior crural muscle in control mice (Intact and not treated with any
235 agonist/antagonist) was ~50% and untreated Ovx mice was lower at ~40% demonstrating that
236 PTP is measurable *in vivo* (Table 1). Further, Ovx mice treated with E_2 had greater *in vivo* PTP
237 than those treated with vehicle (Table 1).

238 In mice implanted with EMG electrodes on tibialis anterior muscle, unpotentiated twitch
239 torque was 0.48 ± 0.11 mN·m and following the tetanic contraction increased to 0.66 ± 0.11
240 mN·m equating to a PTP of 40 ± 15% ($p<0.001$; Fig. 2A, D, F). No change was detected in
241 twitch M-wave amplitude from the unpotentiated to the potentiated twitch contractions (0.57 ±
242 0.22 to 0.57 ± 0.20 mV, respectively; $p=0.935$; Fig. 2B, C, E, F). The disproportionate increase
243 in twitch torque (40%) compared to no change in the corresponding M-wave amplitude (Fig. 2F)
244 indicates that fiber excitability does not contribute to *in vivo* PTP.
245

246 **Effects of estrogenic interventions on *in vivo* PTP**

247 *E_2 (Experiment 1):* To confirm that the difference in PTP between Intact and Ovx mice
248 shown in Table 1 was due to estrogen status rather than age and to pinpoint that the ovarian
249 hormone affecting PTP is E_2, PTP was measured before and after an acute treatment with vehicle
250 or E_2. Changes in PTP from Pre-treatment to Post-treatment, denoted by dashed lines, were not
251 significant in Intact mice treated with either vehicle or E_2 or in Ovx mice treated with vehicle

252 (Fig. 3). However, PTP in Ovx mice significantly increased from 41 ± 7% Pre-treatment to 64 ±
253 19% Post-treatment with E_2 (Fig. 3). This result indicates that acute exposure of muscle to E_2 in
254 an ovarian hormone deficient environment is sufficient to augment *in vivo* PTP in mouse muscle.
255 *ERα (Experiment 2):* Peak torque of unpotentiated twitches did not differ between
256 ERαWT and ERαKO mice (p=0.253; Table 1). Potentiated twitches generated more torque than
257 unpotentiated twitches but PTP did not differ between ERαWT and ERαKO mice (p=0.347; Fig.
258 4 and Table 1), indicating estrogenic modulation of PTP is not through E_2-ERα signaling *in vivo*.
259 *ERβ (Experiment 3):* Peak torque of unpotentiated twitches did not differ among
260 Intact+Veh, Intact+PHTPP, Ovx+Veh, and Oxv+DPN mice (p=0.301). Pre-treatment potentiated
261 twitches generated ~30-80% more torque than unpotentiated twitches (solid symbols, Fig. 5).
262 There was no significant change in PTP following ERβ intervention within any group (Fig. 5).
263 That is, treatment of Intact mice with the ERβ antagonist, PHTPP, did not significantly inhibit
264 PTP nor did the ERβ agonist, DPN, augment PTP in Ovx mice (Fig. 5). These data suggest that
265 estrogenic modulation of skeletal muscle force potentiation *in vivo* is not likely through ERβ.
266 *GPER (Experiment 4):* Peak torques of unpotentiated twitches did not differ among
267 Intact+Veh, Intact+G15, Ovx+Veh, and Ovx+G1 mice (p=0.254). The only group that had a
268 significant change in PTP from Pre- to Post-treatment was Ovx mice treated with the GPER
269 agonist, G1 (PTP increased from 41 ± 10 to 66 ± 21%; Fig. 6). Because acute treatment with a
270 GPER agonist increased *in vivo* PTP of the anterior crural muscles in anesthetized mice, our
271 hypothesis that E_2 mediates its effect on skeletal muscle force potentiation through specific
272 estrogen receptors was supported.
273
274

275 **DISCUSSION**

276 The initial objective of this work was to establish an *in vivo* PTP protocol in hindlimb
277 muscles of anesthetized mice. This was accomplished by using a sensitive and reproducible
278 physiological approach involving the surgical implantation of stimulating electrodes on the
279 common peroneal nerve, a branch of the sciatic nerve innervating the anterior crural muscles.
280 This permitted isometric dorsiflexion torque of unpotentiated twitches to be measured without
281 any prior muscle contractions, followed by a one-second tetanic contraction, and then potentiated
282 twitch torque measurements. Twitch torque following tetanic contraction was enhanced (i.e.,
283 potentiated) and the magnitude of this *in vivo* PTP was 30-70%, similar to *in vitro* PTP values of
284 isolated mouse EDL muscle (26, 32), establishing a viable *in vivo* PTP protocol (Fig. 1). We next
285 reaffirmed historical work demonstrating that PTP results from fiber intrinsic mechanisms rather
286 than electrophysiological changes of the plasmalemma (41). This was done by measuring M-
287 wave amplitudes in pre- and post-tetanic twitches *via* electromyography of the major anterior
288 crural muscle, the tibialis anterior muscle, simultaneously with the torque measurements. While
289 twitch torque increased by ~40%, M-wave amplitude did not differ between the pre- and post-
290 twitches confirming that electrophysiological changes did not contribute to PTP (Fig. 2).

291 The main objective of this work was to test the hypothesis that PTP is enhanced by E_2-ER
292 signaling in skeletal muscle *in vivo*. Our hypothesis was supported by evidence that E_2 enhances
293 PTP, shown in multiple comparisons. First, *in vivo* PTP was greater in mice with intact ovaries
294 compared to Ovx mice, and second, Ovx mice treated with E_2 had greater PTP compared to Ovx
295 mice treated with vehicle (Table 1). The ability of E_2 to acutely (i.e., within 1 h) increase PTP
296 was demonstrated in a third set of measurement in Ovx mice when PTP increased from ~40 to

297 65% in response to E_2 treatment (Fig. 3). The magnitude of the *in vivo* response to E_2 was similar
298 to *in vitro* PTP of isolated EDL muscle from Ovx mice that was augmented by exposure to E_2 in
299 the surrounding bath (32). Important to note is that mice were greater than 4 mo of age at the
300 time of surgical intervention and physiological measurements in studies reported here as well as
301 in the study by Lai and coworkers (32). In contrast, *in vitro* PTP was not responsive to E_2
302 treatment in younger, growing, 2 mo-old ovariectomized female mice (42).

303 After demonstrating that E_2 affects PTP *in vivo*, we next tested the hypothesis that the
304 hormone elicits its effects through specific ERs. PTP did not differ between mice ablated for
305 ERα in skeletal muscle fibers and control littermates, indicating that E_2 does not enhance PTP by
306 signaling through ERα (Fig. 4), consistent with previous *in vitro* results (32). A pharmacological
307 approach using an ERβ antagonist and an ERβ agonist also failed to affect PTP *in vivo* (Fig. 5).
308 These results are inconsistent with previous *in vitro* data showing that myosin pRLC was
309 reduced by siRNA knockdown of ERβ in C2C12 cells (32) and demonstrates the importance of
310 following up *in vitro* results with *in vivo* testing.

311 Activation of an ER that did elicit an increase of *in vivo* PTP was the non-genomic ER,
312 GPER that executes a rapid effect of E_2 (i.e., $\leq$ 1 h; Fig. 6). Acute treatment with the GPER
313 agonist, G1, increased PTP in eight of nine Ovx mice indicating E_2 signals through GPER to
314 elicit PTP. This result supports our hypothesis and is in line with siRNA knockdown of GPER
315 causing reduced pRLC in C2C12 cells (32). GPER has been studied minimally in skeletal
316 muscle, but more so in cardiac and smooth muscle. In a recent review, Groban and coworkers
317 report on the cardioprotective effects of GPER and summarize how GPER activation by E_2 or G1
318 regulates contractility of the heart to preserve diastolic function in females (43). Similarly, GPER
319 activation by G1 increased contractility in smooth muscle, both myometrial cells and uterine

320 strips (44). Interestingly, our results showed a trend ($p=0.073$) for G15 to increase *in vivo* PTP as
321 well, even though G15 is a GPER antagonist as initially reported in breast cancer and uterine
322 epithelial cells (40). G15 has been shown to have GPER antagonistic activity in cardiomyocytes
323 (43, 45), the heart (46), and smooth muscle (44) but to our knowledge G15 has not previously
324 been tested in skeletal muscle. In contrast to non-skeletal muscle tissues and cells, G15 treatment
325 enhanced some of the bone-protecting characteristics of E_2 in Ovx rats indicating that G15 had
326 tissue-specific effects in the skeleton that could be considered agonistic (47). Thus, more work is
327 needed to elucidate GPER agonistic/antagonistic mechanisms in the musculoskeletal system as a
328 whole and identifying targets of G1 and G15 will be important experiments to begin deducing
329 such mechanisms.

330 Few studies have reported on estrogenic effects or sex differences of force potentiation in
331 humans. O'Leary and coworkers showed no difference in PTP of the dorsiflexors between young
332 women and men (48). Post-activation potential (PAP) is a similar enhancement of twitch
333 contractile performance that is studied in humans (49). The conditioning contraction eliciting
334 PAP is voluntary activation of the muscle, as opposed to a tetanic contraction at a high
335 stimulation frequency for PTP. PAP of the plantarflexors was measured in young adult females
336 using oral contraceptives and males (50). PAP did not differ between the sexes in traditionally
337 measured PAP, but when a 10 min cycling warm-up preceded the PAP protocol, PAP was higher
338 in females than males. In another study, PAP was measured in two groups of perimenopausal
339 women; early perimenopausal (follicle-stimulating hormone (FSH) <25 IU/L and irregular
340 menstrual cycles) and late perimenopausal (FSH >25 IU/L) women (51). PAP did not differ
341 between peri- and late-menopausal groups. However, PAP and FSH levels were negatively
342 correlated and authors suggested that the menopausal transition might further reveal reduced

343 PAP as FSH continues to rise and E_2 declines during the full transition to postmenopause. In
344 such studies in humans and in our *in vivo* animal model study, potentiation of twitch torque may
345 also be influenced by compliance of in-series tendons (52), which in turn may be affected by the
346 presence or absence of estrogen (53).

347 Force potentiation is a short-term consequence of muscle contraction triggered primarily
348 by muscle protein phosphorylation *via* kinase activity. A key signaling pathway eliciting PTP
349 involves phosphorylation of myosin RLC by myosin light chain kinase (25) and
350 dephosphorylation by phosphatases, such as myosin light chain phosphatase. This single
351 phosphoprotein and its regulation, however, are not the only mediators of PTP (26, 32).
352 Phosphoproteomic profiling by mass spectrometry showed estrogen deficiency resulted in
353 several differentially phosphorylated sarcomeric proteins and identified alterations of calcium
354 signaling pathways, which could theoretically be related to the functional consequence of PTP
355 (54). Here, we determined that E_2 enhances PTP *in vivo* and that the ER through which the
356 hormone mediates specific effects is GPER. The results provide an important step toward
357 delineating precise mechanisms of signaling underlying force generation and consequently
358 muscle weakness when E_2-ER signaling is disrupted as occurs with aging in females.

359

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370

371 **Data Availability**

372 The data that support the findings of this study are available from the corresponding author upon
373 reasonable request.

374

375 **Disclosures**

376 No conflicts of interest, financial or otherwise, are declared by the authors. The authors indicate
377 no potential conflicts of interest.

378

379 **Author Contributions**

380 GL, CWB, GLW, and DAL conceived and designed research; GL and CWB performed
381 experiments and analyzed data; GL, CWB, GLW, and DAL interpreted results of experiments;
382 GL prepared figures; GL and DAL drafted manuscript; GL, CWB, GLW, and DAL edited and
383 revised manuscript and approved final version of manuscript.

384

385

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552

553

554 **FIGURE LEGENDS**

555

556 **Figure 1. Representative torque tracing from an *in vivo* post-tetanic twitch potentiation**
557 **(PTP) protocol.** The anterior crural muscles of an anesthetized female mouse were stimulated to
558 perform isometric twitch and tetanic contractions through a surgically implanted nerve cuff on
559 the peroneal branch of the sciatic nerve. PTP was calculated as the percent increase from twitch
560 torque prior to the tetanic contraction (Unpotentiated) to the greatest of the two twitch torques
561 following the tetanic contraction (Potentiated), indicated by the dashed lines. Note that the x axis
562 (time) is not to scale.

563

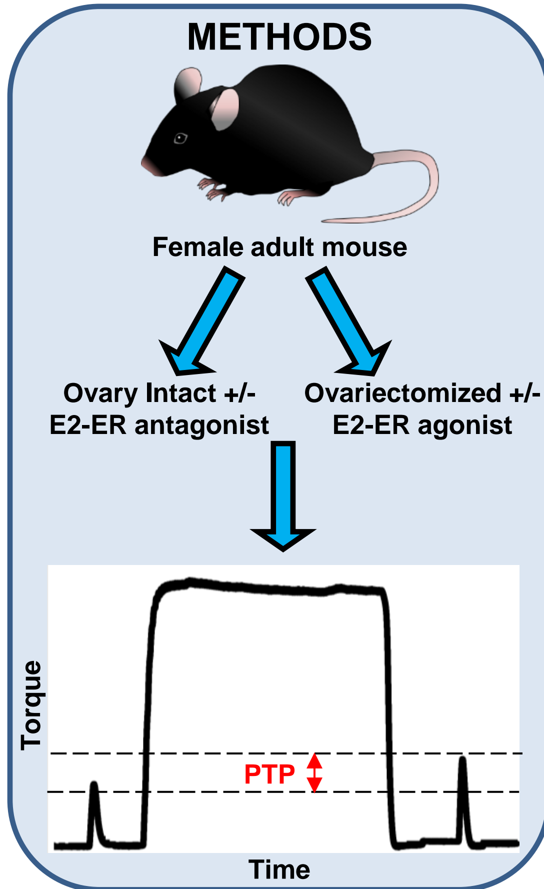
564 **Figure 2. *In vivo* post-tetanic twitch potentiation of anterior crural muscles with**
565 **simultaneous electromyography (EMG) of the tibialis anterior muscle.** (A) Representative
566 twitch torque tracings before (Unpotentiated) and after (Potentiated) a tetanic contraction by
567 anterior crural muscles of an anesthetized female mouse. Tibialis anterior muscle EMG tracings
568 from the (B) Unpotentiated and (C) Potentiated twitches shown in A. (D) Unpotentiated twitch
569 torques averaged 0.48 ± 0.04 mN·m and increased to 0.66 ± 0.04 mN·m for the Potentiated
570 twitches ($p<0.001$). (E) M-wave amplitudes (i.e., RMS) of Unpotentiated and Potentiated
571 twitches were 0.57 ± 0.07 and 0.57 ± 0.06 mV, respectively ($p=0.780$). (F) Change in torque
572 (39.7 ± 4.9% = PTP) and EMG M-wave amplitude (0.9 ± 2.8%) from Unpotentiated to
573 Potentiated twitches. Symbols in D, E, F represent data from individual mice (n=9). Values
574 represented by horizontal bars in D, E, and F are mean ± SD.

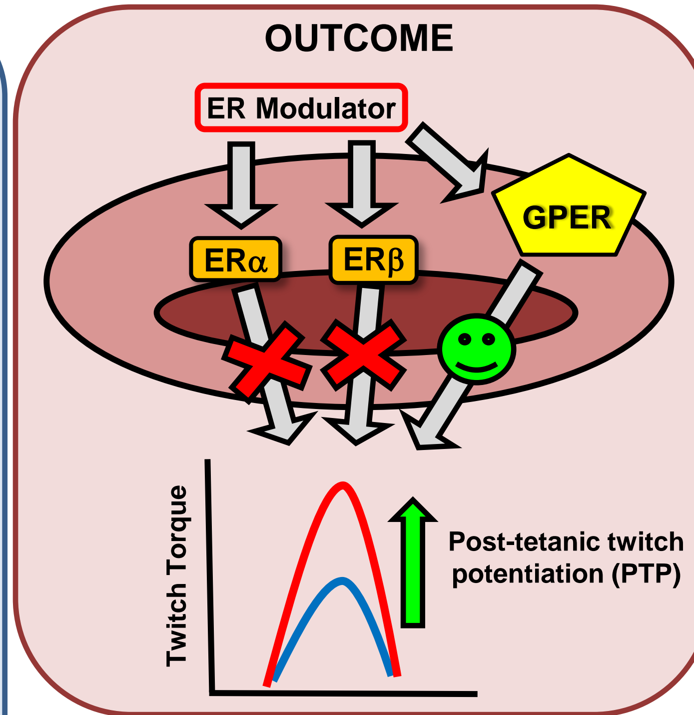
575

576 **Figure 3. *In vivo* post-tetanic potentiation (PTP) of the anterior crural muscles in response
577 to 17-β estradiol (E_2).** PTP was measured in ovary-intact (Intact) or ovariectomized (Ovx) mice
578 before and 1 h after treatment with vehicle (Veh) or E_2. Symbols connected by dashed lines
579 represent data from individual mice (n=6-11). P-values are from paired *t-tests* comparing Pre- to
580 Post-treatments. Only in Ovx mice treated with E_2 did PTP significantly increase.
581
582 **Figure 4. *In vivo* post-tetanic potentiation (PTP) of the anterior crural muscles in female
583 mice ablated for estrogen receptor α specifically in skeletal muscle (ERαKO) and wildtype
584 littermates (ERαWT).** PTP was not significantly different between these groups of mice
585 (p=0.347). Symbols represent data from individual mice (n=7-8). Values represented by
586 horizontal bars are mean ± SD.
587
588 **Figure 5. *In vivo* post-tetanic potentiation (PTP) of the anterior crural muscles in response
589 to estrogen receptor β (ERβ) antagonist and agonist.** PTP was measured in ovary-intact
590 (Intact) mice before and 1 h after treatment with vehicle (Veh) or the ERβ antagonist, PHTPP.
591 PTP was measured in ovariectomized (Ovx) mice before and 1 h after treatment with Veh or the
592 ERβ agonist, DPN. Symbols connected by dashed lines represent data from individual mice
593 (n=4-10). P-values are from paired *t-tests* comparing Pre- to Post-treatments.
594
595 **Figure 6. *In vivo* post-tetanic potentiation (PTP) of the anterior crural muscles in response
596 to G-protein estrogen receptor (GPER) antagonist and agonist.** (A) Representative
597 unpotentiated twitch force tracing from an ovariectomized (Ovx) mouse and potentiated twitch
598 force tracings before and after treatment with the GPER agonist G1. (B) PTP was measured in

599 ovary-intact (Intact) mice before and 1 h after treatment with vehicle (Veh) or the GPER
600 antagonist, G15. PTP was measured in Ovx mice before and 1 h after treatment with the GPER
601 agonist, G1. Symbols connected by dashed lines represent data from individual mice (n=7-10).
602 P-values are from paired *t-tests* comparing Pre- to Post-treatments. Only in Ovx mice treated
603 with G1 did PTP significantly increase.

In Vivo Potentiation of Muscle Torque Enhancement by Estradiol





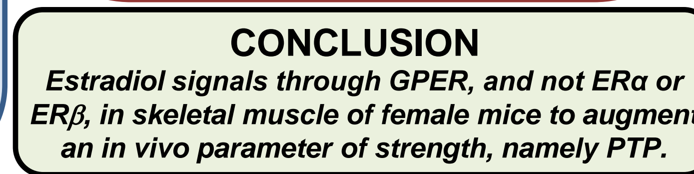


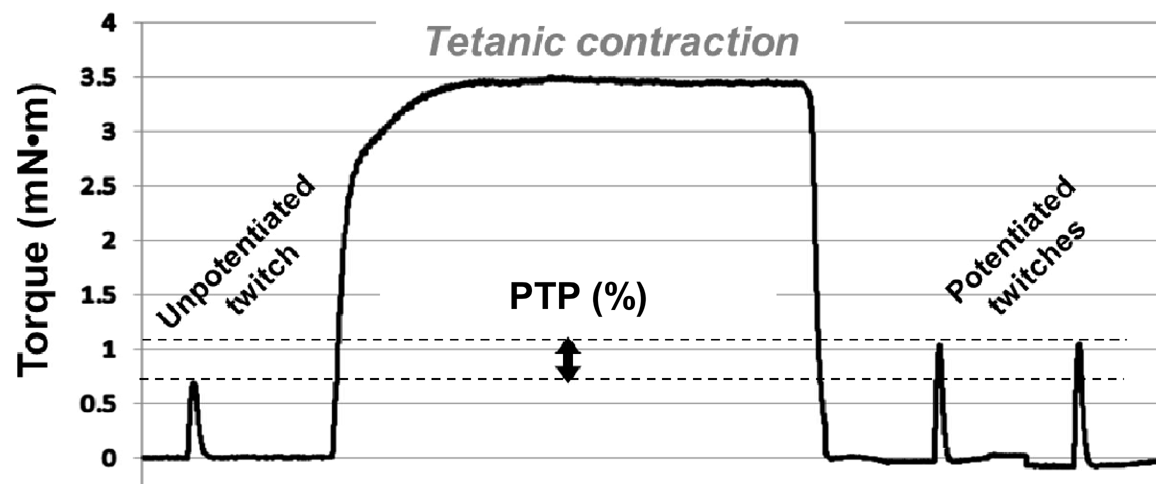
Table 1. *In vivo* physiological parameters of post-tetanic twitch potentiation (PTP) of anterior crural muscles between mice with and without ovaries, between ovariectomized mice without and with acute estradiol treatment, and between mice without and with ablation of the α estrogen receptor specifically in skeletal muscle.

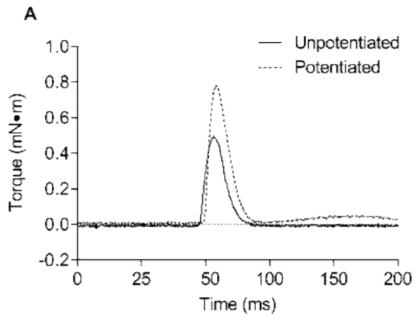
Treatment /Genotype (n)	Age (mo)	Body mass (g)	Unpotentiated twitch torque (mN·m)	Tetanic torque (mN·m)	Potentiated twitch torque (mN·m)	PTP (%)
Intact (23)	4.8 ± 0.8	24.7 ± 0.8	0.55 ± 0.06	2.14 ± 0.26	0.82 ± 0.13	51.2 ± 18.0
Ovx (14)	7.6 ± 1.2*	32.5 ± 1.1*	0.59 ± 0.16	2.30 ± 0.33	0.77 ± 0.04	41.8 ± 9.7*

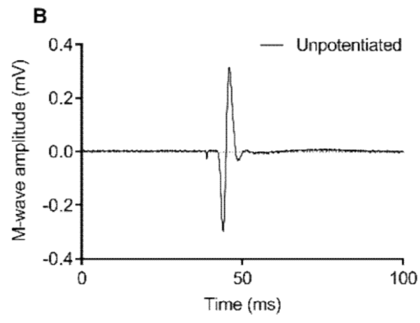
Ovx+Veh (10)	7.2 ± 0.8	32.6 ± 1.0	0.51 ± 0.13	2.38 ± 0.48	0.72 ± 0.04	34.8 ± 15.0
Ovx+E_2 (10)	7.2 ± 0.5	30.5 ± 1.8$^{\#}$	0.52 ± 0.11	2.42 ± 0.50	0.85 ± 0.05$^{\#}$	61.2 ± 19.0$^{\#}$

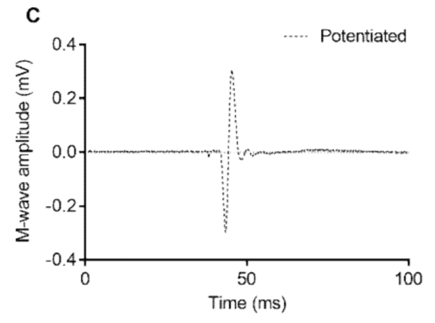
ERαWT (7)	4.5 ± 1.1	24.4 ± 0.3	0.45 ± 0.05	2.18 ± 0.29	0.75 ± 0.07	69.5 ± 6.8
ERαKO (8)	5.1 ± 1.2	23.6 ± 0.9$^{\$}$	0.41 ± 0.08	1.75 ± 0.25$^{\$}$	0.66 ± 0.09$^{\$}$	62.2 ± 18.6

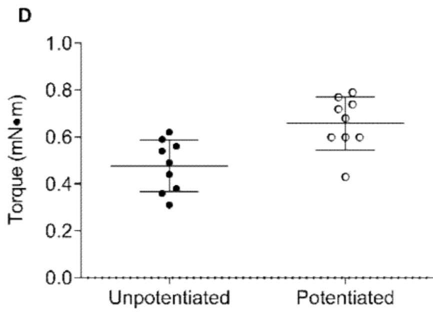
Values are mean ± SD. Intact = ovary intact mice; Ovx = ovariectomized mice; Veh = ethanol; E_2 = 17β-estradiol; ERαWT = wildtype littermate mice; ERαKO = skeletal muscle specific estrogen receptor α knock-out mice. [*]Significantly different from Intact; [#]Significantly different from Ovx+Veh; [$]Significantly different from ERαWT

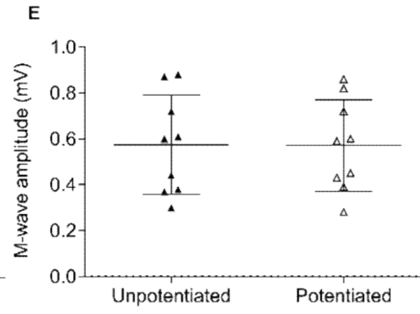

Tetanic contraction
Torque (mN•m)
4
3.5
3
2.5
2
1.5
1
0.5
0
Unpotentiated twitch
PTP (%)
Potentiated twitches

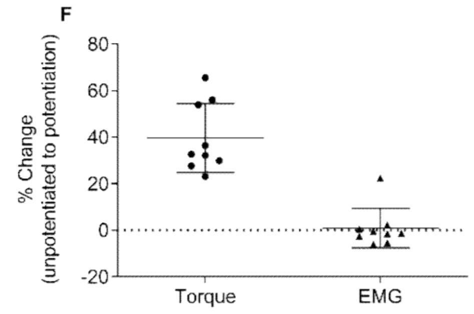

A
Unpotentiated
Potentiated
Torque (mN•m)
Time (ms)

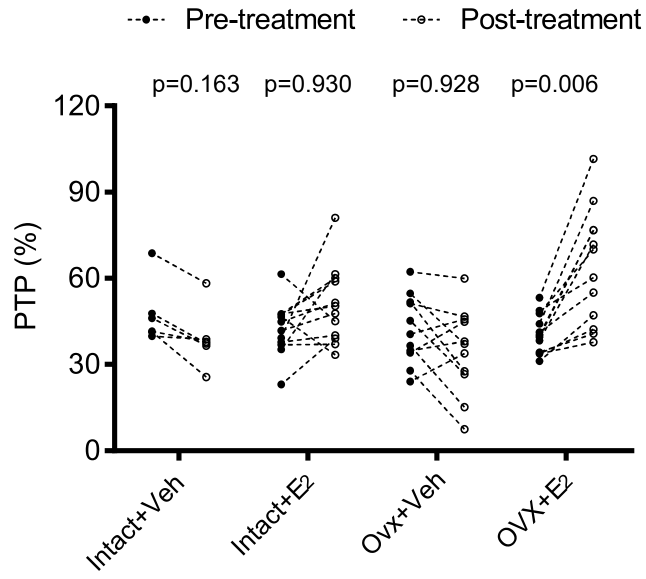

B
Unpotentiated
M-wave amplitude (mV)
Time (ms)


C
Potentiated
M-wave amplitude (mV)
Time (ms)

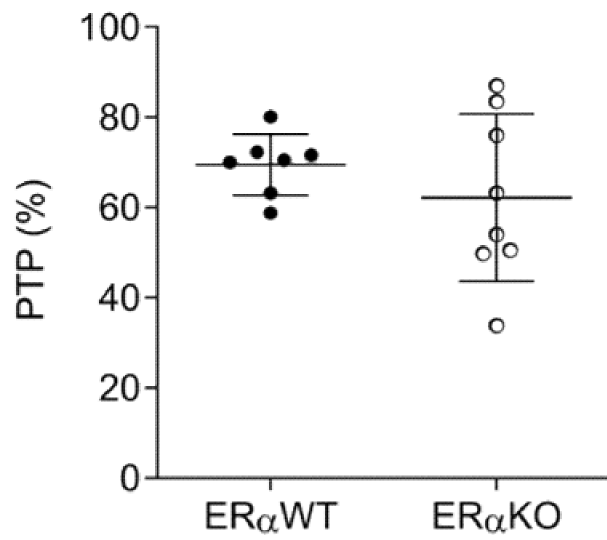

D
Torque (mN•m)
Unpotentiated
Potentiated


E
M-wave amplitude (mV)
Unpotentiated
Potentiated

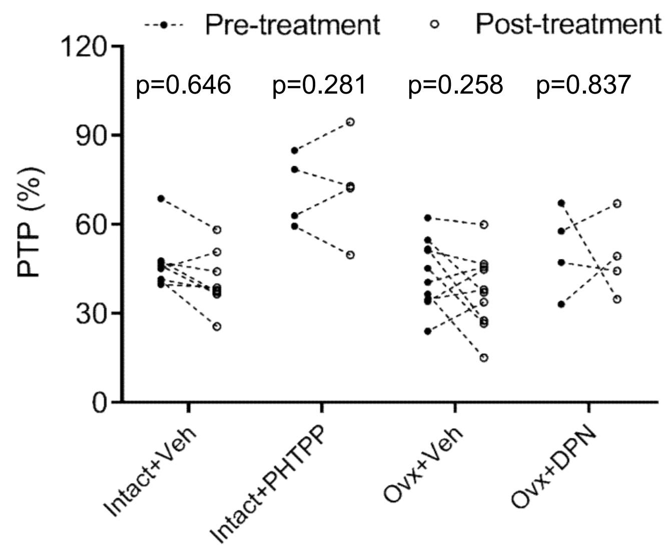

F
% Change
(unpotentiated to potentiation)
Torque
EMG



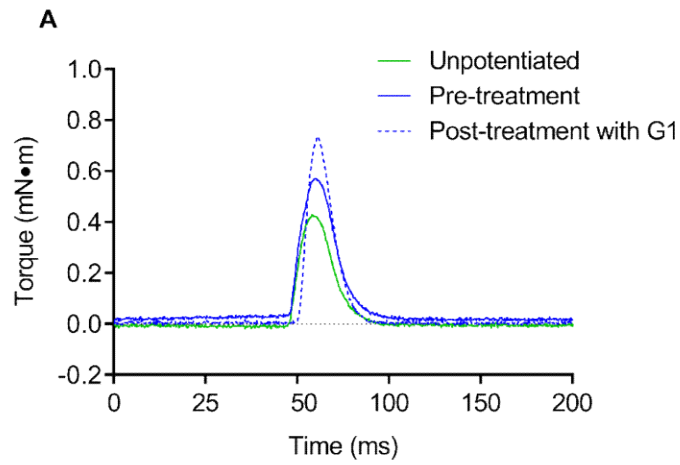
Pre-treatment
Post-treatment
p=0.163
p=0.930
p=0.928
p=0.006
120
90
60
30
0
PTP (%)
Intact+Veh
Intact+E2
Ovx+Veh
OVX+E2

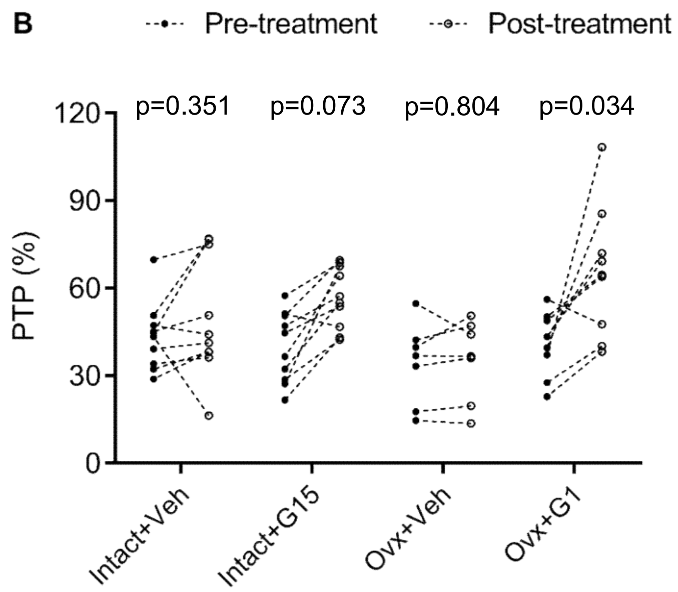


100
80
60
40
20
0
PTP (%)
ERαWT
ERαKO



Pre-treatment
Post-treatment
120
90
60
30
0
PTP (%)
p=0.646
p=0.281
p=0.258
p=0.837
Intact+Veh
Intact+PHTPP
Ovx+Veh
Ovx+DPN


A
Unpotentiated
Pre-treatment
Post-treatment with G1
Torque (mN•m)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0
25
50
100
150
200
Time (ms)


B
Pre-treatment
Post-treatment
p=0.351
p=0.073
p=0.804
p=0.034
PTP (%)
120
90
60
30
0
Intact+Veh
Intact+G15
Ovx+Veh
Ovx+G1