FLAVAN 3-OLS RETARDED DISUSE ATROPHY INDUCED BY HINDLIMB SUSPENSION IN MICE

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ABSTRACT

We found that flavan 3-ols (FL) revealed enhancement of energy expenditure and metabolic changes in skeletal muscle through sympathetic nerve stimulation. On the other hand, β2 adrenaline receptor agonist reported rise in muscle mass. In this study, we examined the effect of ingestion of FL on disuse muscle atrophy induced by hindlimb suspension (HS) in mice. Male C57BL/6J mice were assigned into 4 groups as follows; steady state-distilled water DW (NV), steady state-FL (NF), hindlimb suspension-DW (HV), hindlimb suspension-FL (HF) group. Mice in NV and HV groups were oral administration of distilled water, animals in NF and HF groups were dosed FL (50 mg/kg) for 2 weeks. After treatment, we dissected and weighed tibialis anterior (TA), extensor digitorum longus (EDL), soleus (SOL), gastrocunemius (GASTRO) and measured the expression of two muscle ubiquitin ligases such as ring finger (MuRF)-1 and muscle atrophy F-box (MAFbx) level by western blotting method. The weight of AT, SOL and GASTRO were reduced significantly by hindlimb suspension, but this change was not observed in EDL. In contrast, atrophy in AT, SOL and GASTRO was not observed by the treatment of FL. In addition, mass of EDL was increased significantly by FL ingestion. MuRF-1 and MAFbx level in SOL, which plays a key role in muscle proteolysis, were significantly increased by hindlimb suspension. While there was no significant change on the level of MuRF-1 and MAFbx by the treatment of FL. In conclusion, these results suggested that FL retarded disuse muscle atrophy by proteolysis inhibition.

1. INTRODUCTION (10pt.)

Recently, developed countries face with the problem of aging population with increase of locomotive syndrome patients. Muscle atrophy is one of the cause of locomotive syndrome and it is induced by the activation of ubiquitin proteasome pathway (Okamoto, et al., 2011). On the other hand, we already reported that repeated treatment of FL induced several physiological changes. FL, a group of polyphenolic substances, are found in several plant foods, including catechins and oligomers such as procyanidins. In a previous study, we confirmed that the respiratory exchange ratio, was significantly reduced following repeated supplementation with FL, along with elevation of uncoupling protein (UCP) -1 and UCP-3 protein level in skeletal muscle, such as a key protein in thermogenesis, and β-oxidation related enzyme levels in several tissues (Watanabe et al . 2014). In addition, we also observed that repeated treatment with FL increased mitochondrial DNA copy numbers in skeletal muscle and brown adipose tissue. We also have found that a single dose of FL enhanced energy expenditure (EE) in both feeding and fasting condition. It was also observed the increases in the mRNA expression levels of UCPs and peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) and the protein level of phosphorylated AMPKα in tissues 2 hrs. Moreover, an increase in the plasma adrenaline level was observed 2 hrs after administration of FL (Matsumura et al. 2014). Recently, we confirmed that pretreatment with β2 adrenalin receptor blocker prevented the increases of EE, the mRNA expression of UCP-3, and phosphorylated AMPKα that were induced in the skeletal muscle of mice by a single oral dose of FL (Kamio et al. 2016). These results suggest that the ability of a single oral dose of FL to enhance metabolic activity is mediated by sympathetic nerve stimulation. On the other hand, it was reported that β2 adrenaline receptor agonist such as clenbuterol induced muscle hypertrophy (Joassard, at al., 2013). According to these observation, we examined the effect of FL on the disuse muscle atrophy induced hindlimb suspension (HS) in mice in this study.

2. EXPERIMENT

2.1 MATERIALS

The flavan 3-ols fraction (FL) prepared from cocoa powder was provided by Meiji Co. (Tokyo Japan). The concentration of each flavan 3-ols in this study was (+)-catechin 4.52%, (-)-epicatechin 6.43%, procyanidin B2 3.93%, procyanidin B5 0.85%, procyanidin C1 2.36%, Cinnamtannin A2 1.45%.

2.2 ANIMALS AND DIETS

The study was approved by the Animal Care and Use Committee of the Shibaura Institute of Technology. All the animals received care under the guidelines of this institution. Male C57BL/6J mice 17-19wks were obtained from Charles River laboratories Japan, Inc. (Tokyo, Japan). The diet provided to the animals was MF obtained from the Oriental Yeast Co. Ltd., Tokyo, Japan.

2.3 EXPERIMENTAL METHOD

Thirty two mice were fed basal diet for 7 days and then we divided animals into 4 groups as steady state-distilled water (DW) treatment as NV, steady state-50 mg/kg FL treatment as NF, hindlimb suspension-DW as HV, hindlimb suspension-FL as HF for 2 weeks. Photo 1 shows the mice of hindlimb suspension treatment. At the end of this supplementation period, the animals were sacrificed and their tissues were removed and measured weight and snap frozen in liquid nitrogen and stored at -80 ℃ until analysis.

Photo 1. Treatment of hindlimb suspension

2.4. ANALYSIS

We determined ubiquitin ligases (Muscle Ring Finger-1: MuRF-1, Muscle Atrophy F-box protein: MAFbx) level by western blotting. Tissues were homogenized in a microtube with lysis buffer (CelLyticTM MT cell lysis reagent; Sigma Aldrich, Japan) containing a protease inhibitor (Sigma Aldrich, Japan) and 0.2% w/v SDS. Protein concentration was measured by the Bradford method. Protein (20 μg) was separated by SDS-PAGE using a 4-12% Bis-Tris gel, and transferred onto a polyvinylidene difluoride membrane (Life Technologies, California, USA). The membrane was blocked with membrane-blocking reagent (GE Healthcare, Buckinghamshire, UK) for one hour. After blocking, the membrane was incubated with a rabbit polyclonal primary antibody against MuRF-1 (1:500; sc-32920, Santa Cruz Biotechnology, Inc., USA), an antibody against MAFbx (1:500; sc-33782, Santa Cruz Biotechnology, Inc., Santa Cruz, USA) and an antibody against α-tubulin (1:1000; ab4074, Abcam). After the primary antibody reaction, the membrane was incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:100000) for 1 hr. Immunoreactivity was detected by chemiluminescence using the ECL Select Western Blotting Reagent (GE Healthcare, Buckinghamshire, UK). Fluorescence band images were analyzed using Just TLC (SWEDAY, Larkgatan, Sweden) analysis software. Values of ubiquitin ligase were normalized to those of α-tubulin

2.5 STASTISTICAL METHODS

All data were reported as mean ± standard deviation. Statistical analyses were performed by one or two way ANOVA, post hoc comparisons between experimental groups were made by the two-tailed followed by Tukey's test. A probability of $P < 0.05$ was considered to be statistically significant.

3. RESULT

There was no significant difference between treatment groups in body weight and total diet consumption (Fig.1).

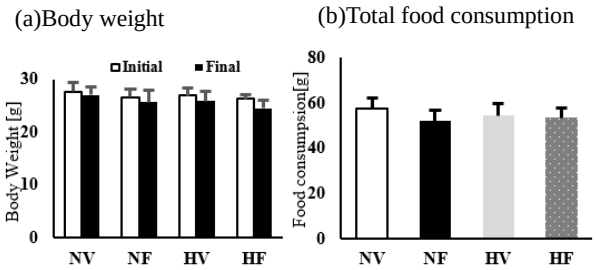

Fig.1 Body weight (a), total food consumption (b). Values are means ± SD, n = 8 per group. Means with different letters are significantly different at $P < 0.05$.

The tissue weight of the animals at the end of feeding period as shown in Table 1. There was no significant difference between the 4 experimental groups on liver, heart, kindney, adrenal gland, spleen, epididymis adipose, perirenal fat, brown adipose, subcutaneous fat. While, the weight of SOL, GASTRO and TA were reduced significantly by NV and NF, but this change was not observed in EDL. Atrophy in SOL, GASTRO and TA was not observed and mass of EDL was increased significantly by FL (Table. 1).

Table.1 Tissue weight

mg/kg	NV	NF	HV	HF
Liver	37646.9±5576.5	40372±4866.7	37304.8±5420.7	40232.8±3712.9
Heart	4750.8±1058.7	4238.3±159.5	4220.5±245.1	4514.8±470
Kidney	12258.3±856	11751.6±612.4	11880.8±821.2	12746.7±1437.7
Adrenal gland	70±18.1	63.6±15.9	77.4±15.3	81.2±19
Spleen	2598±417	2366.2±223.9	2266.3±485.8	2270.4±287
Epiddidymis adipose	17539.1±2578	17191.9±4466.7	16757.9±2209.5	17199.6±3403.4
Perirenal fat	5849.8±1592.4	6192±1889	6289.1±1197.5	5999±1664.3
Brown fat	2774±357.9	2630.5±437.7	3035±540	3043.5±676.9
Subcutaneous fat	7424.4±2118.8	8377.7±1761.5	7733.8±2331.3	9210.9±2545.4
Soleus	778.2±72.7	777.5±116.2	459.4±42.5	649.2±91.4
Gastrocnemius	12662.3±833.5	12855.8±824.9	10574.9±1088	11989.9±642.9
Tibialis anterior	3942.8±301.1	4094.1±185.5	3493.3±185.3	4021.1±273.4
Extensor digitorum longus	912.2±123.1	1099±106.5	879.5±60.3	1095.1±108.4

Values are means ± SD, n = 8 per group. Means with different letters are significantly different at P < 0.05.

The results of the expression level of MuRF-1 and MAFbx in SOL were shown in Fig.2. Both ubiquitin ligases were significant increased by hindlimb suspension treatment. While this change was not observed in the FL treatment group with hindlimb suspension (Fig.2).

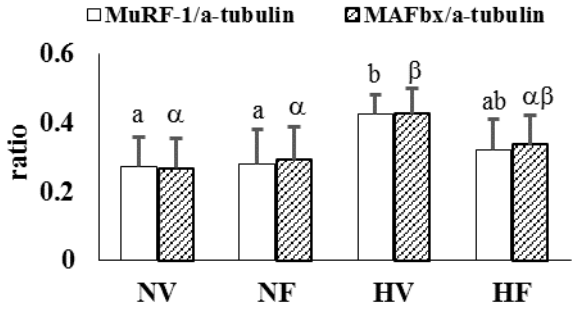

Fig.2 The expression of MuRF-1 and MAFbx in SOL at the end of feeding period. Values are means ± SD, n = 8 per group. There were significant differences between the different character: p<0.05

4. DISCUSION

In this study, we found that the reduction of the muscle mass of AT, SOL and GASTRO by hindlimb suspension was prevented by FL treatment. And also FL increase muscle mass EDL with or without hindlimb suspension (Table 1). In addition, the ubiquitin ligases such as MuRF-1 and MAFbx level in SOL, which plays a key role in muscle proteolysis, were significantly increased by hindlimb suspension. While these changes were disappeared by the ingestion of FL.

It was well known that the mechanical load was arise in sarcomere and from there transmitted to the nuculeus to affect gene expressions (Lange et al. 2005). Recent report suggested that PGC-1α as a transcriptional factor is the key role of muscle biology, because of PGC-1α downregulation was observed in different models of muscle wasting. PGC-1α was suggested to be protect from the atrophy induced by the expression of FoxO (Sandri et al. 2006). It was also known that the two most induced genes are two novel muscle-specific ubiquitin ligases, MAFbx and MuRF1 that are upregulated thorough FoxO (Fig.3). In the present atrophy model, the increase of these ubiquitin ligases are responsible for the increased protein degradation as shown in Fig.2.

On the other hand, we already reported that FL increases PGC-1α in skeletal muscle through catecholamine secreted from the adrenal medulla induced by FL ingestion (Matsumura et al. 2014). In present study showed that FL suppressed the disuse atrophy in SOL, GASTRO and AT, it was suggested to due to β2-adrenomimmic activity of FL. Altogether, FL inhibited FoxO expression through inducing expression of PGC1-α, consequently, reduction of ubiquitin-proteasome system. In addition, it was reported that β-agonists such as clenbuterol, acting through β2-adrenoreceptors, caused muscle hypertrophy (Joassard, at al., 2013). It was suggested that hypertrophy activity of catecholamines was mediated by AKT-mTOR pathway (Kline et al. 2007). Activation of beta receptors is known to also increase intracellular cAMP levels and activates protein kinase A, which may also activate the AKT pathway as shown in Fig.3. FL showed rise of plasma catecholamine mentioned as above and the biological response in skeletal muscle were totally reduced by the pretreatment of β2-adrenaline receptor blocker (Kamio et al. 2016). It was suggested that the elevation of EDL muscle mass observed in this study (Table 1) was also involved β2-adrenomimmic activity of FL. Further studies were needed to elucidation of atrophy prevention and hypertrophy acceleration of FL.

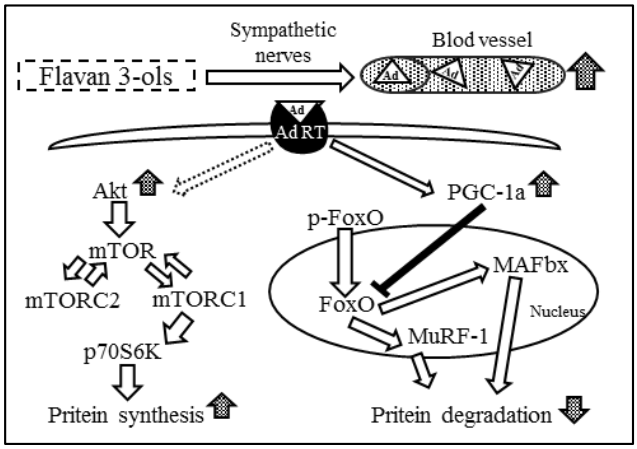

Fig. 3 Scheme for possible mechanisms of atrophy prevention and hypertrophy acceleration induced by FL

5. CONCRUTION

In conclusion, these results suggested that FL retarded disuse muscle atrophy by proteolysis inhibition. It was suggested that these activities of FL involved with β2-adrenomimmic activity. Further studies were needed to elucidation of atrophy prevention and hypertrophy acceleration of FL.

6. REFERENCES

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NOMENCLATURE

Subscripts

FL	: flavan 3-ols
HS	: hindlimb suspension
DW	distilled water
NV	steady state-DW
NF	steady state-FL
HV	hindlimb suspension-DW
HF	hindlimb suspension-FL
TA	tibials anterior
SOL	soleus
EDL	extensor disitorum longus
GASTRO	gastrocnemius
MuRF-1	muscle ring finger-1
MAFbx	muscle atrophy F-box
Ad	adrenaline
RT	receptor
p70S6K	P70S6 kinase

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