Original Research

8-prenylnaringenin promotes recovery from immobilization-induced disuse muscle atrophy through activation of the Akt phosphorylation pathway in mice

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Running head: 8-PN promotes recovery from muscle atrophy *via* Akt pathway

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ABSTRACT

8-Prenylnaringenin (8-PN) is a prenylflavonoid that originates from hop extracts and is thought to help prevent disuse muscle atrophy. We hypothesized that 8-PN affects muscle plasticity by promoting muscle recovery under disuse muscle atrophy. To test the promoting effect of 8-PN on muscle recovery, we administered an 8-PN mixed diet to mice that had been immobilized with a cast to one leg for 14 days. Intake of the 8-PN mixed diet accelerated recovery from muscle atrophy, and prevented reductions in Akt phosphorylation. Studies on cell cultures of mouse myotubes *in vitro* demonstrated that 8-PN activated the PI3K/Akt/P70S6K1 pathway at physiologic concentrations. A cell-culture study using an inhibitor of estrogen receptors and an *in vivo* experiment with ovariectomized mice suggested that the estrogenic activity of 8-PN contributed to recovery from disuse muscle atrophy through activation of an Akt phosphorylation pathway. These data strongly suggest that 8-PN is a naturally occurring compound that could be used as a nutritional supplement to aid recovery from disuse muscle atrophy.

Keywords: Disuse muscle atrophy; 8-Prenylnaringenin; IGF1/PI3K/Akt pathway; phosphorylation; nutritional supplement

INTRODUCTION

Skeletal muscle comprises approximately 40% of the weight of the human body, and is crucial for locomotion, amino acid and glucose metabolism, heat generation, and bone homeostasis (7, 21). Therefore, maintenance of muscle mass has a crucial role in human health.

Muscle unloading because of immobilization, bedridden status, or space flight travel activates protein-degradation pathways such as the ubiquitin-proteasome system, and results in disuse muscle atrophy (DMA) (27, 35). When muscles are released from the conditions responsible for DMA, protein synthesis in skeletal muscle is promoted for recovery of the normal state.

It has been demonstrated that insulin-like growth factor (IGF)-1 enhances phosphorylation of phosphatidylinositol-3 kinase (PI3K), which in turn leads to Akt phosphorylation (4, 34). Phosphorylated Akt represses nuclear translocation of the transcriptional factor forkhead box protein O (Foxo), which is responsible for induction of DMA-related genes such as atrogin-1 in the ubiquitin-proteasome system (36). Phosphorylated Akt also activates the kinase mechanistic target of rapamycin (mTOR), whose downstream targets, 70-kDa ribosomal protein S6 kinase (P70S6K) and eukaryotic inhibition factor 4E-binding protein (4E-BP1), have been shown to activate protein synthesis in skeletal muscle (2, 14). In addition, mTOR signaling is enhanced in

response to the protein kinases ERK1/2 and P90RSK (33, 48). Therefore, it is assumed that activation of these pathways is responsible for recovery from DMA through the promotion of protein synthesis in skeletal muscle (4, 11). In particular, Akt phosphorylation acts as a principal "switching regulator" in the balance between synthesis and degradation of proteins (4, 39). It has been demonstrated that Akt activation is responsible for muscle hypertrophy in transgenic mice in which active Akt is expressed inductively (19). Furthermore, muscle hypertrophy is correlated with phosphorylation of P70S6K1 or 4E-BP1 (2, 48). Exercise and/or amino acid supplementation can activate these signaling pathways to promote protein synthesis in skeletal muscle (12, 18, 28, 48).

8-Prenylnaringenin (8-PN: Fig. 1) is one of the polyphenols found in *Humulus lupulus* L (hop) (41) and brewing products that use hop extracts. 8-PN is considered to be a "phytoestrogen" because it can bind to estrogen receptors (ERs) in a similar manner to soy isoflavones (32). Recently, we found that pre-feeding with 8-PN suppressed loss of muscle mass in denervated mice lacking locomotive activity (24). In that study, 8-PN accumulated in the gastrocnemius muscle (GM) at 2.66–6.44 nmol/g tissue, and caused inhibition of the protein degradation-inducing ubiquitin-proteasome system *via* Akt phosphorylation. Akt phosphorylation was also observed during protein synthesis, suggesting that 8-PN could

promote muscle recovery from DMA. However, no studies have shown that polyphenols, including 8-PN, can improve muscle recovery.

In the present study, we aimed to assess the efficacy of 8-PN for recovery from DMA. Thus, supplementation with an 8-PN-containing diet was started from the recovery phase in mice already suffering from DMA (induced by cast immobilization of a hind limb). At the start of the recovery phase, the cast was removed for release from immobilization, and an 8-PN mixed diet was administered during the recovery phase. We also aimed to determine the effect of 8-PN on the protein-synthesis pathway in muscle tissue *in vivo* and *in vitro* in a muscle cell model (mouse C2C12 myotubes).

MATERIALS AND METHODS

Ethical approval of the study protocol

All experimental protocols were designed in accordance with the guidelines for the care and use of laboratory animals set by the Graduate School of the Institute of Biomedical Sciences, Tokushima University (Tokushima, Japan) and approved by the Committee on Animal Experiments of Tokushima University. To minimize animal suffering, surgery was undertaken under anesthesia using sodium pentobarbital.

Materials

8-PN was synthesized as described in our previous report (16), and its purity was >95%. Information about all other reagents used in this study is provided below.

Animal experiment I: Estimation of loss and recovery of muscle weight by ankle fixation

Seven-week-old male C57/BL6 mice (Japan SLC, Shizuoka, Japan) were housed in a room maintained at 23±1°C on a 12-h/12-h light/dark cycle. The mice had free access to a commercial diet (AIN-93M; Oriental Yeast Company, Tokyo, Japan) and water.

The experimental schedule is shown in Fig. 1. We set an instrument designed to fix the ankle of

114 the right limb of each mouse at the dorsiflexion position in a tube (immobilized condition:
115 “Im”). The tube was used as a cast. To prepare control muscles, the left hind limb remained free
116 from the tube cast (normal condition: “Nom”). The duration of ankle immobilization was 14
117 days.
118
119 At day 14, the recovery period was started by removing the tube cast from the right ankle
120 (reloading: “RL”) and the mice were placed under normal breeding conditions for 20 days (until
121 day 34). Sacrifice was carried at day 14 and 34, and the GM and tibialis anterior muscle (TA)
122 were collected. The extent of reduction in muscle mass was assessed by the ratio of the weight
123 of atrophied muscle (cast side) to the weight of control muscle in each mouse.
124
125 **Experiment II: Effect of 8-PN on recovery from DMA**
126 Experiment II was performed using the same procedures for experiment I described above and
127 shown in Fig. 1. Male C57/BL6 mice or female C57/BL6 mice at 2 weeks after ovariectomy
128 were used. The tube cast was set to the right ankle of each mouse for 14 days. At day 14, the
129 tube cast was released, and the mice were randomly separated into two groups of six. The mice
130 were then supplied with the AIN-93M diet (control group) or an 8-PN (0.05% *w/w*: male mice;
131 0.0005% or 0.00005% *w/w*: female mice) mixed AIN-93M diet (0.05%: PN group; 0.0005%:

132 H-PN group; 0.00005%: L-PN34 group). In the 8-PN mixed diet, the cellulose content was
133 reduced to adjust the composition of the other nutrients. Pellets releasing 17β-estradiol (0.18
134 mg/day) (SE-121; Innovative Research of America, Sarasota, FL, USA) were implanted
135 subcutaneously into the estrogen group in ovariectomized mice. The TA weight was measured at
136 the end of the feeding experiment (day 34). The level of recovery of muscle mass was
137 calculated as the ratio of the weight of recovered muscle (cast side) to the weight of control
138 muscle in each mouse. Samples of tissue and plasma were stored at −80°C until western blotting
139 analysis (24).
140
141 **HPLC analyses**
142 HPLC analyses were carried out according to our previous report (24). Briefly, plasma (10 μL)
143 was incubated with 100 U of β-glucuronidase type H-1 (possessing both β-glucuronidase
144 activity and sulfatase activity) in 0.1 M sodium acetate buffer (pH 5.0; 90 μL) and 50 mM
145 ascorbic acid (20 μL) for 45 min. 8-PN was extracted with ethyl acetate and evaporated using a
146 centrifugal evaporator. The sample was injected into an HPLC–UV detection system
147 (SPD-10AV; Shimadzu, Tokyo, Japan) equipped with a TSK-gel ODS-80Ts HPLC column (150
148 × 4.6 mm; Tosoh, Tokyo, Japan) using a λ_{max} value of 292 nm. In the mobile phase, solvent A
149 was 0.5% phosphoric acid and solvent B was methanol containing 0.5% phosphoric acid. For

8-PN detection, solvent B was 65%. The flow rate was 1.0 mL/min.

Cell culture and sample collection for western blotting analysis

As described in our previous study (24), the mouse myoblast cell line C2C12 (American Type Culture Collection, Manassas, VA, USA) was obtained and its differentiation was initiated with 2% horse serum-containing DMEM for 96 h. The differentiated cells were then desensitized with 0.1% BSA-containing DMEM for 16 h, exposed to 8-PN (0.1–10 μM) or vehicle (0.1% DMSO) for 0.25, 1, or 4 h, and washed with ice-cold HBSS. An ER inhibitor (fulvestrant; AdooQ BioScience, Irvine, CA, USA) at 50 μM was added to the cells at 10 min before PN treatment. The final concentration of DMSO in the medium was set at 0.1%. Cell lysates were collected with 100 μL of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, protease inhibitor (Complete EDTA-free; Roche, Basel, Switzerland), phosphatase inhibitor (PhosSTOP; Roche)). The protein content of each lysate was measured by the Bradford assay (5). Samples were stored at −80°C until western blotting analysis.

Western blotting

Western blotting analyses were carried out as described in our previous study (24). Briefly, samples were separated by 10% SDS–PAGE, and transferred onto polyvinylidene difluoride

membranes (GE Healthcare, Piscataway, NJ, USA). The membranes were then incubated with commercial blocking buffer (Blocking-One P (for phosphorylated proteins) or Blocking-One (for other proteins); Nacalai Tesque, Kyoto, Japan) for 1 h to block non-specific binding. After detection of total protein by Ponceau S staining (0.1% *w/w* Ponceau S in 5% acetic acid aq.), the membranes were incubated for 1 h at room temperature with the following primary antibodies: anti-Akt (pan) (11E7) rabbit antibody, anti-PI3K antibody, anti-phospho-PI3K p85 (Tyr458)/p55 (Tyr199) antibody, anti-phospho-P70S6K (Thr389) (1A5) mouse antibody, anti-GAPDH rabbit mAb, anti-pERK rabbit antibody, anti-ERK42/44 antibody, anti-p4E-BP1 Thr37/46 antibody, anti-p90RSK1 Thr359/Ser363 antibody (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA), anti-phospho-Akt (Ser473) (193H12) rabbit mAb, anti-P90RSK1/2/3 antibody (1:500 dilution; Cell Signaling Technology), or anti-P70S6K1 antibody (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing with TBS containing 0.05% Tween-20 (TBST), the membranes were incubated with peroxidase-labeled secondary antibodies for 1 h. After three washes with TBST, the immunocomplexes were visualized with a chemiluminescence detection kit (ECL prime; GE Healthcare) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Phosphorylated proteins (except for 4E-BP1) were normalized by the total amount of each protein.

186 **Statistical analyses**

187 Data are shown as means ± SE. The data shown in Figs. 3C, 3D, 5, and 6 were analyzed by

188 one-way ANOVA with the Tukey multiple comparison test ($P < 0.05$). The data shown in Figs. 2,

189 3B, and 4 were analyzed by a two-sided Student's *t*-test ($P < 0.05$).

190

191 **RESULTS**

192 **Change in muscle mass in mice with fixation of the right ankle at maximum dorsiflexion**

193 To observe the recovery of muscle mass from immobilization, we developed a new model of

194 DMA by fixing the right ankle at maximum dorsiflexion in a tube cast. The TA of the leg

195 immobilized with the tube cast for 14 days decreased in weight to 66.7% of the initial weight,

196 while the mass of the GM was maintained at 103% (Fig. 2). Over a period of 20 days after

197 reloading (34 days from start of fixation), the weight of the TA increased significantly to reach

198 82.8% of the initial weight ($P = 0.026$). Therefore, we confirmed that our newly developed

199 method was suitable for estimating recovery of muscle mass from DMA.

200

201 **Promotion of recovery of muscle weight after DMA by intake of an 8-PN mixed diet**

202 To estimate the effect of 8-PN on the recovery from DMA induced by cast immobilization, an

203 8-PN mixed diet was administered to mice during the period of reloading (from release of

204 immobilization at day 14). Food intake during the entire experiment and body weight at the
205 final point of dissection did not vary between the control group and the 8-PN mixed-diet group
206 (Fig. 3A, B). At 20 days after reloading (day 34), the control group (RL-C) and 8-PN group
207 (RL-PN) had recovered from DMA. The 8-PN mixed-diet group showed significantly higher
208 muscle weight than the control group (Fig. 3C; ANOVA $P = 4.58 \times 10^{-9}$), suggesting that 8-PN
209 promoted muscle recovery after release from immobilization by the tube cast. The plasma IGF-1
210 concentration in the PN group at day 34 was significantly higher than that in the control group
211 (Fig. 3D; ANOVA $P = 9.68 \times 10^{-11}$).
212
213 **Effect of 8-PN on a protein synthesis-related pathway in the TA**
214 Among the signaling pathways responsible for muscle synthesis upon 8-PN intake, we first
215 detected changes in Akt phosphorylation (Fig. 4). At day 14, immobilization significantly
216 attenuated Akt phosphorylation ($P = 0.004$). At day 34, higher Akt phosphorylation was
217 observed in the normal leg (Nom-C: without immobilization with the control diet for 34 days),
218 while the reloaded leg (RL-C: 14 days of immobilization and 20 days of reloading with the
219 control diet) showed significantly lower phosphorylation of Akt ($P = 0.032$). In contrast, no
220 reduction in Akt phosphorylation was observed in the PN group. RL-C ($P = 0.045$) and RL-PN
221 ($P = 0.012$) showed higher Akt phosphorylation compared with Im-C. Phosphorylation of

222 mTOR (p-mTOR) in RL-C was significantly higher than that in Im-C (P = 0.019).
223 Phosphorylation of mTOR was also increased in RL-PN compared with Im-C, but a significant
224 difference was not found. In addition, the level of p4E-BP1 was increased in RL-PN compared
225 with Im-C. P70S6K1 in RL-PN showed slightly higher phosphorylation compared with Im-C
226 (Fig. 4). These data suggested that immobilization attenuated signaling pathways that included
227 phosphorylation of Akt and 4E-BP1. Phosphorylation of mTOR and P70S6K1 was increased in
228 the recovery phase, and 8-PN accelerated muscle recovery by changing the phosphorylation of
229 Akt, 4E-BP1, mTOR, and P70S6K1 during reloading. No changes were observed in total
230 protein between the interventions (data not shown). Among the above four targets, pAkt and
231 pP70S6K showed different trends compared with the control diet (at day 34), although the
232 results of this animal experiment could not explain the effects of 8-PN on the phosphorylation
233 of these proteins *in vivo*. Thus, we carried out additional cell-culture experiments to clarify the
234 influence of 8-PN on the phosphorylation of the proteins responsible for muscle recovery.
235
236 **Activation of the PI3K/Akt signaling pathway by 8-PN in mouse myotubes**
237 The plasma 8-PN concentrations in 8-PN-fed mice were determined to ascertain the 8-PN
238 concentration applied for the experiments on C2C12 myotubes. The concentrations were found
239 to be 1.19 ± 0.18 μM at day 34 in the 8-PN-fed mice. We detected changes in phosphorylation

240 of Akt, P70S6K1, and PI3K in C2C12 myotubes. 8-PN increased Akt phosphorylation without
241 changing the total level of Akt (non-phosphorylated and phosphorylated Akt) in C2C12
242 myotubes (Fig. 5). Treatment with 8-PN for 1 h elicited significant activation of Akt
243 phosphorylation (ANOVA $P = 0.003$). Phosphorylation of PI3K, Akt, and P70S6K1 was
244 increased upon 8-PN treatment without changes in the total amounts of these proteins, or those
245 of GAPDH (Fig. 6). 8-PN (0.1 or 1 μM) activated PI3K phosphorylation, while P70S6K1 (0.1
246 or 1 μM) and 8-PN (1 or 10 μM) increased Akt phosphorylation.
247
248 **Involvement of estrogenic activity in the contribution of 8-PN to recovery of muscle mass**
249 Fulvestrant is an ER inhibitor. Fulvestrant suppressed 8-PN-induced phosphorylation of Akt and
250 P70S6K1 in C2C12 cells (Fig. 7). In ovariectomized mice, 8-PN promoted recovery of muscle
251 mass in the H-PN34 group (0.0005% 8-PN group at day34) in comparison with the C34 group
252 (control-diet group at day34) (Fig. 8). Administration of 17β-estradiol also enhanced recovery
253 of muscle mass, because the muscle mass in the E34 group (Estrogen tablet group at day 34)
254 was significantly higher than that in the C34 group. Therefore, it is likely that the estrogenic
255 activity of 8-PN is involved in its promotion of recovery of muscle mass from an atrophied
256 condition.
257

DISCUSSION

Muscle atrophy is caused by immobilization, space flight, or aging. Food components that can promote muscle recovery are undoubtedly helpful for human health. The present study demonstrated that 8-PN derived from foods and beverages can promote recovery of muscle mass after DMA.

We developed an appropriate model to estimate muscle recovery. Experiments involving suspension or denervation of rodent tails have generally been used as models of DMA. However, access to a diet and water during tail suspension is difficult, and neurologic disorders can occur in denervated animals. These conditions hinder estimations of the effects of diet or drugs on DMA. In humans, immobilization with a cast can reflect the realities of DMA, but it is difficult to create the same extent of fixation throughout experiments. The method developed in the present study can be used to estimate how an agent promotes protein synthesis under DMA in mice. We fixed the right ankle of each mouse at maximum dorsiflexion with a tube, which acted as a cast. This technology is currently awaiting a patent (number #2015-074324). The region of atrophied muscle is dependent on the position and degree of flexion (9). We fixed the ankle of the mice at maximum dorsiflexion in the tube cast. This position of fixation induced muscle loss not in the posterior region of the leg (GM), but in the anterior region of the leg (TA). Normal

276 mobilization (a fundamental physical condition for protein synthesis) of the hind limb was
277 observed at 2 days after reloading. The mass of the TA of the atrophied leg was increased at 20
278 days after reloading (Fig. 2), suggesting the promotion of protein synthesis upon physical
279 activity. Our method for targeting promotion of the recovery of muscle mass by release of
280 cast-based immobilization appears able to accurately reflect human rehabilitation after cast
281 immobilization, bed rest, or space flight. Therefore, this model can be suitable for estimating
282 muscle recovery after atrophy. It should be noted that the phosphorylation of mTOR, ERK, and
283 P70S6K1 in Nom-C at 34 days was unexpectedly higher than that in Nom-C at 14 days. The leg
284 without a cast at 34 days would be affected by mobilization signals occurring in RL-C, because
285 the exercise (mobilization) condition affects the protein synthesis pathway through the secretion
286 of hormonal factors (8, 30). However, comparisons between a leg with a cast and a leg without a
287 cast in the same body can avoid the influences of nutrient intake, and this model therefore
288 appears suitable for estimating the effect of 8-PN on muscle recovery from DMA.
289
290 Epigallocatechin gallate and resveratrol applied to tail-suspended mice have been shown to
291 regulate muscle mass by suppressing an apoptotic pathway and activating the proliferation of
292 satellite cells (1, 3). In those studies, epigallocatechin gallate and resveratrol affected the
293 degradation and synthesis of muscle because supplementation was started at the initial point of

294 suspension (1, 3). Indeed, it has also been demonstrated that these polyphenols downregulate
295 the protein-degradation pathway during the development of muscle atrophy (13, 46, 47). Here,
296 we focused on the specific effect of 8-PN on muscle during the recovery phase. 8-PN
297 supplementation was started at the beginning of the recovery (reloading) period. This is the first
298 report showing that a natural polyphenol can elicit muscle recovery after DMA onset. The total
299 intake of the diet was identical among the groups (Fig. 3A), meaning that no nutrients other than
300 8-PN affected the muscle recovery differently between the control-diet group and the 8-PN
301 group. The plasma concentration of 8-PN reached >1 μM in our study, and muscular
302 accumulation of 8-PN at 2.66–6.44 nmol/g tissue was observed in our previous study (24).
303 Intake of an 8-PN diet during the recovery period appeared to enable accumulation of 8-PN in
304 the TA, and revealed a promotive effect. 8-PN "lodges" in myotubes through its prenyl-group,
305 and is not excreted from myotubes *via* ATP-binding cassette transporters (24). Prenylated
306 quercetin also accumulates in tissue (including skeletal muscle) after its continuous intake (23,
307 42). Thus, prenylation of flavonoids is advantageous for accumulation in skeletal muscle. In
308 addition, greater amounts of 8-PN compared with non-prenylated molecules were found to
309 accumulate in skeletal muscle *in vivo* and activate Akt phosphorylation (24). The major
310 polyphenol catechin can activate PI3K phosphorylation in myotubes, and its effect is enhanced
311 by acylation (43). These observations may suggest that modification of polyphenols by a

312 hydrophobic functional group (e.g., prenylation) can enhance their biologic effects in skeletal
313 muscle.
314
315 8-PN promoted recovery of muscle mass even though the food intake and weight increase were
316 identical between the 8-PN-diet group and the control-diet group (Fig. 3). Thus, the
317 phenomenon induced by 8-PN was not derived from the balance of nutrient intake and/or
318 expenditure of total energy, but instead arose through changes in the synthesis and/or
319 breakdown of muscle. IGF-1 secretion was activated by 8-PN (Fig. 3), although IGF-1 signaling
320 was lost during DMA because unloading stress triggers IRS-1 degradation (26). Therefore, 8-PN
321 exerted its effect on skeletal muscle directly. pAkt, pmTOR, pP70S6K1, and p4E-BP1 were
322 increased in the RL-C muscle (Fig. 4). These phosphorylations are activated by exercise (10,
323 22), meaning that reloading trigged this signaling pathway. In the 8-PN group, these proteins
324 were also activated during reloading, but their activation was similar to that in the control-diet
325 group. Amino acids such as leucine activate protein-signaling pathways to synthesize proteins in
326 normal muscle (17), while 8-PN (or other polyphenols) that suppress muscle atrophy do not
327 increase muscle mass under normal conditions (24, 25). Ascertaining the additional effect of
328 8-PN on normal mobility (reloading) when the TA has almost reached a plateau in its normal
329 muscle mass would be difficult. In contrast, 8-PN enhances Akt phosphorylation in acutely

330 atrophied muscle (24). These findings imply that 8-PN could accelerate these signaling
331 pathways during recovery at an early stage. The Akt phosphorylation pathway plays an
332 important part in protein synthesis during recovery from DMA (4, 39). A reduction in the level
333 of Akt phosphorylation in the TA with a cast led to acceleration of protein degradation by
334 immobilization (Fig. 4). Akt phosphorylation was relatively stable in the 8-PN group at day 34
335 because the TA had almost reached a plateau of protein synthesis. The extent of Akt
336 phosphorylation in this group was sufficient to maintain the muscle mass because the level was
337 similar to that in Nom-C at day 14. P70S6K1 phosphorylation is significantly correlated with
338 muscle recovery from atrophy (6). The phosphorylation status of P70S6K1 in the 8-PN group
339 tended to be different from that in the control group. This phosphorylation state was responsible,
340 at least partly, for the protein synthesis in the RL-PN group. The phosphorylation detected in
341 our study could not have occurred simultaneously. Time-course experiments are required to
342 assess the time-dependent changes in the phosphorylation of each signaling molecule.
343
344 To estimate the effect of 8-PN at physiologic concentrations, we first measured the mean plasma
345 concentration in test mice and determined it to be 1.19 μM. Rad *et al.* (29) showed that 8-PN
346 circulates at ≤0.1 μM in human plasma after single ingestion of 8-PN at 750 mg. A plasma
347 concentration of >1 μM 8-PN was detected at 24 h after a single ingestion of 8-PN in a rodent

study (24). That rodent study suggested that the presence of an active aglycone, together with its conjugated metabolites, in the circulation after oral ingestion of 8-PN (24). Thus, we applied an aglycone of 8-PN (0.1–10 μM) to cultured cells. 8-PN at these concentrations was able to activate PI3K/Akt/P70S6K1 phosphorylation within 1 h (Fig. 6). The maximum cellular accumulation of 8-PN in C2C12 cells was accomplished within 1 h, and its accumulation was maintained until 24 h (24). These data suggest that 8-PN accumulated in muscle cells activates a pPI3K/Akt/P70S6K1 pathway. Promotion of a protein-synthesis signaling pathway involving phosphorylation of Akt followed by phosphorylation of P70S6K1 is activated by amino-acid supplementation and/or resistance exercise (15, 24, 37). 8-PN may boost the promoting effects of nutrients and exercise on protein synthesis in skeletal muscle.

8-PN is a phytoestrogen *in vitro* and *in vivo* (29, 32). In ovariectomized rats, 8-PN was shown to have similar effects to estradiol (31). It has been reported that estrogen can affect the maintenance of skeletal muscle regardless of sex (38, 40). ERβ activation leads to the growth and regeneration of muscle (45), and 8-PN can bind to ERβ (32). 17β-estradiol activates PI3K/Akt phosphorylation *via* ERs in murine myotubes (44), and increases the number of satellite cells in muscle after exercise *via* PI3K phosphorylation (but not IGF-1 secretion) (20). Fulvestrant diminished the effects of 8-PN (i.e., enhancement of Akt and P70S6K1

366 phosphorylation (Fig. 7)). In addition, the increased muscle mass after 8-PN consumption was
367 reproduced by administration of 17β-estradiol (Fig. 8). Thus, PI3K phosphorylation is possibly
368 triggered by binding of 8-PN to ERβ. The mechanism of action for 8-PN-dependent acceleration
369 of muscle recovery from DMA may be related to its estrogenic activity.
370
371 Our data confirmed that 8-PN accelerates muscle recovery after DMA onset. Furthermore, 8-PN
372 was found to activate the phosphorylation of Akt, 4E-BP1, and P70S6K1 during reloading in
373 mice. At physiologic concentrations, 8-PN activated the phosphorylation of PI3K, Akt, and
374 P70S6K1 in myotube cells, and this 8-PN-dependent activation of phosphorylation seemed to be
375 associated with its estrogenic activity. As basic nutritional supplementation and exercise are
376 critical for muscle recovery, taking 8-PN as a supplement could enhance the effects of these
377 factors during rehabilitation after DMA. The effect of 8-PN on muscle recovery could provide
378 reference information for developing supplements supporting muscle maintenance.
379
380 ***Perspectives and Significance***
381 This is the first study to demonstrate the promoting effect of a dietary flavonoid on recovery
382 from disuse muscle atrophy in rodents. A cure for atrophy of skeletal muscle can help toward
383 improvement of quality of life by suppression of metabolic disorders. The effect is different

from that of glucocorticoids (which strengthen anabolism of skeletal muscle), because the 8-prenylnaringenin (8-PN) used in this study only exerted a promoting effect under the atrophied condition. The present study provides: (i) basic information on the development of nutritional therapy by flavonoids; and (ii) a candidate that can boost the effect of rehabilitation of skeletal muscle. Flavonoids other than 8-PN that possess estrogenic activity may also exert a similar effect, because the promoting effect of 8-PN appeared to be associated with its estrogenic activity. 8-PN is a prenylated flavonoid. Prenylation may improve the effect of natural products (including flavonoids) under development for the treatment of muscle atrophy. Demonstration of a synergistic effect between 8-PN supplementation and exercise or amino-acid supplementation is crucial to establish utility in clinical practice.

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402

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Figure legends

Figure 1. (A) Structure of 8-PN. (B) Experimental schedule: day 0 to day 14 was the atrophy-development period; day 14 to day 34 was the recovery period. To induce atrophy, the right hind leg of each mouse was immobilized with a tube cast, and the left hind leg remained free from the tube cast. At day 14, the tube cast was removed from the right leg. At day 14 and day 34, muscles were collected to estimate the level of atrophy.

Figure 2. Muscle atrophy and recovery from DMA in the right hind leg of mice. DMA was induced by immobilization using a tube cast (see Fig. 1). The muscle masses after 14 days of immobilization (Im) and 20 days of reloading (RL) are shown in the GM (A) and TA (B). These levels were calculated using the ratio of atrophied muscle (with cast) to normal muscle (without cast). Data are means ± SE ($n = 4$). *$P < 0.05$, significant difference compared with Im.

566

567 Figure 3. Effect of intake of an 8-PN mixed diet on recovery of muscle mass from DMA. The

568 immobilization with the cast lasted for 14 days, and the mice were then released from the cast.

569 Recovery was estimated at day 34. (A) Mean intake of food per day in all experiments. (B)

570 Body weight at the end of the experiment (day 34). (C) Muscle mass in the TA. These levels

571 were calculated using the ratio of atrophied muscle (with cast) to normal muscle (without cast).

572 (D) Plasma concentration of IGF-1. Im-C: at day 14; RL-C: at day 34 (after recovery from

573 immobilization) with the control diet; RL-PN: at day 34 (after recovery from immobilization)

574 with the 8-PN mixed diet. Different letters represent significant differences ($P < 0.05$) by

575 one-way ANOVA with the Tukey multiple comparison test.

576

577 Figure 4. Effect of an 8-PN mixed diet on a protein-synthesis pathway in the TA. The

578 phosphorylation of target proteins was evaluated by western blotting. (A) Typical images of

579 western blotting. (B) Density analysis for the phosphorylation level within each total protein.

580 Data are means ± SE. *$P < 0.05$, significant difference between Nom and another group. $^{\dagger}P <$

581 0.05, significant difference between Im-C and RL. Nom: without tube cast; Im: with tube cast;

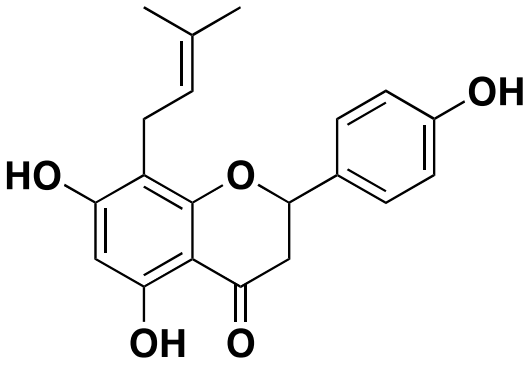
582 RL: reloading; C: control diet; PN: 8-PN diet.

583

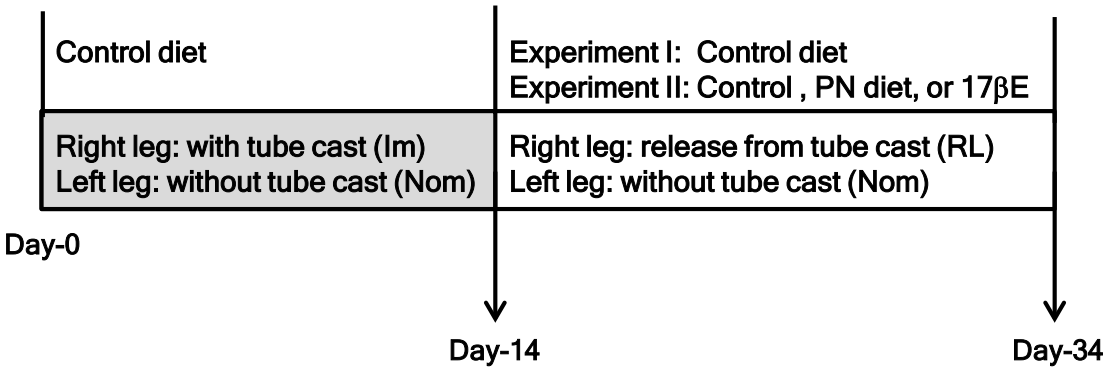
584 Figure 5. Enhancement of Akt phosphorylation by 8-PN in C2C12 myotubes. Murine C2C12
585 myotubes were treated with 8-PN (1 μM) for 0.25–4 h. Phosphorylated Akt was determined by
586 western blotting. (A) Typical images of western blotting. (B) Band densities for phosphorylated
587 Akt. Data are means ± SE (n = 3). Different letters represent significant differences ($P < 0.05$)
588 by ANOVA with the Tukey multiple comparison test.
589
590 Figure 6. Effects of 8-PN on phosphorylation of PI3K, Akt, and P70S6K1 in murine C2C12
591 myotubes. (A) Typical images of western blotting. (B–D) Band densities for phosphorylated
592 PI3K (B), phosphorylated Akt (C), and phosphorylated P70S6K1 (D). Data are means ± SE (n =
593 3). Different letters represent significant differences ($P < 0.05$) by ANOVA with the Tukey
594 multiple comparison test.
595
596 Figure 7. Contribution of estrogenic activity of 8-PN to Akt/P70S6K phosphorylation. Murine
597 C2C12 myotubes were treated with 8-PN (1 μM) for 1 h in the presence of an ER inhibitor
598 (fulvestrant). (A) Typical images of western blotting. (B) Band densities for phosphorylated
599 proteins. Data are means ± SE (n = 3). Different letters represent significant differences ($P <$
600 0.05) by ANOVA with the Tukey multiple comparison test.
601

602 Figure 8. Effects of ovariectomy and estradiol supplementation on muscle mass of atrophied
603 mice. An 8-PN (0.0005% *w/w*: H-PN group; 0.00005% *w/w*: L-PN group) mixed diet or pellets
604 releasing 17β-estradiol (E) were supplied to mice. The muscle mass in the TA was calculated
605 using the ratio of atrophied muscle (with cast) to normal muscle (without cast). Different letters
606 represent significant differences ($P < 0.05$) by ANOVA with the Tukey multiple comparison
607 test.

A

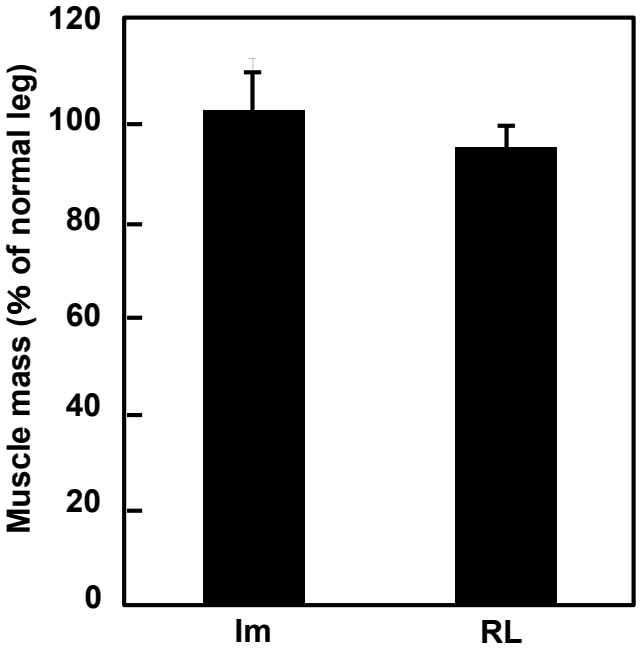

HO
O
OH
OH
O

B

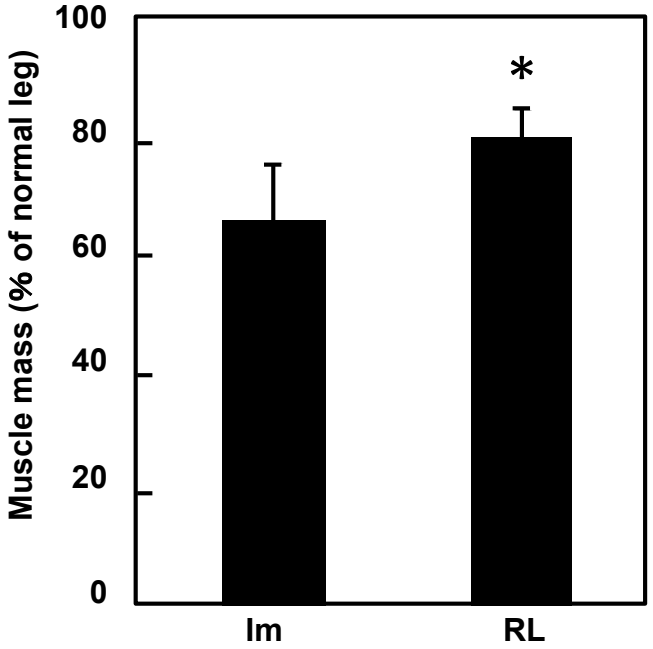

Control diet
Experiment I: Control diet
Experiment II: Control , PN diet, or 17βE
Right leg: with tube cast (Im)
Left leg: without tube cast (Nom)
Right leg: release from tube cast (RL)
Left leg: without tube cast (Nom)
Day-0
Day-14
Day-34

F. 1

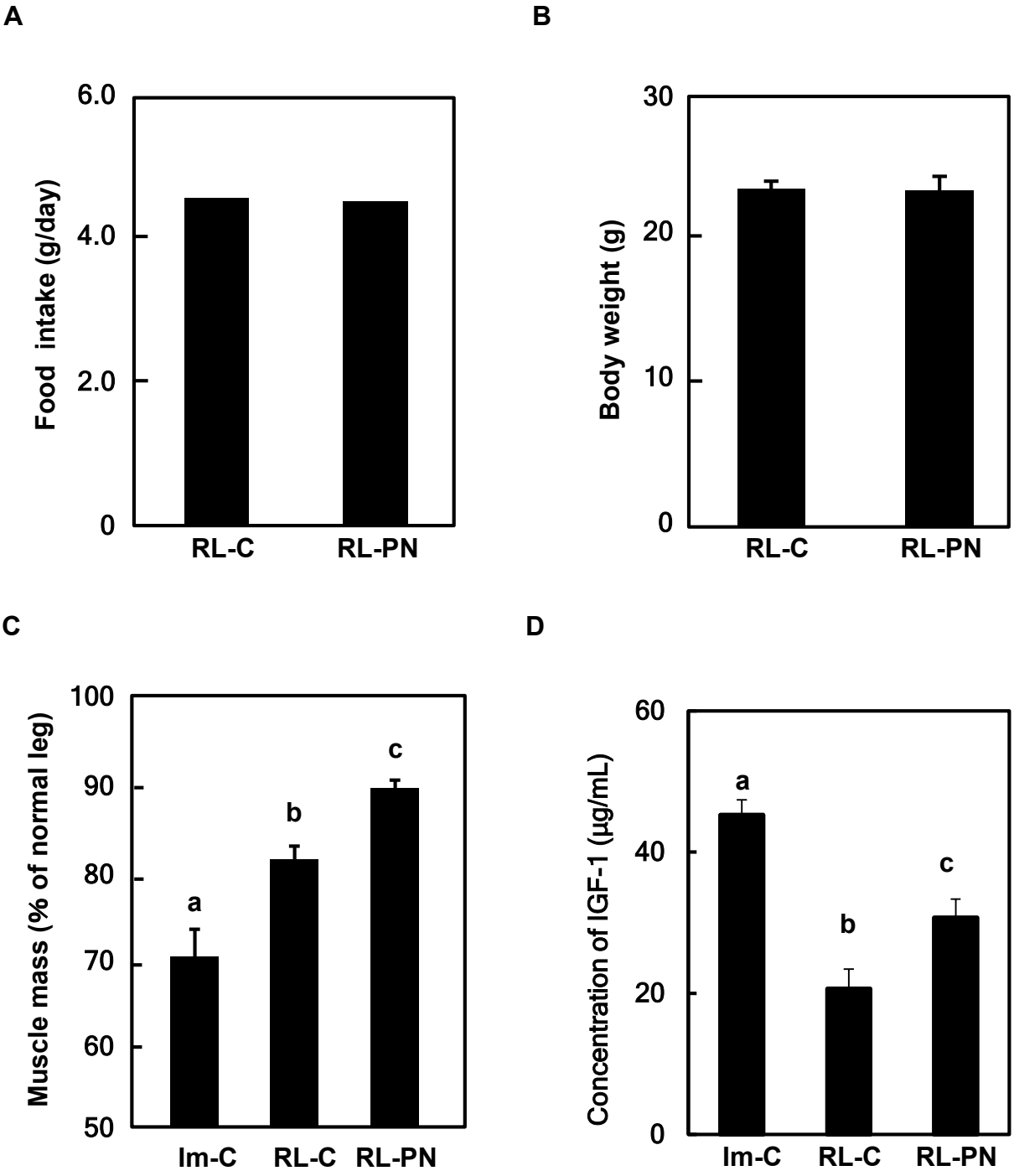
A


120
100
80
60
40
20
0
Muscle mass (% of normal leg)
Im
RL

B

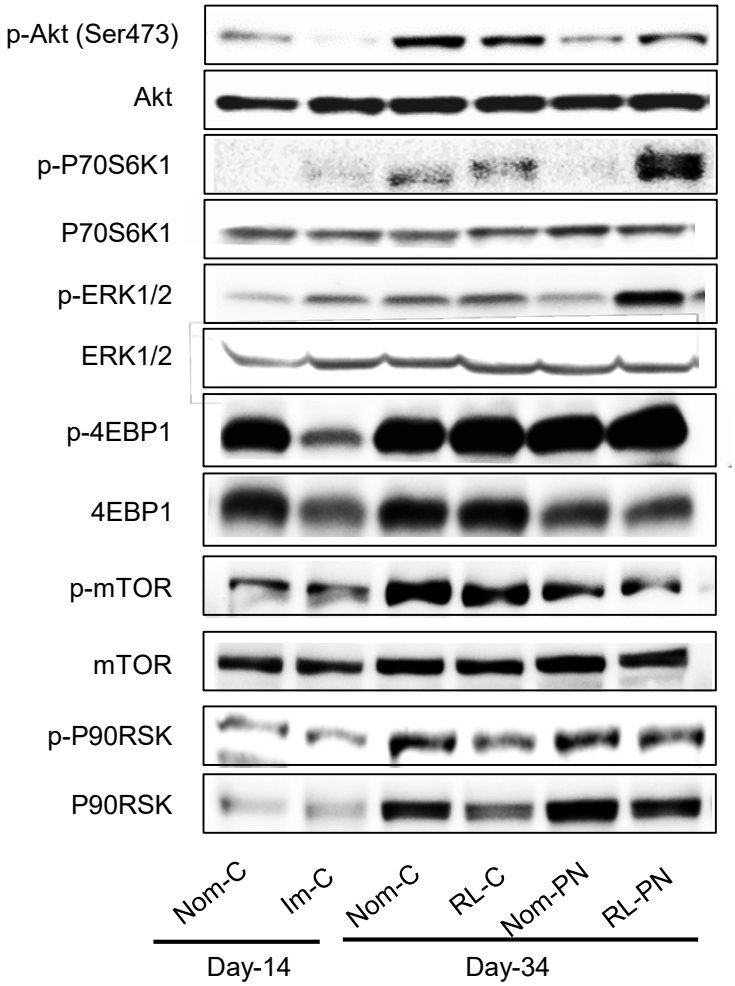

100
80
60
40
20
0
Muscle mass (% of normal leg)
*
Im
RL

F. 2


A
6.0
4.0
2.0
0
Food intake (g/day)
RL-C
RL-PN
B
30
20
10
0
Body weight (g)
RL-C
RL-PN
C
100
90
80
70
60
50
Muscle mass (% of normal leg)
a
b
c
Im-C
RL-C
RL-PN
D
60
40
20
0
Concentration of IGF-1 (μg/mL)
a
b
c
Im-C
RL-C
RL-PN

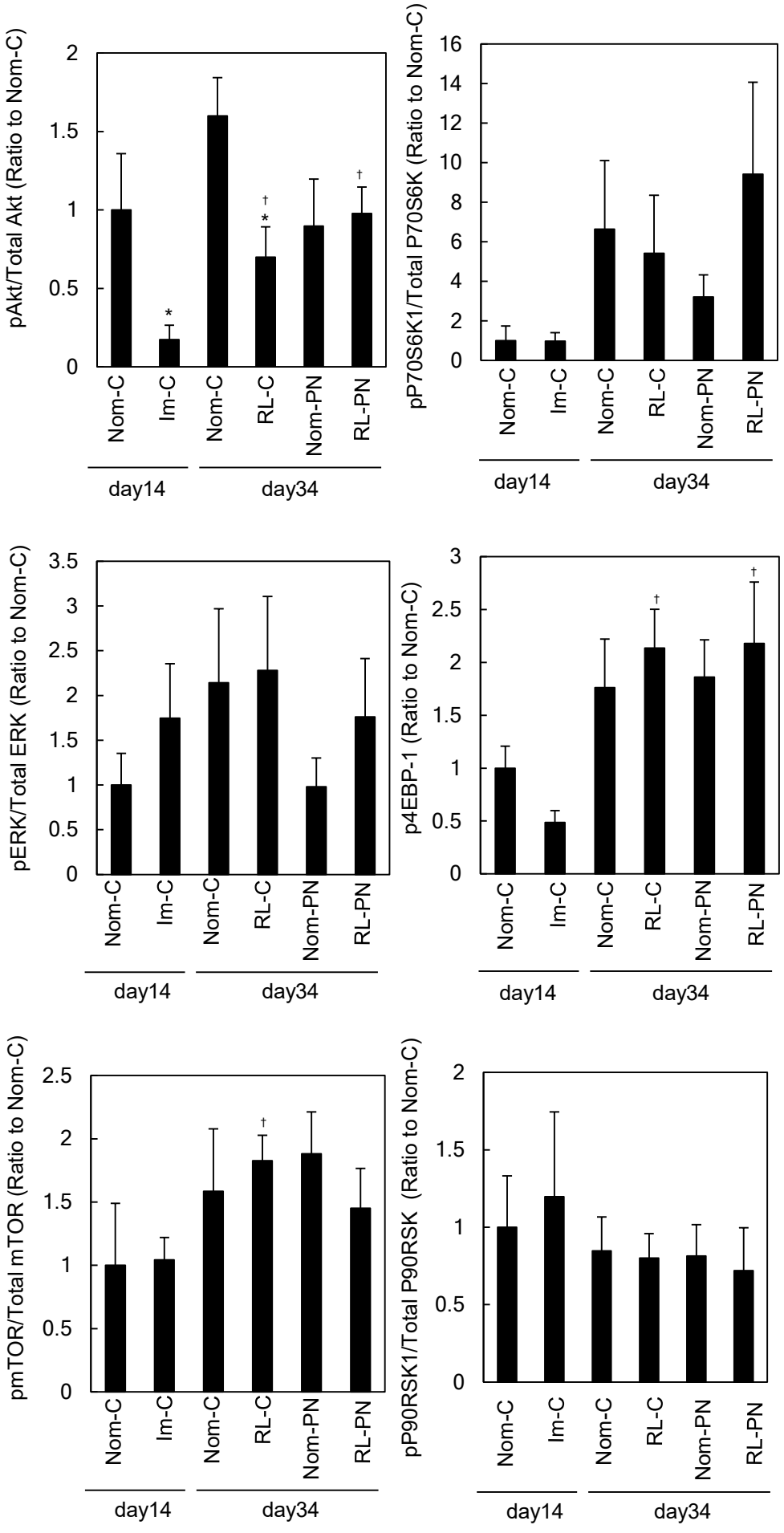
F. 3

A


p-Akt (Ser473)
Akt
p-P70S6K1
P70S6K1
p-ERK1/2
ERK1/2
p-4EBP1
4EBP1
p-mTOR
mTOR
p-P90RSK
P90RSK
Nom-C
Im-C
Nom-C
RL-C
Nom-PN
RL-PN
Day-14
Day-34

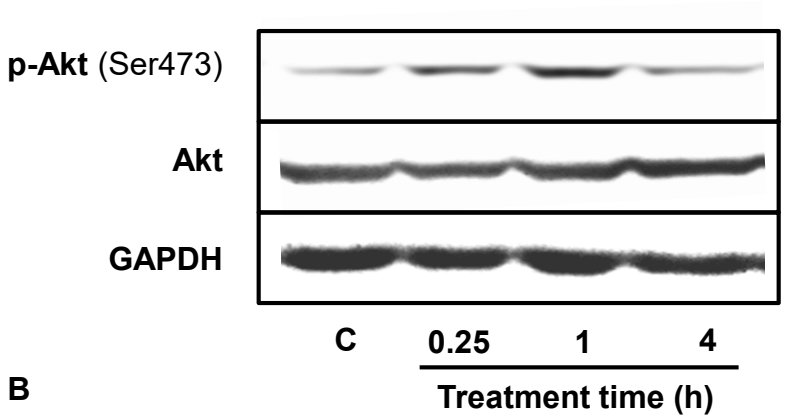
F. 4

B

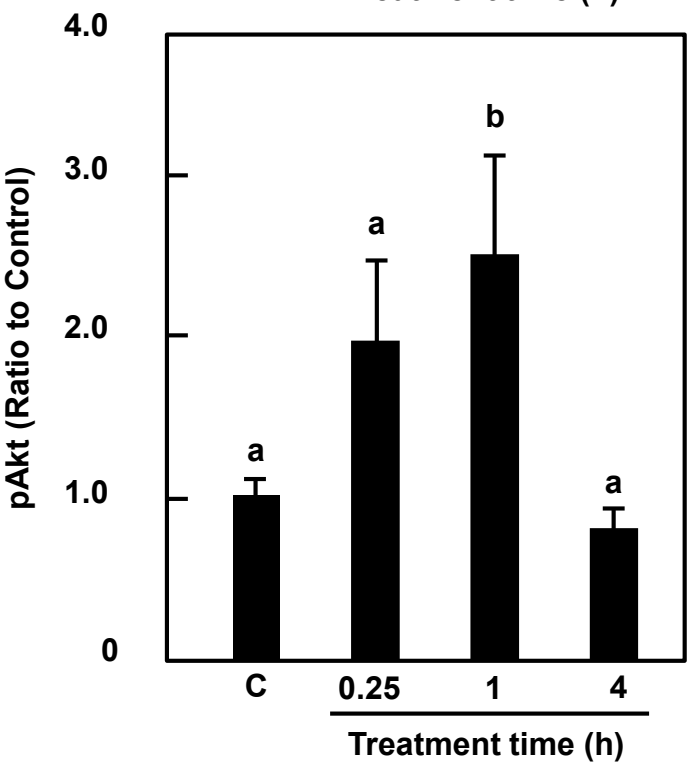


FigureContinued (F. 4)

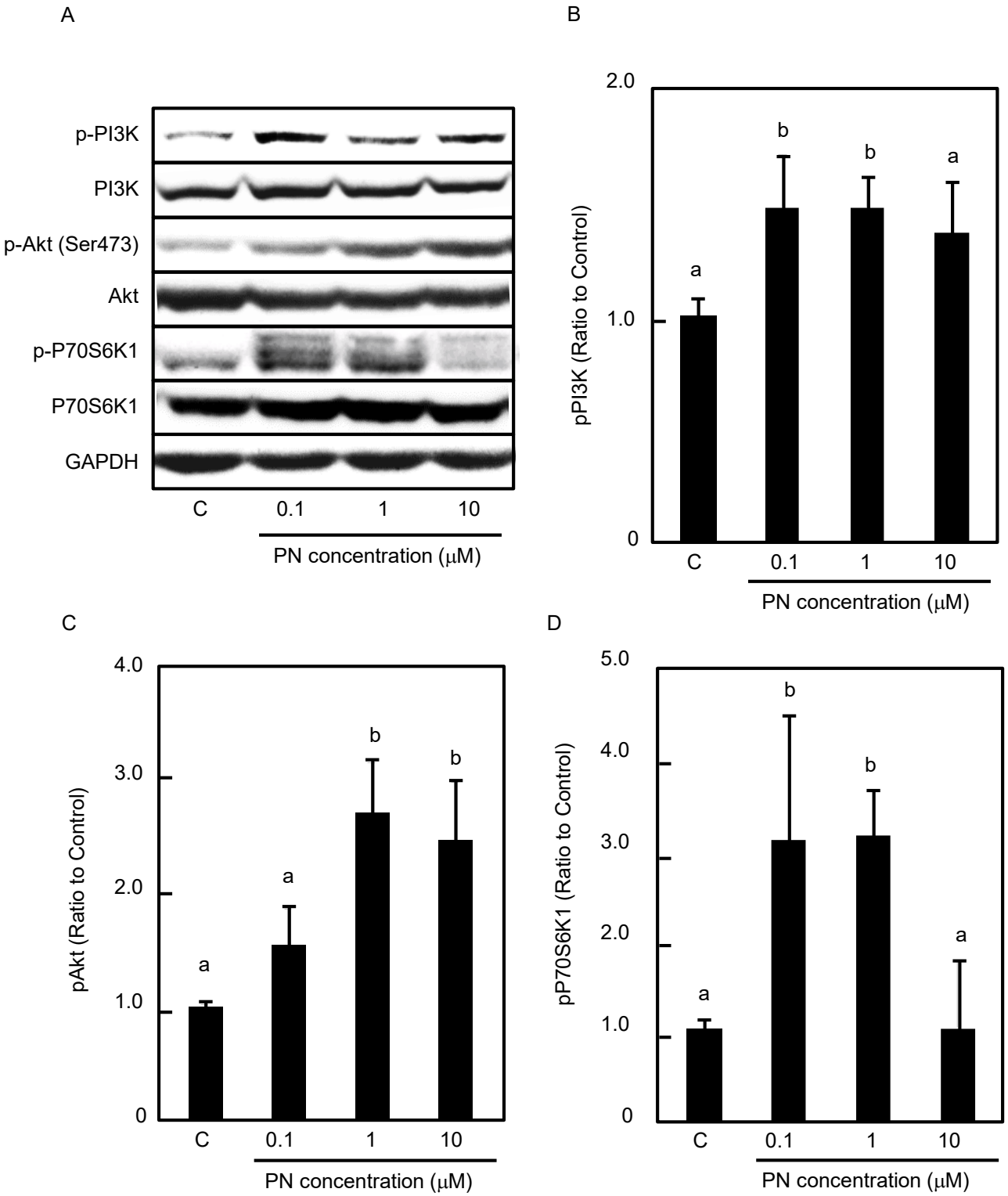
A


p-Akt (Ser473)
Akt
GAPDH
C
0.25
1
4
Treatment time (h)

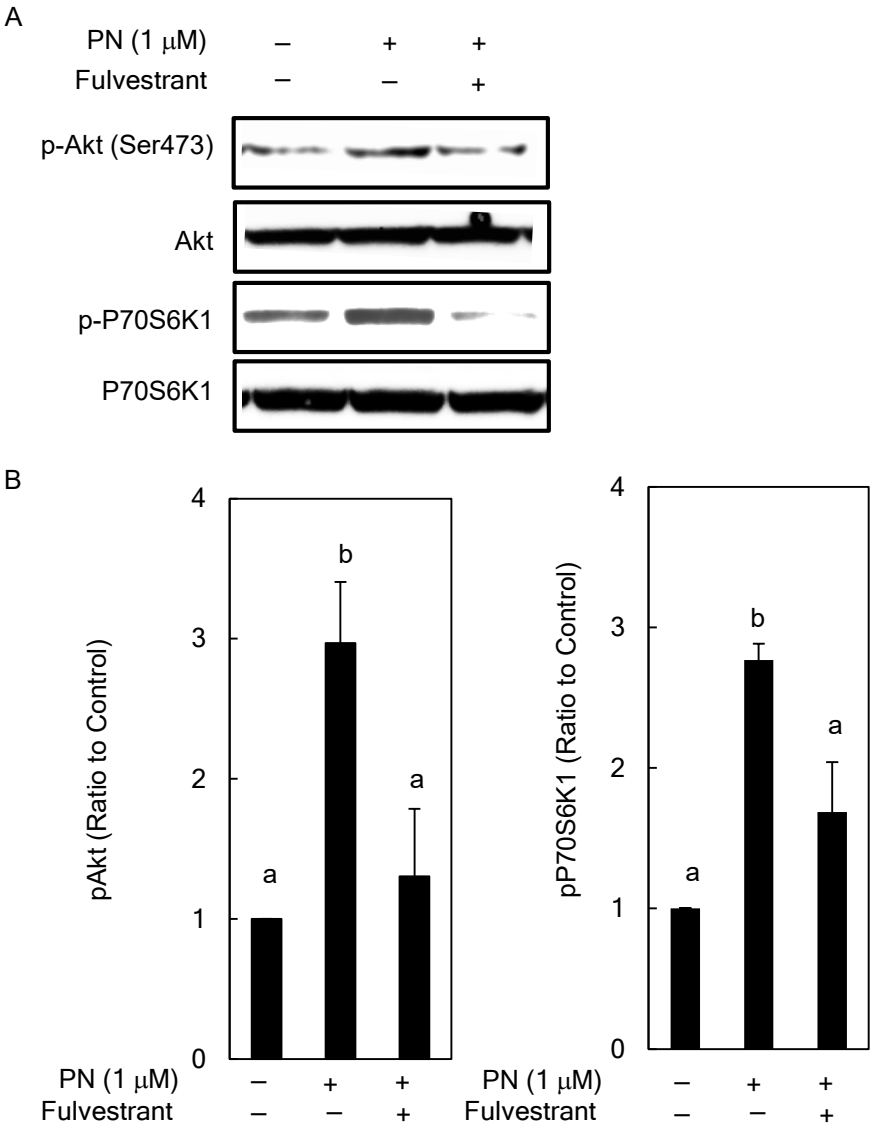
B

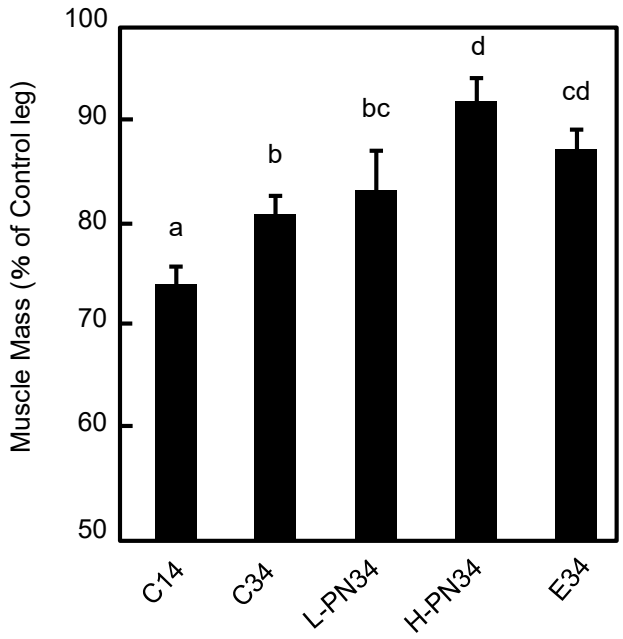

4.0
3.0
2.0
1.0
0
pAkt (Ratio to Control)
a
a
b
a
C
0.25
1
4
Treatment time (h)

F. 5


A
p-PI3K
PI3K
p-Akt (Ser473)
Akt
p-P70S6K1
P70S6K1
GAPDH
C
0.1
1
10
PN concentration (μM)
B
pPI3K (Ratio to Control)
2.0
1.0
0
a
b
b
a
C
0.1
1
10
PN concentration (μM)
C
pAkt (Ratio to Control)
4.0
3.0
2.0
1.0
0
a
a
b
b
C
0.1
1
10
PN concentration (μM)
D
pP70S6K1 (Ratio to Control)
5.0
4.0
3.0
2.0
1.0
0
a
b
b
a
C
0.1
1
10
PN concentration (μM)

F. 6


A
PN (1 μM) – + +
Fulvestrant – – +
p-Akt (Ser473)
Akt
p-P70S6K1
P70S6K1
B
pAkt (Ratio to Control)
0
1
2
3
4
a
b
a
PN (1 μM) – + +
Fulvestrant – – +
pP70S6K1 (Ratio to Control)
0
1
2
3
4
a
b
a
PN (1 μM) – + +
Fulvestrant – – +


Muscle Mass (% of Control leg)
100
90
80
70
60
50
a
b
bc
d
cd
C14
C34
L-PN34
H-PN34
E34