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# Repeated oral administration of flavan-3-ols induces browning in mice adipose tissues through sympathetic nerve activation

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Abstract

We previously found increases in uncoupling protein (Ucp)-1 transcription in brown adipose tissue (BAT) of mice following a single oral dose of flavan 3-ols (FL), a fraction of catechins and procyanidins. It was confirmed that these changes were totally reduced by co-treatment of adrenaline blockers. According to these previous results, FL possibly activates sympathetic nervous system (SNS). In this study, we confirmed the marked increase in urinary catecholamine (CA) s projecting SNS activity following a single dose of 50 mg/kg FL. In addition, we examined the impact of the repeated administration of 50 mg/kg FL for 14 days on adipose tissues in mice. In BAT, FL tended to increase the level of Ucp-1 along with thermogenic transcriptome factors, such as peroxisome proliferator-activated receptor γ coactivator (*PGC*)-*1*α and PR domain-containing (*PRDM*)*1*. Transcription of browning markers, such as *CD137* and transmembrane protein (*TMEM*) 26 in addition to *PGC-1α* were increased in epididymal adipose (eWAT) by FL. A multilocular morphology with cell size reduction was shown in the inguinal adipose (iWAT), together with increasing the level of Ucp-1 following administration of FL. These results suggest that FL produces browning in adipose through activation of the SNS.

Keywords: flavan-3-ols; adipose; browning; catecholamine; sympathetic nerve

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## 1. Introduction

Flavan-3-ols (FL), as a mixture of catechins and B-type oligomer procyanidins (Figure1b and c), are enriched in cocoa [1, 2], apple [3, 4], grape seeds, [5, 6] and red wine [7, 8] . The ingestion of FL-rich foods could have a significant potential for managing cardiovascular health [9–11]. Many intervention studies have suggested that the intake of FL results in beneficial alterations in the metabolism such as a significant decrease in plasma LDL with an increase in HDL [12, 13] and rise in glucose tolerance [14–16].

A part of the catechins are absorbed in the gastrointestinal tract and, subsequently, are metabolized in intestinal epithelial cells or the liver. Therefore, unchanged forms of them are nearly absent in blood or tissues [17]. In addition, B-type oligomer procyanidins are rarely absorbed from the gut into the blood [18–20]. Almost all oligomers ingested from foods move into the colon, and a part of them are degraded by the microbiome. It has been reported that the gut microbiome and metabolites are altered by repeated ingestion of FL, and these changes suggest its possible contribution to improvements in metabolism [21–25]. In addition, acute metabolic changes, such as improvements in glucose and insulin tolerance, were shown to follow after a single intake of FL-rich food [26, 27]. These previous results point out the necessity of discussions from both the acute and chronic sides of the physiological alterations induced by FL ingestion.

In general, catecholamine (CA)s, which are secreted from the end of the sympathetic nerve or adrenal medulla to the effector tissues or blood following SNS hyperactivation, are known to

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induce a large metabolic alteration through the activation of adrenaline receptors [28]. SNS activation, following cold stress or treatment of adrenaline agonists, induces to enhance non-shivering thermogenesis through the activation of mitochondrial uncoupling protein (Ucp)-1 in BAT, has also been well investigated [29].

We previously found that a significant increase in energy expenditure along with *UCP-1* mRNA expression in BAT was observed a few hours after a single oral dose of FL in mice [30]. In addition, we also confirmed that these changes were completely reduced by co-treatment with FL and β3 adrenaline blockers. [31]. The β3 adrenaline receptor is specifically expressed in BAT and causes thermogenesis. Therefore, our previous results in these metabolic changes suggest that FL possibly activates SNS.

Recently, it was reported that the activation of SNS induces browning of white adipose tissue (WAT) such as the conversion of WAT into beige adipocytes or promotion of differentiation from mesenchymal stem cells to beige fat [32]. Brown-like adipocytes (beige adipocyte), characterized a multilocular morphology and the expression of Ucp-1, was found in WAT after SNS activation following cold exposure [33]. In addition, it is well known that CAs secreted from adrenal medulla are excreted into the urine, and the change in SNS activity can be estimated by the amount of CAs in the urine [34].

In the present study, we first determined the CAs concentration in 24 h urine following a single

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oral dose of FL in order to confirm SNS activation. Next, we examined the effects of the repeated oral dose of FL on mouse adipose tissues, such as BAT, epididymal (eWAT), and inguinal white adipose (iWAT), by the measurement of thermogenic-related protein and gene expressions in addition to histological observation in iWAT.

## 2. Materials and methods

### 2.1. Materials

FL from cocoa were prepared according to Natsume et al.[35] The FL contained 4.56% (+)-catechin, 6.43% (-)-epicatechin, 3.93% procyanidin B2, 2.36% procyanidin C1, and 1.45% cinnamtannin A2 (Figure1 a-c). To determine a reference value, we also ascertained the polyphenol concentration in this fraction using the Prussian blue method, and it showed a value of 72.3%.

### 2.2. Animals and diets

The study was approved by the Animal Care and Use Committee of Shibaura Institute of Technology (Permit Number: AEA19016). We used C57BL/6J 13 week old mice, weighing 21–26 g obtained from Charles River Laboratories Japan, Inc. (Tokyo, Japan). In the acclimation period, four mice were placed in a cage and kept in a temperature-regulated room (23–25 °C)

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with controlled lighting (12/12 h light/dark cycles) and freely accessible water and diet. Basal diet (MF®) was obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan).

### 2.3. Effect of a single dose of FL on the excretion of CAs in urine

After being fed a basal diet for 14 days, the mice were divided randomly into two treatment groups as follows: vehicle (3% Tween 80 in distilled water) ($n$ = 8); 50 mg/kg FL ($n$ = 8). Animals were placed in individual metabolic cages (Figure1e) and were able to access food and water freely. Following a 48 h habituation period, 24 h urine, both pre- and post-oral administration of vehicle or FL, was collected using 20 μL of a 2.5 mol/L HCl containing tube as shown in Figure 1d. The oral administration of vehicle or FL was carried out between 10:00 and 11:00 AM.

### 2.4. Analyses of CAs in urine

We determined the concentration of urinary CAs such as noradrenaline (NA), adrenaline (AD) and their metabolites by treatment with the enzyme (sulfatase from Helix pomatia Type H-2, Sigma–Aldrich, St. Louis, USA) according to the previous report [36]. Briefly, the urine was heated for 10 min following incubation with 500 U/ml of enzyme at 37 ℃ for one hour. After addition of isoprenaline (Sigma–Aldrich, St. Louis, USA) as the internal standard, CAs were purified

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using a Monospin PBA (GL sciences, Tokyo Japan). The HPLC system (Prominance HPLC System Shimazu Corporation, Kyoto, Japan) consisted of a quaternary pump with a vacuum degasser, thermostatted column compartment, autosampler, and equipped with an electrochemical detector (ECD 700 S, Eicom Corporation, Kyoto, Japan). A reverse-phase column (Inertsil ODS-4 ; 250 × 3.0 mm ID, 5 µm, GL Sciences, Tokyo, Japan) was used, and the column temperature was maintained at 35 ℃. The HPLC mobile phase was 24 mM acetate–citrate buffer （pH 3.5)-$CH_3CN$ (100/14.1, v/v). The mobile phase flow rate was 0.3 mL/minute, and the injection volume was 20 µL. The eluents were detected and analyzed at 500 mV. Excretion of CAs were expressed as a ratio with urinary creatinine concentration measured using a LabAssay Creatinine (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan).

2.5. Effect of the repeated oral administration of FL on mouse adipose tissues

After being fed a basal diet for 14 days, mice were divided randomly into two treatment groups as follows: vehicle (3% Tween 80 in distilled water, $n = 11$); 50 mg/kg FL ($n = 11$). Each group was gavage administration with vehicle or 50 mg/kg bw FL for 14 days as shown in Figure 1f. At the end of the treatment period, to avoid suffering and the influence of anesthesia on blood CA dynamics, all the animals were sacrificed via decapitation by skilled researchers according to the experimental procedures.

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BAT, eWAT, and iWAT (Figure 3, Figure 4, and Figure 5) were collected from each mouse by dissection. BAT, eWAT, and 6 out of 11 of iWAT were snap-frozen in liquid nitrogen and stored at –80 °C until analysis. The other 5 out of 11 iWAT were used as frozen sections for histological observation.

## 2.6. Quantitative RT-PCR analysis

Total RNA was prepared from BAT and iWAT using TRIzol Reagent (Life Technologies, California, USA) and eWAT using QIAzol (Qiagen, USA) according to the manufacturers' instructions. Briefly, 10 μg of total RNA was reverse-transcribed in a 20 μL reaction volume with High-Capacity cDNA Reverse Transcription Kits (Life Technologies, California, USA). Real-time reverse-transcription PCR was performed using 50 ng total cDNA in the StepOne™ Real-Time PCR System (Life Technologies, California, USA). The primer and probe sequences were selected using a Taqman™ Gene Expression Assay (Life Technologies, California, USA) and the following genes: Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Mm99999915_g1), *β-actin* (Mm02619580_g1), *UCP-1* (Mm01244861_m1), *peroxisome proliferator-activated receptor γ coactivator* (*PGC)-1α* (Mm01208835_m1), *PR domain-containing* (*PRDM)16* (Mm00712556_m1), *CD137* (Mm00441899_m1), *transmembrane protein (TMEM)26* (Mm01173641_m1), and *T-Box Transcription Factor (TBX)-1* (Mm00448949) from Life

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Technologies; *GAPDH* and *β-actin* were used as internal controls. The buffer used in the systems was THUNDER BIRD Prove qPCR Mix (TOYOBO, Tokyo, Japan). The PCR cycling conditions were 95 °C for 1 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

2.7. 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 using the Bradford method. Protein (20 μg) was separated by SDS-PAGE using a 10–20% 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 α-tubulin (1:800; GR3224374-1, Abcam) and an antibody against Ucp-1 (1:1000; GR286375-2, Abcam). After the primary antibody reaction, the membrane was incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:100,000) for one hour. 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. The ratio of Ucp-1 to α-tubulin for

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each animal was calculated.

### 2.8. Histological analysis of iWAT

iWAT was fixed using Softmount (192-10301, FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan). Ten-micrometer-thick histological sections were cut and stained with hematoxylin and eosin (H&E). All observations were performed with an uplight microscope (CX41LF, OLYMPUS CORPRATION). Histological observation was carried out following H&E-stained slides at a magnification of 40 × using a Camera Control Pro 2, Nikon.

### 2.9. Data analysis and statistical methods

The data are expressed as means and standard deviations. Statistical analyses were performed using two-way ANOVA, followed by the post hoc Dunnett's test or the Student's *t*-test . $p < 0.05$ was considered significant, and $p < 0.1$ was considered to tend significance.

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## 3. Results

### 3.1. Twenty-four hours urinary CAs both pre-and post-oral administration of FL in mice

The results of excreted CAs and their metabolites in 24 h urine pre and post a single dose of vehicle or 50 mg/kg FL are shown in Figure 2. NA in the 24 h urine was almost similar levels of both the pre-and post-vehicle group and the pre-FL group; in contrast, a single oral dose of FL significantly increased NA excretion in urine (Figure 2a). Nearly similar results were obtained for the AD levels in urine as shown in Figure 2b. The total CAs, which is the sum of NA and AD, are shown in Figure 2c. A marked increase in total CAs excretion was induced by a single oral administration of FL (Figure 2c).

### 3.2. Effect of repeated oral doses of FL adipose tissues in mice

The results for thermogenic-related protein and gene expression in BAT are shown in Figure 3. The level of Ucp-1 tended to increase in BAT by the repeated administration of FL (Figure 3b). FL treatment significantly increased the mRNA expression of *UCP-1*, *PGC-1α,* and *PRDM16* in BAT (Figure 3c). In eWAT, there was no difference in the levels of Ucp-1 between the experimental groups (Figure 4b). In addition, *CD137* and *PGC-1α* were significantly upregulated, and TMEM26 also tended to increase in eWAT by the repeated gavage treatment of FL (Figure 4c). In histochemical observations, a multilocular morphology along with a reduction

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in cell size were shown in iWAT following FL administration (Figure 5b). The level of Ucp-1 also increased significantly in iWAT in the FL group compared to the vehicle group (Figure 5c). *PRDM16* mRNA expression was significantly increased in the FL group (Figure 5d).

## 4. Discussion

The activation of BAT and the formation of brown-like adipocytes called beige adipocytes, within WAT, has focused much attention as a therapeutic target for obesity and its complications [37]. These adipose tissues have attracted special interest because of their ability to dissipate energy and their possible ability to differentiate themselves from white adipocytes [38]. It was well investigated that browning of adipose tissue was induced by cold exposure [39], exercise [40], and caloric restriction [41]. These results indicate that browning is produced by the activation of the SNS [42]. SNS innervating adipose such as BAT or beige adipose plays a key role in promoting non-shivering thermogenesis.

Recently, it has been reported that dietary polyphenols, such as catechin [43] and resveratrol [44], were reported to activate BAT and possibly induce thermogenesis. We previously found that a single oral dose of FL induced enhancement of energy expenditure along with increased mRNA expression of *UCP*-1 in BAT [30]. These alterations induced by a single dose of FL were totally reduced by co-administration of a specific β3 blocker, SR52930 [31]. In addition, Choo et al. also reported that a body fat-suppressive effect following ingestion of green tea catechin was inhibited

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by the supplementation of a β blocker [43]. According to these previous results, it was suggested that administration of polyphenols activate SNS, resulting in thermogenesis through the β3 adrenergic receptor expressed in BAT.

In the present study, we first determined the amount of CAs excreted into urine following a single oral dose of FL. A significant increase in CAs in 24 h urine was observed following administration of FL (Figure 2). Determination of urinary CAs has been used to assess SNS activity inducing by the stressors in mammals [45]. Food components, as well as many physiological stress conditions (exercise, cold, and posture), are influencing plasma CAs levels and the physiological processes. It is also known that 60–70% of circulating CAs in plasma are sulfate conjugated forms, consequently, they are excreted in the urine[46]. Therefore, increasing CAs and their metabolites, following a single oral dose of FL, was considered the result in activation of SNS induced by FL.

Next, we examined the effect of the repeated oral doses of FL on adipose in mice. In the result of this study, the level of Ucp-1 tended to increase with the upregulation of its mRNA in BAT following the repeated oral doses of FL (Figure 3a,b). It has been well investigated that activation of the β3 receptor expressed in BAT or beige adipose induces the activation of the cAMP/PKA pathway, consequently upregulating *PGC1α* through the activation of p38 mitogen-activated protein kinase (MAPK) [47]. PGC1α is known to widely express in BAT, stimulating *UCP-1*

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expression and increasing the number of mitochondria with oxidative capacity, therefore essential for thermogenesis [48]. It has also been reported that PRDM16 and PGC1α were the transcription factors related to thermogenesis. They work together to induce the development of BAT phenotype by β3 activation [49]. In our present results, FL upregulated these two transcription factors (Figure 3c). These results suggest that the thermogenic formation of BAT is induced by repeated oral doses of FL.

It has been reported that beige adipose characterized by Ucp-1 expression and mitochondrial generation is developed in WAT in response to SNS activation [50]. Beige adipose represents specific markers and also has thermogenetic ability through activation of Ucp-1 similar to BAT. In particular, a distinct sub-population of WAT resident progenitors, which express the markers CD137 and TMEM26 on their surface, show a greater ability to differentiate into beige cells [51]. In the result of this study, there was a difference in response to FL between two types of WAT such as perigonadal eWAT and subcutaneous iWAT. Repeated 14-day gavage treatment of 50 mg/kg FL significantly increased Ucp-1 levels along with a multilocular morphology characterized as beige fat in iWAT (Figure 4). In contrast, upregulation of browning markers, such as *CD137* and *TMEM26*, was observed, but no changes in Ucp-1 levels was observed in eWAT even following the treatment of FL (Figure 3).

It was reported that mild SNS activation recruits different precursor cells, such as $Myh11^{+}$ or

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SMA$^+$ vascular smooth muscle cells [52], and Ebf2$^+$ or Pdgfrα$^+$ adipogenic cells [53] into iWAT, can be differentiated into beige adipocytes. If the SNS inactivation occurs, SNS-induced beige adipocytes lose Ucp-1 expression[54]. De-activated beige cells have a white adipose-like morphology but can be re-activated by an additional bout of β-adrenergic signaling [55]. It was also reported that the differentiation of Pdgfrα$^+$ adipogenic cells in eWAT was regulated by SNS activation, resulting in repeated differentiation white to beige or beige to white [56]. iWAT are reported to have highly susceptible to browning even with mild stimulation; in contrast, the eWAT of male mice are quite resistant to browning [55]. The differences in their response to FL observed in this study may occur in the differentiate ability difference between eWAT and iWAT. In the present study, we confirmed that FL activates SNS, resulting in the browning of adipose, but their mechanism of action is still unknown.

## 5. Conclusion

In conclusion, the results of this study showed that oral administration of FL activated SNS, consequently induced the activation of BAT, and promoted browning of WAT. It was suggested that the effect of FL was more sensitive on the subcutaneous iWAT than visceral eWAT. The mechanisms for the activation of SNS induced by oral administration of FL or FL-rich foods remain unclear; further elucidation through additional studies is needed.

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Author Contributions:

Conceptualization, N.O.; Data curation, Y.I. and O.M.; Formal analysis, T.F. and Y.F.; Investigation, Y.I., O.M., T.T., M.H., and M.O.; Methodology, Y.I., O.M., and Y.F.; Project administration, Y.F. and N.O.; Supervision, Y.F. and N.O.; Validation, T.F.; Visualization preparation, Y.I. and O.M.; Writing, N.O.

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Conflicts of Interest:

The authors declare no conflict of interest.

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Figure Legend

**Figure 1.** Composition of flavan 3-ols fraction(FL) and scheme of experimental procedures. Composition of flavan 3-ols fraction (FL, a), structure of catechins (b), structure of B-type procyanidins(c), scheme for the experimental procedure to determine urinary catecholamines (CAs) following a single dose of FL(d), photo of the metabolic cage (e), scheme for the experimental procedure to examine the effect of repeated administration of FL on adipose in mice.

**Figure 2.** Twenty-four hours urinary catecholamines (CA) both pre-and post-oral administration of vehicle or 50 mg/kg flavan 3-ols (FL) in mice. Noradrenaline (NA, a), adrenaline (AD, b), and total CAs (c).The values represent the mean ± standard deviation (each group, $n = 8$). ** $p < 0.01$, *** $p < 0.001$ (two-way ANOVA, followed by the post hoc Dunnett's test).

**Figure 3.** The effect of repeated oral administration of 50 mg/kg flavan 3-ols (FL) on mice

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brown adipose tissue (BAT). Photo of BAT (a), Ucp-1 levels determined by western blotting (b), mRNA expressions of *UCP-1*, *PGC-1α* and *PRDM16* (c). The values represent the mean ± standard deviation (each group, $n$ =11). # $p < 0.1$, ** $p < 0.01$, * $p < 0.05$, ** $p < 0.01$ (Student's $t$-test).

**Figure 4.** The effect of repeated oral administration of 50 mg/kg flavan 3-ols (FL) on mice epididymal adipose (eWAT). Photo of eWAT (a), Ucp-1 levels determined by western blotting (b), mRNA expressions of *UCP-1*, *PGC-1α* , *PRDM16*, *CD137*, *TMEM26* and *TBX-1* (c).The values represent the mean ± standard deviation (each group, $n$ =11). # $p < 0.1$, ** $p < 0.01$, * $p < 0.05$, ** $p < 0.01$ (Student's $t$-test).

**Figure 5.** The effect of repeated oral administration of 50 mg/kg flavan 3-ols (FL) on mice inguinal adipose (iWAT). Photo of iWAT (a), histochemical observation of iWAT with arrows point to multilocular morphology(b), Ucp-1 levels determined by western blotting (c), mRNA expressions of *UCP-1*, *PGC-1α*, *PRDM16*, *CD137*, *TMEM26* and *TBX-1* (d). The values represent the mean ± standard deviation (each group, $n$ =6). # $p < 0.1$, ** $p < 0.01$, * $p < 0.05$, ** $p < 0.01$ (Student's $t$-test).