Eicosapentaenoic acid enhances skeletal muscle hypertrophy without altering the protein anabolic signaling pathway

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Summary

This study aimed to examine the effect of eicosapentaenoic acid (EPA) on skeletal muscle hypertrophy induced by muscle overload and the associated intracellular signaling pathways. Male C57BL/6J mice were randomly assigned to oral treatment with either EPA or corn oil for 6 weeks. After 4 weeks of treatment, the gastrocnemius muscle of the right hindlimb was surgically removed to overload the plantaris and soleus muscles for 1 or 2 weeks. We examined the effect of EPA on the signaling pathway associated with protein synthesis using the soleus muscles. According to our analysis of the compensatory muscle growth, EPA administration enhanced hypertrophy of the soleus muscle but not hypertrophy of the plantaris muscle. Nevertheless, EPA administration did not enhance the expression or phosphorylation of Akt, mechanistic target of rapamycin (mTOR), or S6 kinase (S6K) in the soleus muscle. In conclusion, EPA enhances skeletal muscle hypertrophy, which can be independent of changes in the AKT–mTOR–S6K pathway.

Keywords eicosapentaenoic acid □ Hypertrophy □ protein synthesis □ mTOR protein

Introduction

Skeletal muscles perform important functions in both physical movements and metabolic regulation. Decline in muscle protein synthesis, increase in protein degradation, impairment of neuromuscular integrity, and metabolic disorders contribute to the loss of muscle mass strength (Cruz-Jentoft *et al.* 2010). Sarcopenia, which is defined as the age-related loss of muscle mass and strength, is a growing concern in the aging society. Nutrition and physical exercise can be strategically used to overcome age-related protein synthesis impairment and slow the progression of sarcopenia (Dickinson *et al.* 2013, Robinson *et al.* 2018). Skeletal muscle mass primarily depends on the dynamic relationship between protein synthesis and degradation (Schiaffino *et al.* 2013). Proteins and amino acids, especially branched-chain amino acids and anabolic hormones (i.e., insulin), stimulate protein synthesis; however, sarcopenia involves resistance to this system, which is called anabolic resistance (Burd *et al.* 2013).

Eicosapentaenoic acid (EPA) is an ω-3 polyunsaturated fatty acid with various health benefits. ω-3 polyunsaturated fatty acids exhibit anti-inflammatory effects and prevent cardiovascular disease (De Caterina *et al.* 2011, Trebaticka *et al.* 2017). They may exert their biological effects through the following mechanisms: release of bioactive mediators; direct effect on ion channels; direct action on membranes, which requires incorporation into the phospholipid layer of the plasma membrane; and activation of G protein-coupled receptor 120, an ω-3 polyunsaturated fatty acid receptor (De Caterina *et al.* 2011, OH *et al.* 2011, White *et al.* 2014).

Supplementation with ω-3 polyunsaturated fatty acids can increase muscle mass and function and exert anti-sarcopenic effects (Gray *et al.* 2018, Ochi *et al.* 2018). Supplementation with dietary ω-3 fatty acids or fish oil increases muscle mass or strength (Da Boit *et al.* 2017, Rodacki *et al.* 2012, Smith *et al.* 2015) and muscle protein synthesis (Smith *et al.* 2011a, Smith *et al.* 2011b) in human subjects. Activation of protein anabolic signaling by ω-3 polyunsaturated fatty acids has been demonstrated in steer (Gingras *et al.* 2007), rats (Kamolrat *et al.* 2013a), and C2C12 myotubes (Kamolrat *et al.* 2013b). In contrast, McGlory recently demonstrated that fish oil supplementation suppresses resistance exercise and protein feeding-induced increase in anabolic signaling through the Akt–S6 kinase (S6K) pathway, which did not affect muscle protein synthesis in young men (Mcglory *et al.* 2016). These data suggest the involvement of anabolic signaling-dependent and anabolic signaling-independent mechanisms in the effect of EPA on muscle protein synthesis. Additionally, ω-3 polyunsaturated fatty acids attenuated protein catabolism in skeletal muscles in rodents with cancer cachexia (Whitehouse *et al.* 2001a), sepsis (Khal *et al.* 2008), and arthritis (Castillero *et al.* 2009) and during immobilization (You *et al.* 2010). Furthermore, treatment with EPA or docosahexaenoic acid suppresses protein degradation in C2C12 cells (Smith *et al.* 2005, Smith *et al.* 1999).

In the present study, we examined the effect of EPA on muscle protein synthesis by evaluating compensatory muscle growth in mice, which can involve multiple mechanisms (Spangenburg *et al.*

2009). We investigated the effect of EPA alone, whereas most previous studies had evaluated the effect of ω-3 polyunsaturated fatty acids in the form of fish oil.

Materials and methods

Animals

All experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals of Nagoya University. Male C57BL/6J mice (8 weeks of age) were obtained from Chubu Kagakushizai Co., Ltd (Nagoya, Japan). After a week of acclimation, the mice were randomly distributed into 2 groups: the control group, which was fed standard chow (Oriental Yeast Co., Ltd., Tokyo, Japan) containing 6% corn oil (Ajinomoto Co., Inc., Tokyo, Japan), and the EPA group, which was fed standard chow containing 6% EPA (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan). The feed was prepared daily. The mice were maintained in a 12:12 h reversal light–dark environment at 23°C and supplied with feed and water *ad libitum*.

Materials

EPA ethyl ester (>98%) was kindly donated by Mochida Pharmaceutical Co., Ltd. We purchased primary antibodies against phospho-Akt (Ser473), phospho-S6 kinase (Ser371), and S6 kinase (49D7) from Cell Signaling Technology, Inc. (Beverly, MA, USA) and antibodies against Akt 1/2/3 (H-136) from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Enhanced chemiluminescence (ECL) western blotting detection reagents were obtained from GE Healthcare UK Limited (Buckinghamshire, UK).

Overload-induced muscle hypertrophy

Overload-induced muscle hypertrophy is the model used to examine molecular and cellular mechanisms that regulate muscle growth (Spangenburg *et al.* 2009). The procedure for the overloading study is presented in Fig. 1. Hypertrophic muscle growth was evaluated, as described previously (Makanae *et al.* 2013, Serrano *et al.* 2008). Briefly, mice were anesthetized using an intraperitoneal injection of sodium pentobarbital (50 mg/kg). The gastrocnemius muscle of the right hindlimb was surgically removed to induce compensatory hypertrophy of the soleus and plantaris muscles through functional overloading. An incision was made through the skin, and the Achilles tendon was exposed in the left hind legs (sham-operated), which were used as controls. After 1 or 2 weeks of overloading, the muscles and epididymal fats were dissected under anesthesia, and the mice were sacrificed. The wet weight of the muscles was measured; subsequently, the muscles were frozen in liquid nitrogen and stored at −80°C until analysis.

Insulin tolerance test

At 4 weeks, an insulin tolerance test (ITT) was conducted to assess global insulin sensitivity. Blood was collected from the tail tip. Mice that were fasted for 5 h were weighed, and insulin (0.5 UI/kg body weight; Novorapid, Novo Nordisk A/S, Bagsvaerd, Denmark) was injected intraperitoneally. Blood glucose was measured before insulin injection and 20, 40, and 60 min after the injection.

Insulin signaling in muscle

Insulin (0.5 UI/kg) was injected intraperitoneally, and the soleus muscles were extracted after 10 min of injection. The muscles were frozen using liquid nitrogen and stored at −80°C until analysis.

Western blotting

The muscles were homogenized in ice-cold homogenization buffer (50 mM HEPES, pH 7.4; 150 mM NaCl; 1.5 mM $MgCl_2$; 0.01% trypsin inhibitor; 10% glycerol, 1% Triton X-100; and 2 mM phenylmethylsulfonyl fluoride). The lysates were incubated on ice for 1 h and centrifuged at 3873× *g* for 30 min at 4°C. The supernatants were stored at −20°C until analysis. Protein concentrations in the samples were determined using a protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). The lysate was solubilized in 2× loading sample buffer (0.125 M Tris-HCl, pH 6.8; 10% 2-mercaptoethanol; 4% sodium dodecyl sulfate; 20% glycerol; and 0.01% bromophenol blue) and boiled at 100°C for 5 min. For each sample, 10 μg of protein extract was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 20 mA. The proteins were transferred to polyvinylidene difluoride membranes (EMD Millipore Corporation, Billerica, MA, USA) through semi-dry transfer at 25 V for 60 min. Each membrane was blocked with 5% nonfat dry milk for 1 h and rinsed with 1× phosphate-buffered saline (PBS) containing 0.1% Tween 20 before the blots were incubated with a 1:1000 dilution of the primary antibodies overnight at 4°C. Subsequently, the blots were washed in 1× PBS with 0.1% Tween 20, followed by incubation with a 1:1000 dilution of goat anti-rabbit IgG (H+L)–horseradish peroxidase

conjugated antibody (Bio-Rad Laboratories Inc., Hercules, CA, USA) or human-serum-adsorbed and peroxidase-labeled goat anti-mouse IgG (H+L) antibody **(**KPL, Gaithersburg, MD, USA**)** for 1 h at room temperature. Immunoreactive bands were detected using ECL detection reagents, and band intensity was quantified using the ImageJ densitometry software (National Institutes of Health, Bethesda, MD, USA). The individual control/overload data points were divided by the mean value for the control/overload group; thus, the mean value for the normalized control/overload group was 1 with variability. The density of the protein band for the control/sham-operated, EPA/overload, and EPA/sham-operated groups was expressed as the fold change of the density of the control/overload values (Siriguleng *et al.* 2018).

Statistical analysis

All values are expressed as the mean ± SD. Differences were analyzed using Student's unpaired or paired *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's test. One-way ANOVA analysis was performed among the 4 groups (control/overload, control/sham-operated, EPA/overload, and EPA/sham-operated). Differences with $p<0.05$ were considered statistically significant. All analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Effect of 4-week administration of EPA on body weight, muscle weight, epididymal fat, and food intake

As shown in Table 1, the food intake during the 4 weeks and the amount of epididymal fat after 4 weeks of EPA administration were lower in the EPA-administered group than in the control group. Neither body weight nor muscle weight was different between the two groups.

Effect of 4-week administration of EPA on insulin sensitivity

The fasting blood glucose level in the EPA group was significantly lower than that in the control group (Fig. 2a). The ITT showed that the blood glucose level 20 min after insulin injection was significantly lower in the EPA group than in the control group (Fig. 2b), suggesting that EPA administration increased systemic insulin sensitivity. However, the phosphorylation (Ser473) and protein expression of Akt in the soleus muscles after 10 min of intraperitoneal insulin injection were similar between the control group and the EPA group (Fig. 2c). The Akt–mechanistic target of rapamycin (mTOR)–S6K signaling in the soleus muscles of the fasted mice was not different between the control and the EPA groups after 4 weeks of EPA administration (Fig. 3).

Effect of EPA on the weight of the soleus and plantaris muscles in overload-induced muscle hypertrophy

We examined the effect of EPA administration on the growth of overloaded muscles for 1 or 2 weeks. To evaluate the time course of muscle growth, we measured the muscle weights at 1 and 2 weeks of overloading. The overloaded muscles were significantly heavier than the sham-operated leg muscles in all groups for both the soleus and plantaris muscles (Fig. 4). In addition, the soleus muscles from the overloaded legs of mice in the EPA group were heavier than that in the control group at 2 weeks of overloading but not at 1 week of overloading (Fig. 4a). The plantaris muscle weight in the overloaded leg was not significantly different among the groups at both 1 and 2 weeks of overloading (Fig. 4b). Table 2 presents the changes in body weight, overloaded leg muscle weight, epididymal fat weight, and total food intake after 1 or 2 weeks of overload. Body weight, epididymal fat weight, and food intake per day were significantly lower in the EPA group than in the control group at both 1 and 2 weeks of overloading. The weights of the tibialis anterior and extensor digitorum longus muscles in the overloading leg were not significantly different among the groups at both 1 and 2 weeks of overloading.

Effect of EPA on overload-induced anabolic signaling in soleus muscle

We evaluated the skeletal muscle cell signaling pathway associated with protein synthesis in the soleus muscles. The phosphorylation (Ser473) and protein expression of Akt, mTOR, and S6K were examined (Fig. 5). The protein expression and phosphorylation of Akt (Ser473), mTOR, and S6K (Ser371) were higher in the soleus muscles of the overloaded legs, compared to that in

184 the sham-operated legs, and were not significantly different between the control and EPA groups at

185 both 1 and 2 weeks of overload (Fig. 5).

186

187 **Discussion**

188 The principal finding in the present study was that EPA administration can enhance muscle
189 growth induced by muscle overload *in vivo*. To the best of our knowledge, this is the first report on
190 the effects of EPA on compensatory muscle hypertrophy. The AKT–mTOR–S6K signaling
191 pathway for protein synthesis was not affected by EPA administration. Although epidemiological
192 studies and studies on human subjects, animal models, and skeletal-muscle cell lines indicate the
193 role of EPA in the regulation of muscle weight, the mechanisms underlying this effect remain
194 unclear (Gray *et al.* 2018, Ochi *et al.* 2018).

195 We hypothesized that improvement in insulin sensitivity enhances muscle protein synthesis
196 because insulin is a major anabolic hormone. The beneficial effect of ω-3 fatty acids on insulin
197 sensitivity, which improves glucose metabolism, has been reported in animal models of obesity
198 and diabetes; however, this effect remains controversial in human studies (Lalia *et al.* 2016).
199 Recently, ω-3 fatty acid administration resulted in an increase in muscle protein synthesis, the
200 anabolic response to insulin and amino acid infusion, in healthy young and middle-aged people
201 (Smith *et al.* 2011b) and older adults (Smith *et al.* 2011a). Smith et al. additionally reported that
202 insulin and amino acid-induced phosphorylation of AKT, mTOR, and S6K, the major signal
203 molecules associated with skeletal-muscle protein synthesis, was enhanced after supplementation
204 with ω-3 fatty acids; neither the basal rate of muscle protein synthesis nor signaling element
205 phosphorylation was altered in response to ω-3 fatty acid administration (Smith *et al.* 2011a, Smith

206 *et al.* 2011b). Enhancement of amino acid-induced protein synthesis by ω-3 fatty acids has also
207 been reported in C2C12 cells, with increased S6K phosphorylation (Kamolrat *et al.* 2013b). In the
208 present study, the mice administered EPA for 4 weeks exhibited a decrease in fasting blood glucose
209 levels and an improved response in the ITT compared with the control mice, which indicates that
210 EPA improved insulin sensitivity. The following overloading experiments demonstrated enhanced
211 muscle growth of the soleus muscle. However, phosphorylation of the signaling molecules
212 associated with muscle protein synthesis was not altered, suggesting that the increase in soleus
213 growth observed in the present study is not caused by the enhancement of anabolic signaling.
214 In catabolic states, the anti-inflammatory effects of EPA possibly have crucial functions in
215 the reduction of muscle degradation. The role of EPA in the regulation of the ubiquitin–proteasome
216 pathway has been demonstrated in pathological states such as cancer (Whitehouse *et al.* 2001a),
217 starvation (Whitehouse *et al.* 2001b), hyperthermia (Smith *et al.* 2005), and sepsis (Khal *et al.*
218 2008). Administration of EPA downregulated muscle TNF-α, which activates nuclear factor-kB
219 (NF-κB), the major transcription factor for the ubiquitin–proteasome pathway, in a rat model of
220 arthritis (Castillero *et al.* 2009) and a mouse model of Duchenne muscular dystrophy (Machado *et*
221 *al.* 2011). Additionally, the effects of EPA on TNF-α, NF-κB, and the proteasome pathway have
222 been demonstrated in C2C12 myoblasts and myotubes (Smith *et al.* 2005, Smith *et al.* 1999,
223 Huang *et al.* 2011, Magee *et al.* 2008). In the present study, we observed a lower amount of
224 epididymal fat in the EPA group than in the control group. A lower amount of epididymal fat is

associated with reduced inflammation (Sato *et al*. 2010, Figueras *et al*. 2011). However, the anti-inflammatory effect of EPA is usually observed in obese models but not in normal models (Itoh et al. 2012). Furthermore, it has been demonstrated that ω-3 fatty acids can increase muscle mass in healthy people (Smith *et al.* 2011b) and healthy animals (Gingras *et al.* 2007) without activation of the catabolic system. In the present study, although the lack of inflammatory marker analysis limits our argument, it is unlikely that the anti-inflammatory effects of EPA enhanced the growth of soleus in the healthy mice.

In the present study, we observed the enhancement effect of EPA on overload-induced muscle hypertrophy only in the soleus muscle, a primarily type I muscle, but not in the plantaris muscle, a primarily type II muscle. This effect was in contrast to the effect of prior chronic aerobic exercise on overload-induced muscle hypertrophy, in which the effect was only observed in the plantaris muscle (Siriguleng *et al.* 2018). Type II muscles are more sensitive to the effects of various physiological and pathological conditions than type I muscles (Holecek *et al.* 2017, Koopman *et al.* 2006, Muthny *et al.* 2008). Thus, we hypothesized that enhanced hypertrophy through EPA administration would be observed in the plantaris muscle. We observed a significant increase or a tendency toward increase in the phosphorylation of AKT ($p<0.01$), mTOR ($p=0.08$), and S6K ($p=0.05$) in the plantaris muscles of the overloaded legs in the EPA group compared to those in the control group (data not shown). These data indicate that administration of EPA potentially augments the AKT–mTOR–S6K pathway, which can be associated with increase in

insulin sensitivity. However, the muscle growth of plantaris was not affected by EPA administration, indicating that our hypothesis was not true in the present study. The AKT–mTOR–S6K pathway in the soleus muscles was not affected despite the EPA-induced enhancement in soleus muscle growth. In summary, the present results suggest that a different mechanism or signaling pathway is involved in EPA-induced muscle hypertrophy.

Compensatory muscle hypertrophy is regulated in several steps. The IGF–Akt–FoxO signaling pathway plays a major role in this type of muscle growth; however, the precise mechanisms remain to be clarified (Schiaffino *et al.* 2011, Schiaffino *et al.* 2013). The present results, which demonstrate that the AKT–mTOR–S6K signaling was not affected, suggest that this pathway does not play a role in enhancing soleus muscle growth. Recently, the involvement of satellite-cell recruitment and the role of IL-6 signaling have been demonstrated (Serrano *et al.* 2008). Furthermore, the autophagy–lysosome system and ubiquitin–proteasome system need to be appropriately regulated during these processes (Schiaffino *et al.* 2013). These complicated systems are regulated by the intracellular signal transduction system in the skeletal muscles.

Conclusion

EPA enhances growth of the soleus muscle without affecting anabolic signaling. Although the mechanism underlying this effect remains unclear, our findings suggest that EPA or fish oil may be promising prophylactic agents against decline in physical strength in healthy people.

Conflicts of interest

The authors have no potential conflicts of interest.

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Figure legends

Figure 1. The sequence of the study procedure for functional overloading.

Figure 2. Effect of EPA administration on insulin sensitivity.

Fasting glucose levels after 4 weeks of EPA administration (a). Insulin tolerance test (ITT) was performed after 4 weeks of EPA administration (*$p<0.05$) (b). Phosphorylation (c) and protein expression (d) of Akt in the soleus muscles were analyzed by western blotting. Representative immunoblots are displayed in the top panels. Control group (n=6); EPA group (n=7). Data are expressed as the mean ± SD.

Figure 3. Effect of EPA administration on the Akt–mTOR–S6K pathway in soleus muscles.

Phosphorylation and protein expression of Akt, mTOR, and S6K in the soleus muscles after 4 weeks of EPA administration were analyzed by western blotting. Representative immunoblots are displayed in the top panels. Control group (n=6); EPA group (n=7). Data are expressed as the mean ± SD. The density of the protein band of the EPA groups was expressed as the fold change in the density with respect to the mean of the Control group values.

Figure 4 Effect of EPA administration on muscle weight after 1 or 2 weeks of overloading. Weight of the soleus muscles (a) and plantaris muscles (b) of functionally overloaded legs or sham-operated legs was measured after 1 or 2 weeks of overloading. Control group (1 week: n=6; 2 weeks: n=6); EPA group (1 week: n=8; 2 weeks: n=6); 1W, Overload of 1 week; 2W, Overload of 2 weeks. Significant differences between the overloaded and sham-operated legs in each group after 1 or 2 weeks of

overloading (*p<0.05, ***p<0.001, ****p<0.0001) and between the overloaded legs in the control and EPA groups ($^{\&\&}$p<0.01) are indicated. Statistical analysis of the differences between 1 week and 2 weeks of overloading was not performed. Data are expressed as the mean ± SD.

Figure 5. Effect of EPA administration on the Akt–mTOR–S6K pathway in the soleus muscles during overloading. Phosphorylation and protein expression of Akt, mTOR, and S6K after 1 or 2 weeks of overloading in the soleus muscles were analyzed by western blotting. Control group (1 week: n=6; 2 weeks: n=6); EPA group (1 week: n=8; 2 weeks: n=6); 1W, Overload of 1 week; 2W, Overload of 2 weeks. Significant differences between overloaded and sham-operated legs after 1 or 2 weeks of overloading (*p<0.05, **p<0.01) are indicated. Statistical analysis of the differences between 1 week and 2 weeks of overloading was not performed. Data are expressed as the mean ± SD.

Table 1. Body weight, weight of muscles, and epididymal fat weight after 4 weeks of EPA administration

	Control (n=6)	EPA (n=7)
Body weight (g)	27.9 ± 0.8	27.5 ± 0.5
Weight of muscles (mg)		
Gastrocnemius	146 ± 5	147 ± 8
Plantaris	23.4 ± 1.5	23.1 ± 2.5
Soleus	10.9 ± 0.6	10.6 ± 0.4
Tibialis anterior	50.7 ± 2.5	52.9 ± 2.6
Extensor digitorum longus	12.3 ± 0.6	12.1 ± 0.9
Epididymal fat weight (mg)	493 ± 89	298 ± 78***
Food intake per day (g/day)	3.71 ± 0.07	3.47 ± 0.09***
Total food intake (g)	107.58 ± 1.91	101.01 ± 2.74***

Data are expressed as mean ± SD.
Statistical difference vs. the Control group (***p<0.001)

Table 2. Body weight, weight of muscles, and epididymal fat weight after 1 week or 2 weeks of overloading

	1 week of overloading		2 weeks of overloading	
	Control (n=6)	EPA (n=8)	Control (n=6)	EPA (n=6)
Body weight (g)	25.9 ± 0.9	24.8 ± 1.0*	28.5 ± 1.6	26.4 ± 0.9**
Weight of muscles of overloaded legs (mg)				
Tibialis anterior	45.6 ± 3.3	46.1 ± 3.7	43.9 ± 5.6	47.5 ± 2.6
Extensor digitorum longus	11.1 ± 0.9	11.2 ± 0.7	11.1 ± 0.8	10.2 ± 0.9
Weight of muscles of overloaded legs (mg)/body weight (g)				
Tibialis anterior	1.71 ± 0.12	1.84 ± 0.13	1.57 ± 0.25	1.79 ± 0.16
Extensor digitorum longus	0.42 ± 0.16	0.45 ± 0.15	0.40 ± 0.21	0.39 ± 0.18
Epididymal fat weight (mg)	449 ± 13	199 ± 5***	309 ± 32	210 ± 11***
Epididymal fat weight (mg)/body weight (g)	16.77 ± 4.18	7.91 ± 1.79***	11.06 ± 1.01	7.91 ± 0.39**
Food intake per day (g/day)	3.10 ± 0.11	2.96 ± 0.15*	3.52 ± 0.08	3.44 ± 0.05*
Total food intake (g)	110.29 ± 4.79	105.23 ± 6.89	148.08 ± 3.23	144.58 ± 2.08*

Data are expressed as mean ± SD.

Statistical difference vs. the Control group in 1 week or 2 weeks of overloading (*p<0.05, **p<0.01, ***p<0.001)

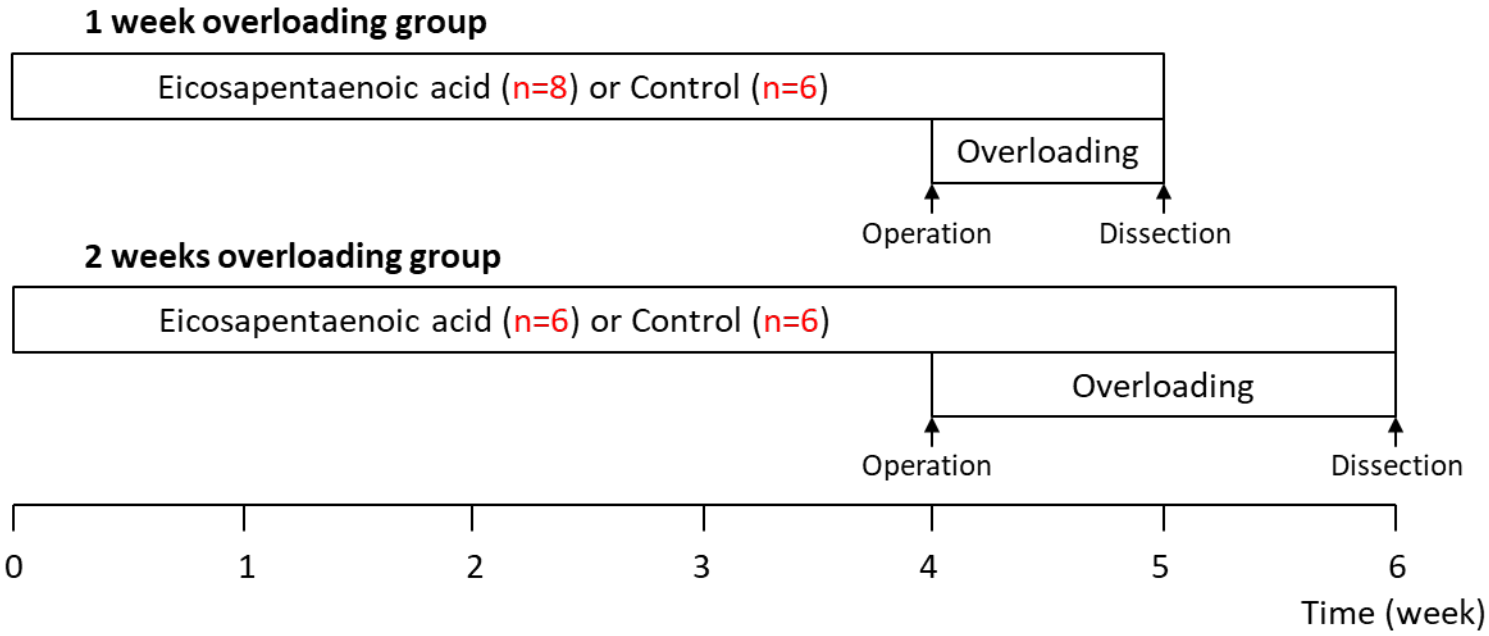
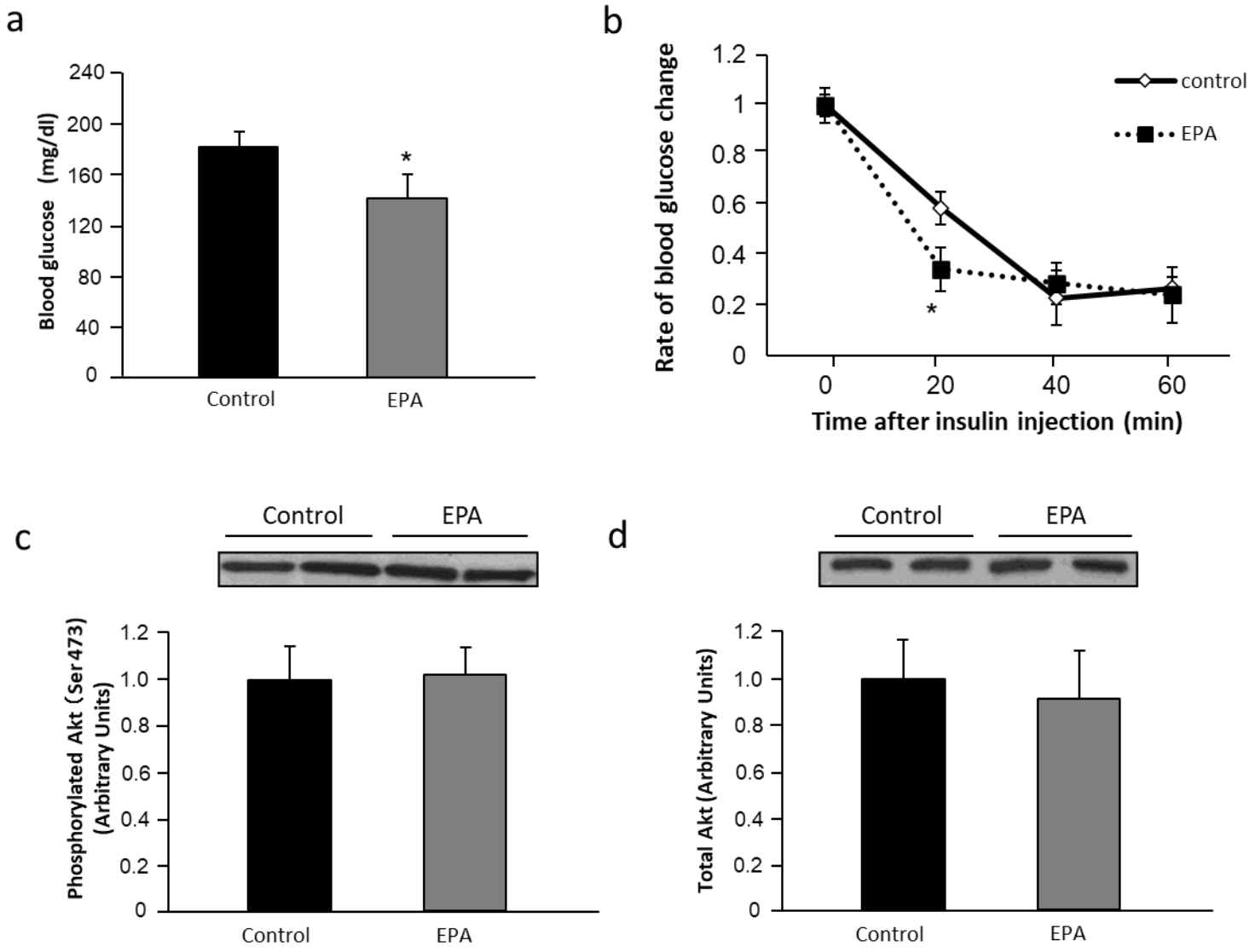


Figure 1. SIRIGULENG et al.



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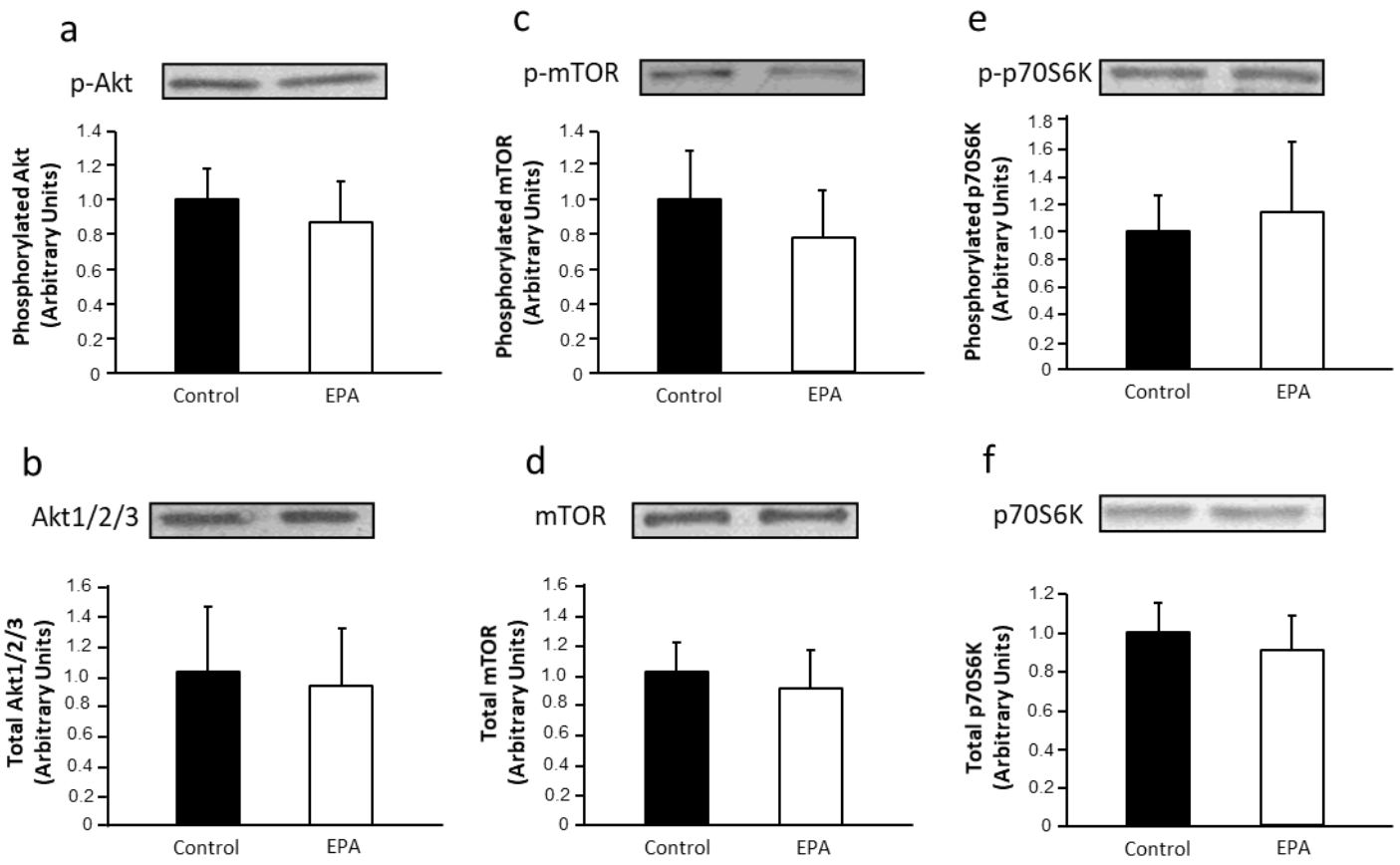

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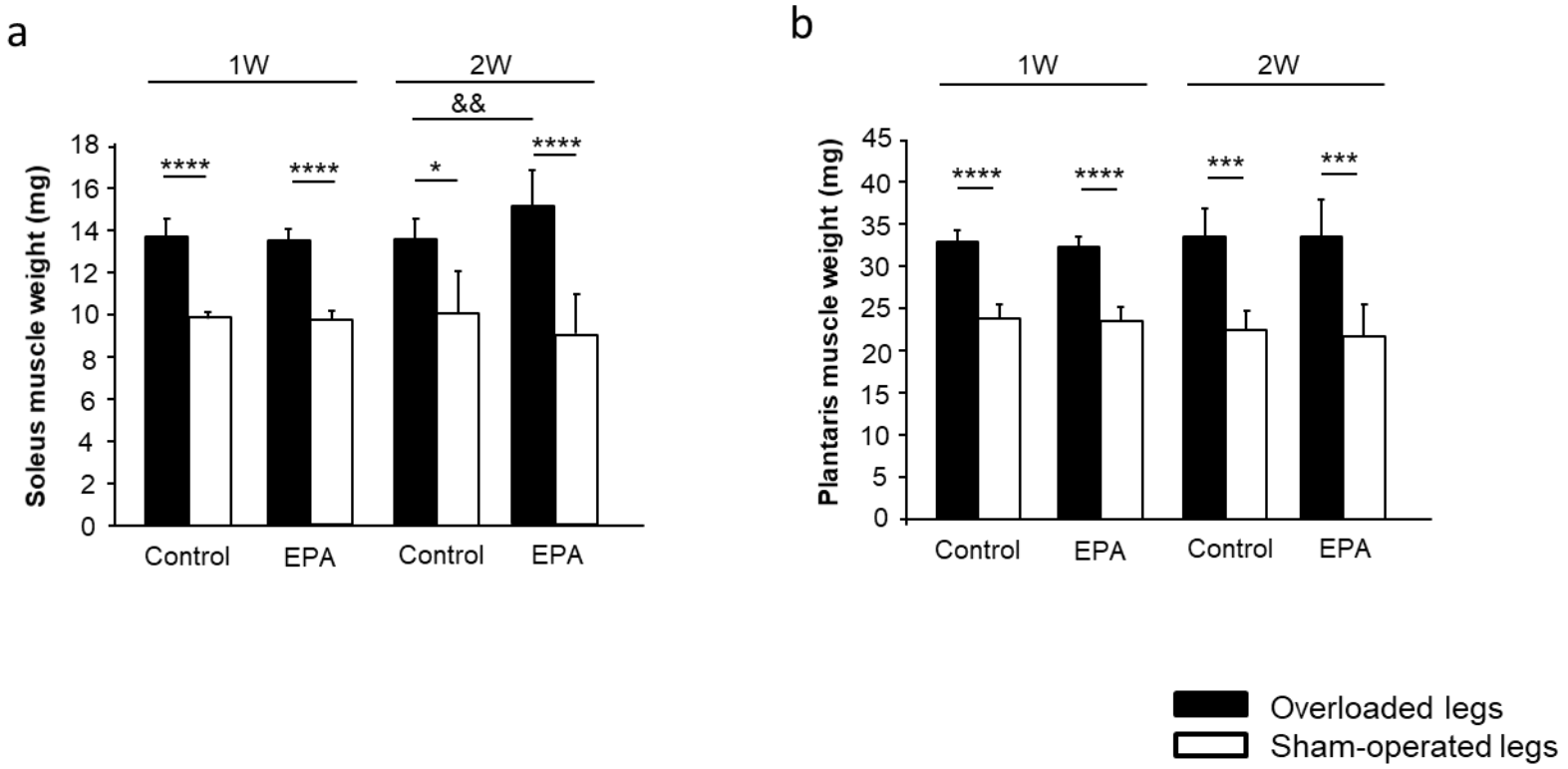

Figure 4. SIRIGULENG et al.

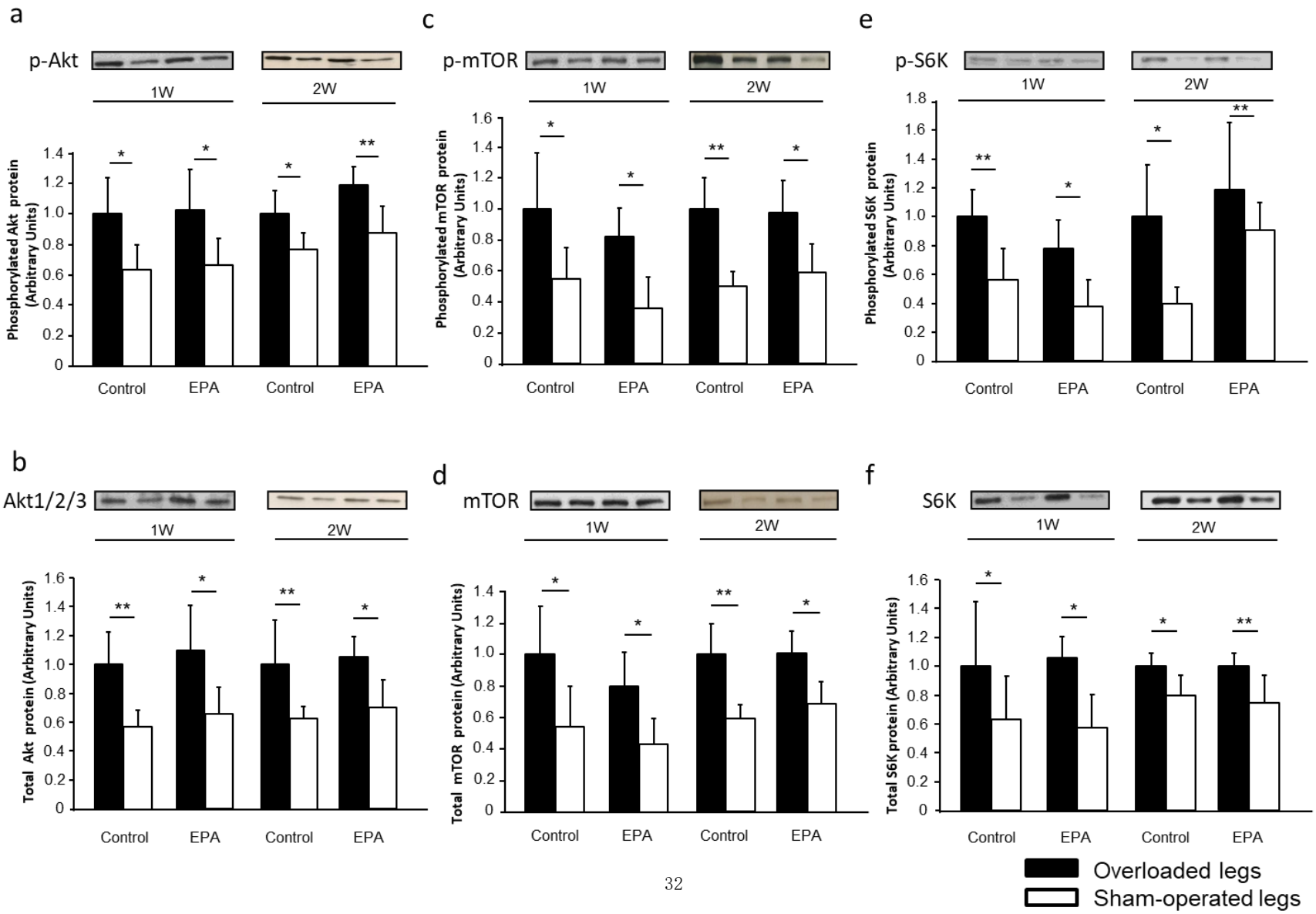

Figure 5. SIRIGULENG et al.