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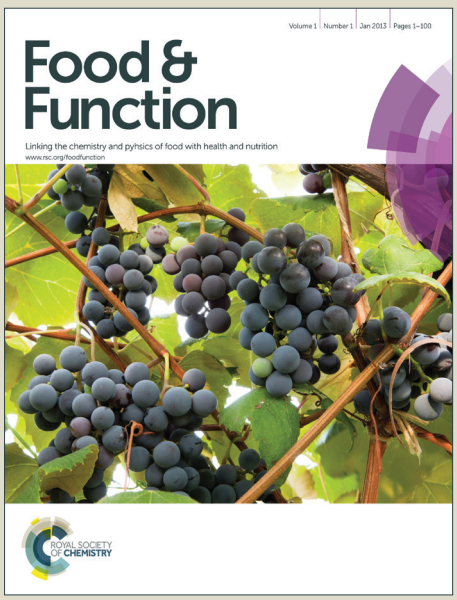

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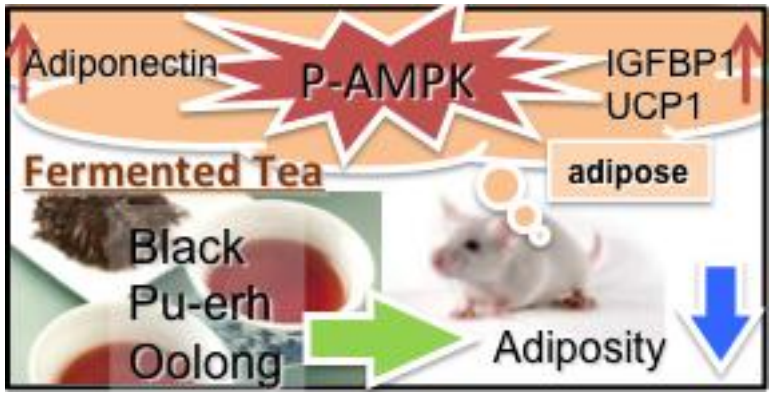

Oolong, black and pu-erh tea suppresses adiposity in mice via activation of AMP-activated protein kinase

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Abstract

It is well known that tea has a variety of beneficial impacts on human health, including anti-obesity effects. It is well documented that green tea and its constituent catechins suppress obesity, but the effects of other tea on obesity and its potential mechanisms involved are not yet fully understood. In this study, we investigated suppression of adiposity by oolong, black and pu-erh tea and characterized the underlying molecular mechanism *in vivo*. We found that consumption of oolong, black or pu-erh tea for a period of one week significantly decreased visceral fat without affecting body weight in male ICR mice. On a mechanistic level, consumption of tea increased phosphorylation of AMP-activated protein kinase (AMPK) in white adipose tissue (WAT). This was accompanied by the induction of WAT protein levels of uncoupling protein 1 and insulin-like growth factor binding protein 1. Our results indicate that oolong, black and pu-erh tea, in particular black tea, suppresses adiposity via phosphorylation of the key metabolic regulator AMPK and increases browning of WAT.

Keywords: acetyl-CoA carboxylase (ACC), AMP-activated protein kinase (AMPK), black tea, insulin-like growth factor binding protein 1 (IGFBP-1), pu-erh tea, uncoupling protein (UCP),

Introduction

With the exception of water, tea is the most consumed beverage in the world. There are four types of tea, namely, green tea (non-oxidized), oolong tea (partially oxidized), black tea (fully oxidized) and pu-erh tea (oxidized and fermented). Although catechins and their derivatives are the main polyphenolic components in tea, their levels vary with the degree of oxidation and/or fermentation. Caffeine level also varies during the production process of tea. A variety of recent cell, animal, and human studies have described the effects of tea, and in particular green tea[1-3], in preventing obesity and ameliorating energy consumption. Specifically, they have demonstrated that green tea, or components thereof, reduce adipocyte differentiation and proliferation, lipogenesis, fat mass, body weight, fat absorption, appetite, and plasma levels of triglycerides, free fatty acids, cholesterol, glucose, insulin, and leptin[1, 2, 4]. In contrast, however, the effects of other tea on these parameters are not well elucidated.

AMPK is an important therapeutic target for drugs in the prevention and treatment of obesity and diabetes mellitus[5], such as metformin and the thiazolidinediones[6, 7]. Activation of AMPK suppresses various anabolic processes, including fatty acid synthesis, cholesterol synthesis, and gluconeogenesis, and activates a variety of catabolic processes, such as fatty acid uptake and oxidation, and glucose uptake. AMPK is itself modulated by the adipokines leptin and adiponectin, which are pivotal regulators of whole-body energy metabolism[8-10]. Collectively, these results indicate that stimulation of AMPK is important for the prevention and treatment of diabetes and obesity.

Obesity results from an imbalance between energy uptake and expenditure, and excess dietary fat intake is one of the primary etiologic factors in the development of

this disease. In the last two decades, uncoupling proteins (UCPs) have attracted considerable interest in the context of energy metabolism and obesity [11, 12]. UCP-1 plays an important role in energy expenditure by regulating fat oxidation and heat generation in brown adipose tissue (BAT) and, moreover, UCP-2 and UCP-3 are also implicated in regulation of energy metabolism and the development of obesity[13, 14]. UCP-1 is involved in the formation of brown phenotype in white adipose tissue (WAT). Certain environmental and chemical stimuli, such as cold exposure and peroxisome proliferator-activated receptor γ (PPARγ), promote browning of WAT[15-19]. Recently, certain food components increase UCP-1 expression in WAT: For example, fucoxanthin from edible seaweed, *Undaria pinnatifida*, increases UCP-1 protein expression in WAT[19]. We also showed that a polyphenol-rich black soybean seed coat extract induces both mRNA and protein expression of UCP-1 in WAT[20]. These results indicate that browning of WAT accompanied by up-regulation of UCP-1 expression may contribute to reducing WAT weight.

In adipocytes, phosphorylation of AMPK activates glycerol-3-phosphate acyl transferase-1 and carnitine palmitoyltransferase-1, the principal enzymes in fatty acid metabolism, and inhibits phosphorylation of acetyl CoA carboxylase (ACC), a rate limiting enzyme for lipogenesis[21]. Moreover, phosphorylation of AMPK in WAT has been shown to result in upregulated expression of the gene encoding insulin-like growth factor binding protein-1 (IGFBP-1)[22]. IGFBP-1 is known to suppress sucrose-induced increases in body weight, adipose tissue weight, and serum leptin levels, and to inhibit IGF-1 induced adipocyte differentiation[23]. In preadipocytes, IGF-1 has been shown to activate the cAMP-response element binding protein via the IGF-1 receptor, resulting in up-regulation of PPARγ and CCAAT/enhancer binding protein (C/EBP) α expression[24].

Moreover, activation of AMPK reportedly inhibits adipocyte differentiation through repression of C/EBPβ, C/EBPδ, C/EBPα and PPARγ[25]. Collectively, these observations support the assertion that activated AMPK plays a pivotal role in ameliorating symptoms of obesity by stimulating fatty acid oxidation, adipocyte differentiation and energy metabolism. We previously found that oolong, black and pu-erh tea activates both insulin- and AMPK-dependent signaling pathways to induce glucose transporter 4 translocation in the skeletal muscle of ICR mice and to improve glucose intolerance in these animals[26]. In this study, we demonstrated that oolong, black and pu-erh tea inhibits adiposity *in vivo* by stimulating AMPK, and thereby upregulating levels of energy expenditure-related factors.

Experimental

Materials

Blood total-cholesterol and triglyceride levels were measured using commercially available kits from Wako Pure Chemical Industries (Osaka, Japan). For western blotting, anti-PPARγ-goat IgG, anti-C/EBPα-goat IgG, anti-UCP-1 goat IgG, anti-ACCα-rabbit IgG, anti-mouse IgG, anti-goat IgG, and anti-rabbit IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-AMPK rabbit IgG, anti-p-AMPK rabbit IgG antibodies, and anti-p- ACCα-rabbit IgG antibodies were from Cell Signaling Biotechnology (Tokyo, Japan); and anti-UCP-2 rabbit IgG antibody was purchased from Biolegend (Kyoto, Japan). Blocking-One solution was from Nacalai Tesque (Kyoto, Japan). Polyvinylidene difluoride (PVDF) membrane and ImmunoStar LD were products of GE Healthcare Bio-Science Co., (Piscataway, NJ, USA) and Wako Pure Chemical Industries, respectively. All other reagents used were of the highest grade

116 available from commercial sources.

117

118 **Preparation of tea**

119 Oolong, black, and pu-erh tea leaves were purchased from a local market in Kobe
120 city. Regarding oolong pu-erh tea, leaves were pulverized by a coffee mill and adjusted
121 the size of leaves through a 65-mesh sieve (0.291 mm). Tea was freshly prepared as
122 follows. Briefly, 2 g of tea leaves were extracted in 100 ml of boiled water for 2 min.
123 For oolong and pu-erh tea, the tea leaves were washed with 100 ml of boiled water for
124 15 seconds before extraction. The resulting extracts were cooled to room temperature
125 and used for experiments within a day.

126

127 **Measurement of total polyphenols**

128 The total polyphenol content in each tea was measured by the Folin-Ciocalteu
129 method [15]. Briefly, 10 µl of tea and 50 µl of folin-ciocalteu reagent were mixed with 790
130 µl of distilled water. After 1 min, 150 µl of 20% (w/v) aqueous Na_2CO_3 solution was
131 added, mixed, left at room temperature in the dark for 120 min. The absorbance of the
132 mixture was measured at 750 nm. The total polyphenol content was calculated from a
133 calibration curve using gallic acid as a standard compound.

134

135 **Measurement of catechins by liquid chromatography-tandem mass spectrometry**
136 **(LC-MS/MS) and caffeine by a high-performance liquid chromatography (HPLC)**

137 Tea extract was first centrifuged at 10,000 ×*g* for 10 min at 4°C. The resulting
138 supernatant was then subjected to reverse-phase HPLC and quadrupole tandem mass
139 spectrometry (4000 Q TRAP, AB SCIEX, Foster City, CA, USA). Catechins and

theaflavins were analyzed by LC-MS/MS according to our previous method[26]. Caffein were analysed by HPLC as follows: A portion of the extract (10 μl) was injected into a HPLC-UV (SHIMADZU, RF-20A xs series, Kyoto, Japan) equipped with a Supelco Discovery HS-PEG column (i.d. 4.1 × 250 mm, 5 μm, Sigma-Aldrich) and maintained at a constant temperature of 37 °C. The gradient system used solvent A [0.1% (v/v) phosphoric acid] and solvent B [0.1% (v/v) phosphoric acid-acetonitrile], and was programmed for 0-20 min (B 15-20%), 20-21 min (B 20-15%), and 21-31 min (B 15%) at a detection rate of UV 280 nm. Under these experimental conditions, caffeine was detected at 9.7 min (data was not shown).

Animal treatments

Two animal experiments with the same feeding design were carried out according to the 'Guidelines for the Care and Use of Experimental Animals' at Kobe University Rokkodai Campus (Permission # 21-07-02). Male ICR mice (4 weeks old for Experiment 1 and 6 weeks old for Experiment 2) were obtained from Japan SLC (Shizuoka, Japan) and maintained in a temperature-controlled room (23 ± 2°C) with a 12:12-h light/dark cycle (lights on at 9:00 am). Sixteen mice were given free access to tap water and commercial chow (Rodent lab diet EQ from Japan SLC) and were acclimatized for 7 days before the experiments. The mice were divided into four groups (n=5 mice/group for Experiment 1 and n=4 mice/group for Experiment 2), which were provided olong tea, black tea, pu-erh tea, or water, respectively, to drink for 7 days. All animals were given free access to commercial chow over the 7 days. Feces were collected during the experimental period in Experiment 2. Mice were sacrificed on day 7 without fasting, and perirenal, epididymis, mesenteric and subcutaneous (for only

Experiment 1) WAT was collected. In Experiment 1, another five mice were sacrificed on day 0, and mesenteric WAT and BAT were collected. Adipose tissue was washed in 1.15% (w/v) KCl, immediately frozen in liquid nitrogen, and stored at –80°C until use for western blot analysis.

Measurement of plasma and adiponectin lipids levels

Blood samples were collected and centrifuged at 9,600 ×*g* for 10 min at 4°C. The supernatant was collected and used as plasma for measurement of total-cholesterol and triglyceride levels using the appropriate commercial kit as described above. Plasma adiponectin levels were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Mouse/Rat High Molecular Weight Adiponectin ELISA Kit, Shibayagi, Gunma, Japan).

Western blot analysis

Tissue lysate was prepared from WAT as previously described[27] and used to detect protein levels of PPARγ, C/EBPα, IGFBP-1, UCP-1, UCP-2, ACCα, phosphorylated ACCα (p-ACCα), AMPK, and phosphorylated AMPK (p-AMPK). The protein bands were visualized using ImmunoStar LD (Wako) and detected with a light-Capture II (Atto Corp., Tokyo, Japan). The density of specific bands was determined using ImageJ image analysis software.

Preparation of mRNA and real time PCR analysis

An aliquot of WAT (60 mg each) was homogenized with 300 µl of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) by Polytron® homogenizer. The homogenate

was incubated for 5 min at room temperature and centrifuged at 12,000 × *g* for 15 min at 4°C. The supernatant containing RNA was mixed with 60 µl of chloroform and incubated at room temperature for 10 min. Then the mixture was centrifuged at 12,000 × *g* for 15 min at 4°C. The aqueous phase was transferred to another microtube. To precipitate the RNA, 180 µl of isopropyl alcohol was mixed, and incubated for 10 min at room temperature. The mixture was centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatant was discarded and the RNA pellet was dried at room temperature. The pellet was dissolved in 30 µl of deionized distilled water. After ethanol precipitation of the final elute, total RNA was re-dissolved in 30 µl of deionized distilled water. The quality and concentration of total RNA were measured by spectrophotometry. The RNA samples were purified by digesting the residual DNA using DNase I (Roche, Basel, Switzerland) according to the manufacturer's instructions. The DNase-treated RNA (5.0 µl) was reverse transcribed to cDNA in a reaction mixture (final volume, 20 µl) using ReverTra Ace® qPCR RT Kit (Toyobo Co., Ltd. Osaka, Japan). cDNA was then subjected to quantitative real-time PCR amplification using SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan). The forward and reverse primers used were as follows: Accα [forward 5'-tttcactgtggcttctccag-3′ and reverse 5'-tgcatttcactgctgcaata-3′], Adipoq [forward 5'-gaacttgtgcaggttggatg-3′ and reverse 5'-tgcatctcctttctctccct-3′], UCP-1 [forward 5'-ctgcactggcactacctagc-3' and reverse 5'-aaaggactcagccctgaaga-3'] and β-actin [forward 5'-ggtcatcactattggcaacg-3′ and reverse 5'-tccatacccaagaaggaagg-3′]. Reactions were run in a real-time PCR system (TaKaRa PCR Thermal Cycler Dice, Takara Bio). Relative gene expression levels were calculated by the comparative CT method[28], using the expression of the β-actin gene as an internal control. The results are expressed as the fold-change relative to the expression levels in the water group.

212

213 **Extraction and measurement of fecal lipids**

214 After freeze drying, feces were pulverized to powder in a mortar and pestle. An
215 aliquot of 200 mg fecal powder was suspended in 3 ml of distilled water. The
216 suspension was vortex mixed for 3 min with 4 ml chloroform/methanol=2:1 (v/v) and
217 centrifuged at 3,000×g for 10 min. The chloroform layer was collected and washed with
218 0.88% KCl (w/v) and centrifuged again at 3,000×g for 10 min. The chloroform layer
219 was collected and dried using a centrifugal thickener. For measuring of total lipid
220 content, the feces were weighed then dissolved in isopropyl alcohol containing 10%
221 (w/v) Triton X-100. Total cholesterol and triglyceride content were measured in the
222 solubilized lipids using the appropriate commercial kits.

223

224 **Statistical analysis**

225 The results are presented as the mean ± standard error (SE). Differences among
226 each group were analyzed using Dunnett's test. The level of significance was set at $p <$
227 0.05.

228

229 **Results**

230 **Total polyphenol content and polyphenol composition of oxidaized tea**

231 The total polyphenol content was measured by the Folin-Ciocalteu method. As
232 hown in Table 1, the total polyphenol content was similar in oolong and pu-erh tea (223
233 and 348 mg/l, respectively), while black tea contained a higher polyphenol content (893
234 mg/l). The composition of catechins and theaflavins (TFs) in each tea was determined
235 by LC-MS/MS. Regarding catechins, oolong tea mainly contained EGCg, GC and EGC,

and black tea mainly contained EGCg and ECg (Table 1). On the other hand, pu-erh tea contained much lower levels of catechins, particularly gallate-type catechins than oolong and black tea. The total amount of catechins was similar in oolong and black tea, but its content in pu-erh tea was approximately 30-40% lower than that in oolong and black tea. Black tea significantly contained abundant TFs, particularly TF and TF3g. These results the abundance of catechins and TFs was the greatest in black tea and the lowest in pu-erh tea. Caffeine content was similar in oolong, black and pu-erh tea (291, 338 and 341 mg/l, respectively).

Effect of oolong, black and pu-erh tea on body weight, food and beverage intake, and WAT weight

There was no significant difference in food and beverage intake between the control group and any of the three tea groups in both Experiment 1 and Experiment 2 (Table 2). Similarly, there was no effect of any tea used in this study on body weight, with the exception of a slight decrease in body weight in the black tea group that failed to reach statistical significance. In contrast, relative to the control group, consumption of black tea affected significant reductions in the weight of retroperitoneal, epididymal and mesenteric WAT and tended to reduce in the weight of subcutaneous tissue in Experiment 1 (Table 2). Oolong and pu-erh tea also affected weak reductions in the weight of WAT. In Experiment 2, the same trend was observed: all WAT were decreased by tea. From the results in Experiment 1, we calculated the lean body mass and obtained no difference among the mice groups. This indicated that the reduction of WAT by tea did not affect an increase in the weight of other tissues or organs.

Effect of oolong, black and pu-erh tea on lipid content of plasma and feces

Given the effect of tea on adiposity, we next evaluated their impact on plasma and fecal lipid content. In Experiment 1, black tea significantly decreased plasma cholesterol level. In Experiment 2, all tea groups significantly lowered plasma cholesterol level compared with water group (Table 3). None of the tea had any effect on plasma triglyceride level in both experiments. Since tea significantly decreased WAT weight, we investigated the excretion of lipids to feces in Experiment 2. However, excretion of lipids in feces did not changed by tea (Table 3).

Effect of oolong, black and pu-erh tea on the phosphorylation of AMPK

We previously showed that intake of oolong, black and pu-erh tea for a period of 7 days enhanced AMPK phosphorylation in the skeletal muscle of mice[26]. Therefore, we investigated whether oxidized and/or fermented tea exerted the same effect in WAT and BAT. Significant increases in the levels of p-AMPK in mesenteric WAT were found in response to consumption of black and pu-erh tea, but not oolong tea in both experiments (Figure 1). In Experiment 1, we also found that all tea significantly increased in the levels of p-AMPK in subcutaneous WAT and BAT (Figure 1A). Used tea had no effect on expression level of AMPK in both WAT and BAT. We further investigated the expression and phosphorylation levels of ACCα as one of the downstream target of AMPK in Experimental 1. As shown in Figure 2, black tea slightly increased the phosphorylation level of ACCα without significance. On the other hand, black and pu-erh tea significantly increased mRNA expression of ACCα, although these tea did not increase protein expression of ACC (Figure 2).

Effect of oolong, black and pu-erh tea on the expression of UCPs and IGFBP-1

Since UCP proteins have been linked to energy consumption in adipose tissues, we next determined effect of oolong, black and pu-erh tea on the expression levels of UCP in mesenteric WAT. As shown in Figure 3A, consumption of black tea, but not water, significantly increased protein expression level of UCP-1 in mesenteric WAT compared with non-treated control mice. In the same experiment, we confirmed that protein expression level of UCP-1 in BAT (as a positive control) was drastically higher than that in WAT of non-treated control mice, of which expression level of UCP-1 was weak with almost the same level as that in 3T3-L1 (as a negative control). Relative to the control group, consumption of black and pu-erh tea significantly increased mRNA and protein expression levels of UCP-1 (Figure 3B and 3C). Oolong tea had no effect on expression of UCP-1 in mesenteric WAT. In the case of UCP-2 expression, oolong, black and pu-erh tea showed increasing tendency of UCP-2 protein, but the consistent and significant increase was not observed (Figure 3).

Since the association between IGFBP-1 and body weight, adiposity and hyperglycemia, we next investigated adipose levels of IGFBP-1 in response to consumption of oolong, black and pu-erh tea. Relative to controls, consumption of all three types of tea significantly increased protein expression level of IGFBP-1 in mesenteric WAT in both experiments (Figure 4).

Effect of oolong, black and pu-erh tea on the expression of PPARγ and C/EBPα

PPARγ and C/EBPα have been characterized as master regulators of adipocyte differentiation. In Experiment 1, expression of PPARγ and C/EBPα showed an increasing tendency. In contrast, levels of adipose PPARγ were significantly repressed

in response to consumption of all three types of tea in mesenteric WAT, while significant decreases in adipose levels of C/EBPα were observed in response to consumption of pu-erh tea, but not oolong and black tea in Experiment 2 (Figure 5).

To confirm this opposite result, we measured mRNA expression and plasma levels of adiponectin in Experiment 1. As shown in Figure 6, oolong, black and pu-erh tea significantly increased mRNA expression of adiponectin in mesenteric WAT. Black tea revealed a significant increase in the plasma adiponectin level and oolong and pu-erh tea also revealed the increasing tendency.

Discussion

Tea is abundant in polyphenols such as catechins and theaflavins, which contribute to its various health-promoting effects[29,30]. In this study, we found that consumption in mice of black, oolong and pu-erh tea for a period of one week suppressed adiposity (Table 2) through phosphorylation of AMPK as a key metabolic regulator in mesenteric and subcutaneous WAT and BAT of ICR mice (Figure 1). The activation of AMPK led to increase the protein expression of UCP-1 (Figure 3) and IGFBP-1 (Figure 4). These are well-characterized downstream events of AMPK activation and we observed consistent results in two independent animal experiments. Thus, we assert that activation of AMPK played an important role in suppressing adiposity in mice that consumed oxidized tea, in particular black tea.

While previous studies of the effect of tea or catechins on activation of AMPK have been limited to the muscle or liver of animals, or to cultured adipocytes. For example, we previously reported that black, oolong and pu-erh tea activates AMPK in skeletal muscle of ICR mice to improve glucose intolerance[26]. Similarly, it has been

reported that pu-erh, green, and black tea suppress hyperlipidemia, hyperleptinemia and fatty acid synthesis via activation of AMPK in skeletal muscle of obese rats[31]. In addition, administration of epigallocatechin gallate (EGCg) stimulates p-AMPK and phosphorylated ACC levels in the livers of BALB/c mice, and in 3T3-L1 adipocytes[32]. Moreover, fermented rooibos hot water extract has been shown to inhibit intracellular lipid accumulation via induction of AMPK protein in 3T3-L1 cells[33]. In contrast to these studies, this is to our knowledge the first report that black and pu-erh tea stimulates the phosphorylation of AMPK in mouse mesenteric and subcutaneous WAT, setting off a series of downstream events that result in suppression of adiposity in these animals. As the active compound in tea for activation of AMPK, caffeine is one of the candidates in addition to catechins such as EGCg[32], because caffeine activates AMPK in skeletal muscle[34]. It is, however, unclear whether caffeine also activates AMPK in adipose tissue. In the current study, caffeine content was almost similar, but slightly lower in oolong tea than that in black and pu-erh tea. Significant AMPK activation was observed in mesenteric WAT of mice given black and pu-erh tea. Thus, it is possible that slight differences in the caffeine contents may affect the activation of AMPK.

Members of the UCP family of mitochondrial inner membrane proton transporters play a key role in thermogenesis and energy expenditure in BAT (primarily UCP-1), WAT (UCP-1 and UCP-2) and other tissues [11-14]. Increased UCP-1 expression has been shown to accompany appearance of a BAT-like phenotype in WAT cells (called browning) with overexpressing perilipin[35], a protein predominantly expressed on the surface of lipid droplets in fat cells[36]. To date, there have been certain studies describing the induction by food components of UCP-1 expression in WAT. For example, fucoxanthin from edible seaweed, *Undaria pinnatifida*, increases UCP-1 protein

356 expression in WAT[19]. A study from our group showed that a polyphenol-rich black
357 soybean seed coat extract induced UCP-1 in WAT [20], and catechins have been reported
358 to increase expression of the UCP-1 gene in rat BAT[37]. Our study is the first to show
359 that an intake of black and pu-erh tea increases levels UCP-1 in mesenteric WAT
360 (Figure 3), and leads us to suggest that black and pu-erh tea increases thermogenesis
361 and energy expenditure through promotion of browning of WAT, which will be
362 involved in the reduction of WAT mass. With regard to expression of UCP-2, oxidized
363 tea used in this study showed increasing tendency. Caffeine and epigallocatechin gallate
364 have been reported to increase levels of UCP-2 in rodent BAT and WAT[38,39]. Since
365 activation of AMPK promotes expression of UCPs[40], we suggest that the increase in
366 UCP-1 level by black and pu-erh tea is due, at least in part, to activation of AMPK.
367 Taken together, the current data indicate that black and pu-erh tea increases energy
368 expenditure by activation of AMPK and subsequent induction of UCP-1 protein.
369 In this study, consumption of all types of oxidized tea resulted in increased protein
370 level of IGFBP-1 in mesenteric WAT (Figure 4), which also would be involved in the
371 reduction of WAT mass. IGFBP-1 inhibits the action of IGF in stimulating metabolism
372 and growth, and cross-sectional studies have associated lower levels of IGFBP-1 with
373 the onset of metabolic syndrome and cardiovascular disease[41-43]. Since we have
374 previously shown that administration of green tea promotes IGFBP-1 levels in WAT[3],
375 we speculate that the reduced adiposity in response to consumption of oxidized tea that
376 we observed in the current study is related to up-regulation of IGFBP-1 in WAT.
377 IGFBP-1 is known to be induced by suppression of insulin levels, activation of PPARs,
378 and activation of AMPK[44,45]. Accordingly, although the mechanism by which oxidized
379 tea induced IGFBP-1 in the current study is unclear, we speculate that activation of

AMPK represents a strong possibility.

PPARγ and C/EBPα are master transcriptional regulators of adipocyte differentiation and lipogenesis[21,46]. In the current study, protein expression levels these transcriptional regulators showed opposite results; i.e., they showed increasing tendency by oxidized tea in mesenteric WAT of 5-week old mice, while they were decreased by tea in mesenteric WAT of 7-week old mice. Regarding this inconsistency, we assume that an intake of tea first increases the expression levels of PPARγ and C/EBPα, resulting in the formation of small-size adipocytes to produce adiponectin. Indeed our results demonstrated mRNA and plasama levels of adiponectin were increased by oxidized tea (Figure 6). Subsequently released adiponectin activates AMPK, and then activated AMPK down-regulates the expression of PPARγ and C/EBPα. Response to this sequential mechanism might differ from age of animal and observed expression pattern of PPARγ and C/EBPα were different in the current study. Results from previous studies and the current study suggest a model in which activation of AMPK by tea leads to increase the expression of IGFBP-1 and decrease the expression of PPARγ and C/EBPα in WAT, resulting in the suppression of adiposity. Time-dependent changes in the expression of PPARγ and C/EBPα and their expression levels in different growing stage of animal after intake of tea are unclear, and further experiments are needed to clarify these issues.

It has been reported that polyphenol composition in tea varies among different species and climate[47]. The polyphenol composition in of oolong, black and pu-erh tea showed the almost same as that in our previous report[26]. In the current study, we found that oolong and black tea contained higher amounts of catechins than pu-erh tea (Table 1). The level of GC and EGC were higher in oolong tea than in black tea, but that of

EGCg is almost same in both types of tea. Although EGCg plays an important role in the health-promoting effects of green tea[48], we speculate that the anti-adiposity effect of tea in the current study is not attributable to EGCg because the anti-adiposity effect of black tea is stronger than that of oolong tea. The levels of theaflavins in black tea were much higher than those in either oolong or pu-erh tea as expected. Although the bioavailability of theaflavins is considered to be relatively low, the strong anti-adiposity effect of black tea in the current study may be attributable to theaflavins. Indeed theaflavins have been shown to decrease hepatic lipid accumulation in HepG2 liver cells *in vitro*[49]. In the case of pu-erh tea, since the active compound in its prevention of adiposity is currently unclear, future studies will address this issue. In addition to these tea polyphenols, caffeine is also candidate of active compound. However, caffeine content was almost same level among tea used in the current study (Table 1). It is possible that caffeine may contribute to the suppression of adiposity by oxidized tea, because caffeine and its metabolite are reported to inhibit intracellular lipid accumulation without affecting adipocyte differentiation in 3T3-L1 adipocytes[50].

In conclusion, intake of oolong, black and pu-erh tea for one week suppressed adiposity via activation of AMPK-mediated signaling pathway including an increase of UCP-1 expression as a marker for browning of WAT. Thus, the activation of AMPK is, at least in part, involved in the underling molecular mechanism of anti-adiposity by oxidized tea. Our findings provide scientific evidence for the prevention of obesity by oolong, black and pu-erh tea in addition to green tea.

Acknowledgment

This work was supported in part by Social Coordination Funds for Promoting Science and Technology, Creation of Innovation Centers for Advanced Interdisciplinary Research Area (Innovative Bioproduction Kobe), MEXT, Japan.

Abbreviations

acetyl CoA carboxylase, ACC; AMP-activated protein kinase, AMPK; brown adipose tissue, BAT; CCAAT/enhancer binding protein α, C/EBPα; epigallocatechin gallate, EGCg; epigallocatechin gallate, EGC; insulin-like growth factor binding protein (IGFBP)-1, theaflavin, TF; Peroxisome proliferator-activated receptor γ, PPARγ; Polyvinylidene difluoride, PVDF; uncoupling protein, UCP; white adipose tissue, WAT.

Literature cited

1. S. Wolfram, Y. Wang and F. Thielecke, *Mol Nutr Food Res*, 2006, **50**, 176-87.
2. T.M. Rains, S. Agarwal and K.C. Maki, *J Nutr Biochem*, 2011, **22**, 1-7.
3. M. Ueda and H. Ashida, *J Agric Food Chem*. 2012, **60**, 8917-8923.
4. L. Ostrowska, J. Fiedorczuk and E. Adamska, *Rocz Panstw Zakl Hig.* 2013, **64**, 61-66.
5. D.G. Hardie, S.A. Hawley and J.W. Scott, *Physiol*, 2006, **574**(Pt 1), 5747-5715.
6. N. Musi, M.F. Hirshman, J. Nygren, M. Svanfeldt, P. Bavenholm, O. Rooyackers, G. Zhou, J.M. Williamson, O. Ljunqvist, S. Efendic, D.E. Moller, A. Thorell and L.J. Goodyear, *Diabetes*, 2002, **51** 2074-2081.
7. A. Gruzman, G. Babai and S. Sasson, *Rev. Diabet,* 2009, **6** 13-36.

8. J.R. Speakman and S.E. Mitchell, *Mol Aspects Med*, 2011, **32**, 159-221.
9. B.B. Kahn, T. Alquier, D. Carling and D.G. Hardie, *Cell Metab*, 2005, **1**, 15–25
10. D.G. Hardie, *Endocrinology*, 2003, **144**, 5179–5183.
11. O. Boss, P. Muzzin and J-P. Giacobiono, *Eur. J. Endocrinol,* 1998, **139**, 1-9.
12. D. Ricquier and F. Bouillaud, *Bouillaud Biochem J,* 2000, **345**, 161-179.
13. C. Tiraby, G. Tavernier, F. Capel, A. Mairal, F. Crampes, J. Rami, C. Pujol, J.A. Boutin and D. Langin, *Diabetologia*, 2007, **50**, 2190-2199.
14. N. Chen, R. Bezzina, E. Hinch, P.A. Lewandowski, D. Cameron-Smith, M.L. Mathai, M. Jois, A.J. Sinclair, D.P. Begg, J.D. Wark, H.S. Weisinger and R.S. Weisinger, *Nutr Res*, 2009, **29**, 784-793.
15. C. Vernochet, S.B. Peres, K.E, Davis, M.E. McDonald, L. Qiang, H. Wang, P.E. Scherer and S.R. Farmer, Mol Cell Biol, 2009, 29, 4714-4728..
16. A.Fuster, P.Oliver, J. Sánchez, C. Picó and A.Palou, Comp Biochem Physiol A Mol Integr Physiol, 2009, 153, 106−112.
17. N. Petrovic, T.B. Walden, I.G. Shabalina, J.A. Timmons, B. Cannon and J. Nedergaard, J Biol Chem, 2010 285, 7153-7164.
18. M.L. Bonet, P. Oliver and A. Palou, Biochim Biophys Acta, 2013, 1831, 969-985.
19. H. Maeda, M. Hosokawa, T. Sasgima, K. Funayama and K. Miyashita, Biochem Biophys Res Commun, 2005, 332, 392-397.
20. Y. Kanamoto, Y. Yamashita, F. Nanba, T. Yoshida, T. Tsuda, I. Fukuda, S. Nakamura-Tsuruta and H. Ashida. J Agric Food Chem. 2011, 59, 8985-8993.
21. A. Ejaz, D. Wu, P. Kwan and M. Meydani, *J Nutr.* 2009, **139**, 919–925.
22. M.S. Lewitt, *Biochem Biophys Res Commun,* 2001, **282**, 1126-1131.

476 23. K. Rajkumar, T. Modric and L.J. Murphy, *J Endocrinol*. 1999, **162**, 457-465.
477 24. Y.H. Tseng, A.J. Butte, E. Kokkotou, V.K. Yechoor, C.M. Taniguchi, K.M.
478 Kriauciunas, A.M. Cypess, M. Niinobe, K. Yoshikawa, M. E. Patti and C.R.
479 Kahn, *Nat Cell Biol,* 2005, **7**, 601−611.
480 25. B.B. Kahn, T. Alquier, D. Carling and D.G. Hardie, *Cell Metab,* 2005, **1**, 15–25.
481 26. Y. Yamashita, L. Wang, Z. Tinshun, T. Nakamura and H. Ashida, *J Agric Food*
482 *Chem*. 2012, **60**, 11366-11371.
483 27. Y. Yamashita, M. Okabe, M. Natsume and H. Ashida. *Arch Biochem Biophys,*
484 2012, **527**, 95-104.
485 28. K.J. Livak and T.D. Schmittgen, Methods, 2001,25, 402-408.
486 29. H.H. Chow and I.A. Hakim, *Pharmacol Res*, 2011, **64**, 105-112.
487 30. J.M. Hodgson and K.D. Croft, *Mol Aspects Med*, 2010, **31**, 495-502.
488 31. H.C. Huang and J.K. Lin, *Food Funct*, 2012, 3,170-177.
489 32. T. Murase, K. Misawa, S. Haramizu and T. Hase, *Biochem Pharmacol*, 2009 **78,**
490 78-84.
491 33. M. Sanderson, S.E. Mazibuko, E. Joubert, D. de Beer, R. Johnson, C. Pheiffer, J.
492 Louw and C.J. Muller. *Phytomedicine,* 2014, **21**, 109-117.
493 34. T. Egawa, T. Hamada, X. Ma, K. Karaike, N. Kameda, S. Masuda, N. Iwanaka
494 and T. Hayashi. *Acta Physiol (Oxf)*, 2011, **201**, 227-238.
495 35. T. Sawada, H. Miyoshi, K. Shimada, A. Suzuki and Y. Okamatsu-Ogura, *PLoS*
496 *One,* 2010, **5**, e14006.
497 36. A.S. Greenberg, J.J. Egan, S.A. Wek, N.B. Garty and E.J. Blanchette-Mackie, *J*
498 *Biol Chem,* 1991, **266**, 11341-11346.
499 37. S. Nomura, T. Ichinose, M. Jinde, Y. Kawashima, K. Tachiyashiki and K.

Imaizumi, *J Nutr Biochem,* 2008, **19,** 840-847.

38. A. Kogure, N. Sakane, Y. Takakura, T. Umekawa, K. Yoshioka, H. Nishino, T. Yamamoto, T. Kawada, T. Yoshikawa and T. Yoshida, *Clin Exp Pharmacol Physiol,* 2002, **29**, 391-394.
39. N. Chen, R. Bezzina, E. Hinch, P.A. Lewandowski, D. Cameron-Smith, M.L. Mathai, M. Jois, A.J. Sinclair, D.P. Begg, J.D. Wark, H.S. Weisinger and R.S. Weisinger. *Nutr Res*, 2009, **29**, 784-793.
40. S. Klaus, S. Keipert, M. Rossmeisl and J. Kopecky, *Genes Nutr.* 2012, **7**, 369-386.
41. A.H. Heald, J.K. Cruickshank, L.K. Riste, J.E. Cade, S. Anderson, A. Greenhalgh, J. Sampayo, W. Taylor and W. Fraser, *Diabetologia,* 2001. **44**, 333–339.
42. T. Kalme, M. Seppala, Q. Qiao, R. Koistinen, A. Nissinen, M. Harrela, M. Loukovaara, P. Leinonen and J. Tuomilehto, *J Clin Endocrinol Metab,* 2005, **90**, 1550–1556.
43. S.N. Rajpathak, A.P. McGinn, H.D. Strickler, T.E. Rohan, M. Pollak, A.R. Cappola, L. Kuller, X. Xue, A.B. Newman, E.S. Strotmeyer, B.M. Psaty and R.C. Kaplan, *Growth Horm IGF Res,* 2008, **8**, 166–173.
44. T.G. Unterman, D.T. Oehler, L.J. Murphy and R.G. Lacson, *Endocrinology,* 1991, **128**, 2693−2701.
45. A. Hilding, K. Hall, J. Skogsberg, E. Ehrenborg and M.S. Lewitt, *Biochem Biophys Res Commun*. 2003, **303**, 693-699.
46. Y. Gao, Y. Zhou, A. Xu and D. Wu, *Biol Pharm Bull*, 2008, **31**, 1716–1722.
47. Y.L. Lin, I.M. Juan, Y.L. Chen, Y.C. Liang and J.K. Lin, *J Agric Food Chem,*

1996, **44**, 1387-1394.

48. J.H. Weisburger and F.L. Chung. *Food Chem Toxicol.* 2002, **40**, 1145-1154.

49. C.L. Lin, H.C. Huang and J.K. Lin, *J Lipid Res.* 2007, **48**, 2334-2343.

50. H. Nakabayashi, T. Hashimoto, H. Ashida, S. Nishiumi and K. Kanazawa. *Biofactors.* 2008, **34**, 293-302.

Figure legends

Figure 1. Effects of oolong, black and pu-erh tea on p-AMPK levels in WAT and BAT.

ICR mice were given water (W), oolong tea (O), black tea (B), or pu-erh tea (P) for one week. Lysate was prepared from WAT and BAT and subjected to western blotting analysis to detect p-AMPK and AMPK expression. (A), mesenteric and subcutaneous WAT and BAT in Experiment 1; and (B), mesenteric WAT in Experiment 2. Each panel shows a typical result from five (Experiment 1) or four (Experiment 2) animals. Open, diagonal line, closed, and hatched bars represent the band densities for mice given water, oolong tea, black tea, and pu-erh tea, respectively. Values are means ± SE (n=4 or 5). *Significantly different from the corresponding control (water) group ($p < 0.05$; Dunnett's test).

Figure 2. Effects of oolong, black and pu-erh tea on ACCα expression and p- ACCα levels in mesenteric WAT.

ICR mice were given water (W), oolong tea (O), black tea (B), or pu-erh tea (P) for one week. (A), mRNA was prepared from WAT and carried out real-time PCR to estimate gene expression of ACCα. (B),WAT lysate was prepared and subjected to western blotting analysis to detect p-ACCα and ACCα expression. Each panel shows a typical result from five animals. Open, diagonal line, closed, and hatched bars represent relative (A) gene expression or (B) the band densities of protein for mice given water, oolong tea, black tea, and pu-erh tea, respectively. Values are means ± SE (n=5). *Significantly different from the corresponding control (water) group ($p < 0.05$; Dunnett's test).

555 **Figure 3.** Effects of oolong, black and pu-erh tea on levels of UCP-1 and UCP-2 in
556 mesenteric WAT.
557 ICR mice were given water (W), oolong tea (O), black tea (B), or pu-erh tea (P) for
558 1 week. (A), Lysate was prepared WAT (Cont), BAT and 3T3-L1 adopocytes (L1 cells)
559 and subjected to western blotting analysis to detect protein expression of UCP1. (B),
560 mRNA was prepared from WAT and carried out real-time PCR to estimate gene
561 expression of UCP-1 and β-actin. (C), WAT lysate was prepared and subjected to
562 western blotting analysis to detect protein expression of UCP-1, UCP-2 and β-actin.
563 Each panel shows a typical result from five (Experiment 1) or four (Experiment 2)
564 animals. Open, diagonal line, closed, and hatched bars represent the band densities for
565 mice given water, oolong tea, black tea, and pu-erh tea, respectively. Values are means ±
566 SE (n=4 or 5). *Significantly different from the corresponding control (water) group (p
567 < 0.05; Dunnett's test).
568
569 **Figure 4.** Effects of oolong, black and pu-erh tea on levels of IGFBP-1 in mesenteric
570 WAT.
571 ICR mice were given water (W), oolong tea (O), black tea (B), or pu-erh tea (P) for
572 1 week. WAT lysate was prepared and subjected to western blotting analysis to detect
573 IGFBP-1 and β-actin expression. Each panel shows a typical result from five
574 (Experiment 1) or four (Experiment 2) animals. Open, diagonal line, closed, and
575 hatched bars represent the band densities for mice given water, oolong tea, black tea,
576 and pu-erh tea, respectively. Values are means ± SE (n=4 or 5). *Significantly different
577 from the corresponding control (water) group (p < 0.05; Dunnett's test).
578

Figure 5. Effects of oolong, black and pu-erh tea on levels of PPARγ and C/EBPα in mesenteric WAT.

ICR mice were given water (W), oolong tea (O), black tea (B), or pu-erh tea (P) for 1 week. WAT lysate was prepared and subjected to western blotting analysis to detect PPARγ, C/EBPα and β-actin expression. Each panel shows a typical result from five (Experiment 1) or four (Experiment 2) animals. Open, diagonal line, closed, and hatched bars represent the band densities for mice given water, oolong tea, black tea, and pu-erh tea, respectively. Values are means ± SE (n=4 or 5). *Significantly different from the corresponding control (water) group ($p < 0.05$; Dunnett's test).

Figure 6. Effects of oolong, black and pu-erh tea on adiponectin gene expression levels in mesenteric WAT and released adiponectin levels in plasma.

ICR mice were given water (W), oolong tea (O), black tea (B), or pu-erh tea (P) for one week. (A), mRNA was prepared from WAT and carried out real-time PCR to estimate gene expression of adiponectin. (B), Plasma was prepared and subjected to ELISA to determine released adiponectin levels. Open, diagonal line, closed, and hatched bars represent (A) gene expression or (B) plasma adiponectin levels for mice given water, oolong tea, black tea, and pu-erh tea, respectively. Values are means ± SE (n=5). *Significantly different from the corresponding control (water) group ($p < 0.05$; Dunnett's test).

Table 1. Composition of catechin, theaflavin and caffeine in tea

Composition	oolong tea	black tea	pu-erh tea
Catechins (mg/l)			
C	4.86±0.85	6.86±1.11	3.25±1.23
EC	1.56±1.32	2.22±2.19	1.86±1.10
GC	19.37±5.80	5.61±1.58	4.85±1.22
EGC	13.39±2.01	5.15±0.85	5.86±0.84
ECg	4.32±1.82	9.18±5.46	2.80±1.20
Cg	0.18±0.05	0.20±0.01	0.01±0.01
EGCg	24.64±3.82	25.76±0.74	1.21±0.83
GCg	0.67±0.25	0.10±0.01	0.46±0.05
Total catechins	68.99±2.19	55.08±1.89	20.30±2.48
Theaflavins (mg/l)			
TF	0.24±0.12	20.40±1.25	0.02±0.01
TF3g	0.03±0.01	13.73±0.84	0.01±0.01
TF3'g	0.01±0.01	6.96±0.59	0.01±0.01
TF3,3'dg	0.02±0.01	8.55±0.54	0.02±0.01
Total theaflavins	0.30±0.08	49.64±0.13	0.08±0.01
Catechins plus theaflavins (mg/l)	69.16±2.27	104.72±2.01	20.38±2.49
Total polyphenol* (mEq/l)	223	893	348
Caffein (mg/l)	291±24	338±24	341±1

The catechin and theaflavin composition of tea was determined by LC-MS/MS, while caffeine content was by HPLC. Data are means ± standard deviation (n=3). The total polyphenol content was measured by the Folin-Ciocalteu method. *Total polyphenol content is expressed as mEq gallic acid/l.

C, catechin; EC, epicatechin; GC, gallocatechin; EGC, epigallocatechin; ECg epicatechin gallate; Cg, catechin gallate; EGCg, epigallocatechin gallate; GCg, gallocatechin gallate; TF, theaflavin; TF3g, theaflavin-3-gallate; TF3'g, theaflavin-3'-gallate; TF-3,3'-dg, theaflavin-3,3'-digallate.

Table 2. Effect of oolong, black and pu-erh tea on body weight, food and beverage intake and white adipose tissue (WAT) weight

	water	oolong tea	black tea	pu-erh tea
[Exp. 1]				
Body weight (g)	34.6±0.6	35.2±0.7	32.4±0.3	33.7±0.6
Lean body mass (g)	33.1±0.4	34.2±0.7	31.8±0.3	32.8±0.5
WAT weight (g/ 100 g body weight)				
Retroperitoneal	0.53±0.12	0.20±0.03*	0.16±0.03*	0.29±0.08
Epididymal	1.83±0.28	1.28±0.16	1.03±0.07*	1.36±0.08
Mesenteric	0.63±0.11	0.36±0.06	0.19±0.04*	0.25±0.05*
Subcutaneous	1.23±0.39	1.03±0.08	0.57±0.12	0.87±0.14
Food intake (g/day/head)	5.18±0.23	5.56±1.48	5.59±1.20	5.22±1.11
Beverage intake (ml/day/head)	8.79±1.27	8.48±1.07	7.70±1.76	8.10±1.11
[Exp. 2]				
Body weight (g)	39.3±0.6	38.8±0.6	37.5±0.8	38.4±0.5
WAT weight (g/ 100 g body weight)				
Retroperitoneal	0.77±0.06	0.37±0.06*	0.31±0.03*	0.43±0.07*
Epididymal	1.72±0.13	1.10±0.08*	0.99±0.07*	1.27±0.07*
Mesenteric	0.84±0.03	0.56±0.06*	0.52±0.06*	0.56±0.04*
Food intake (g/day/head)	6.65±0.18	6.55±0.15	5.95±0.23	6.50±0.11
Beverage intake (ml/day/head)	9.46±0.28	10.53±0.44	10.13±0.56	10.13±0.18

ICR mice were given oolong, black and pu-erh tea for one week. Body weight, food and beverage intakes were measured every day. The data are expressed as the mean ± SE.

618 Table 3. Effect of oolong, black and pu-erh tea on blood and fecal lipid concentration
619

	water	oolong tea	black tea	pu-erh tea
[Exp. 1]				
Plasma				
Cholesterol (mg/dl)	91.9±13.0	82.3±6.4	65.8±7.7*	82.8±7.8
Triglyceride (mg/dl)	181.5±17.6	165.9±20.0	153.6±12.1	169.4±17.4
[Exp. 2]				
Plasma				
Cholesterol (mg/dl)	143.2±3.6	130.2±3.9*	123.3±3.0*	124.4±3.1*
Triglyceride (mg/dl)	241.9±6.9	227.2±10.6	229.4±9.8	260±10.6
Feces				
Total lipid（mg/g）	26±1	31±2	24±1	27±3
Cholesterol（mg/g）	5.43±0.17	6.12±0.34	5.58±0.29	5.56±0.37
Triglyceride（mg/g）	7.93±0.34	9.23±0.92	8.04±0.26	7.75±0.25

620 ICR mice were given oolong, black and pu-erh tea for 1 week. During the feeding
621 period, the feces were collected and the lipid content analyzed. Data are expressed as
622 the mean ± SE.

Figure 1

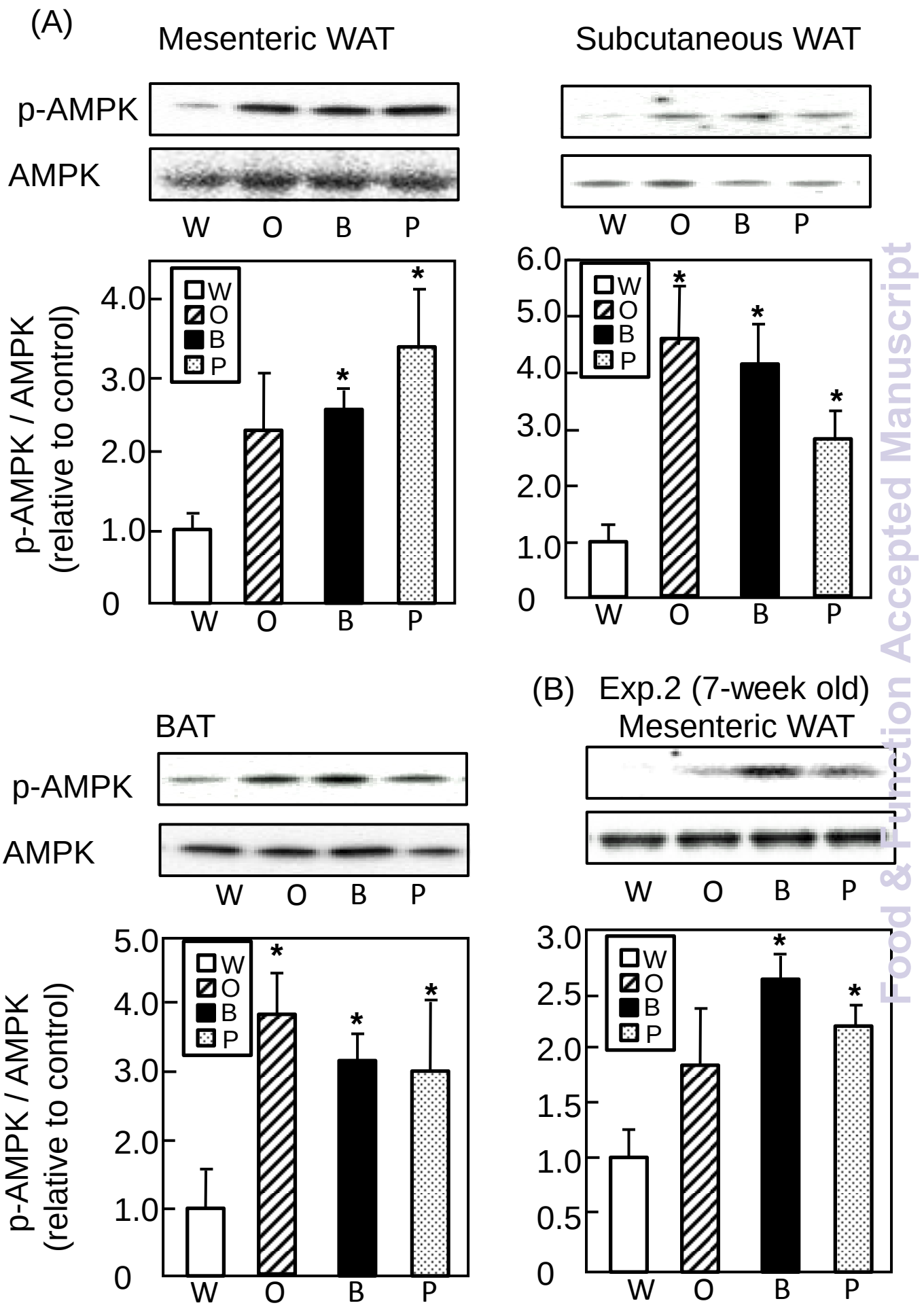


Figure 2

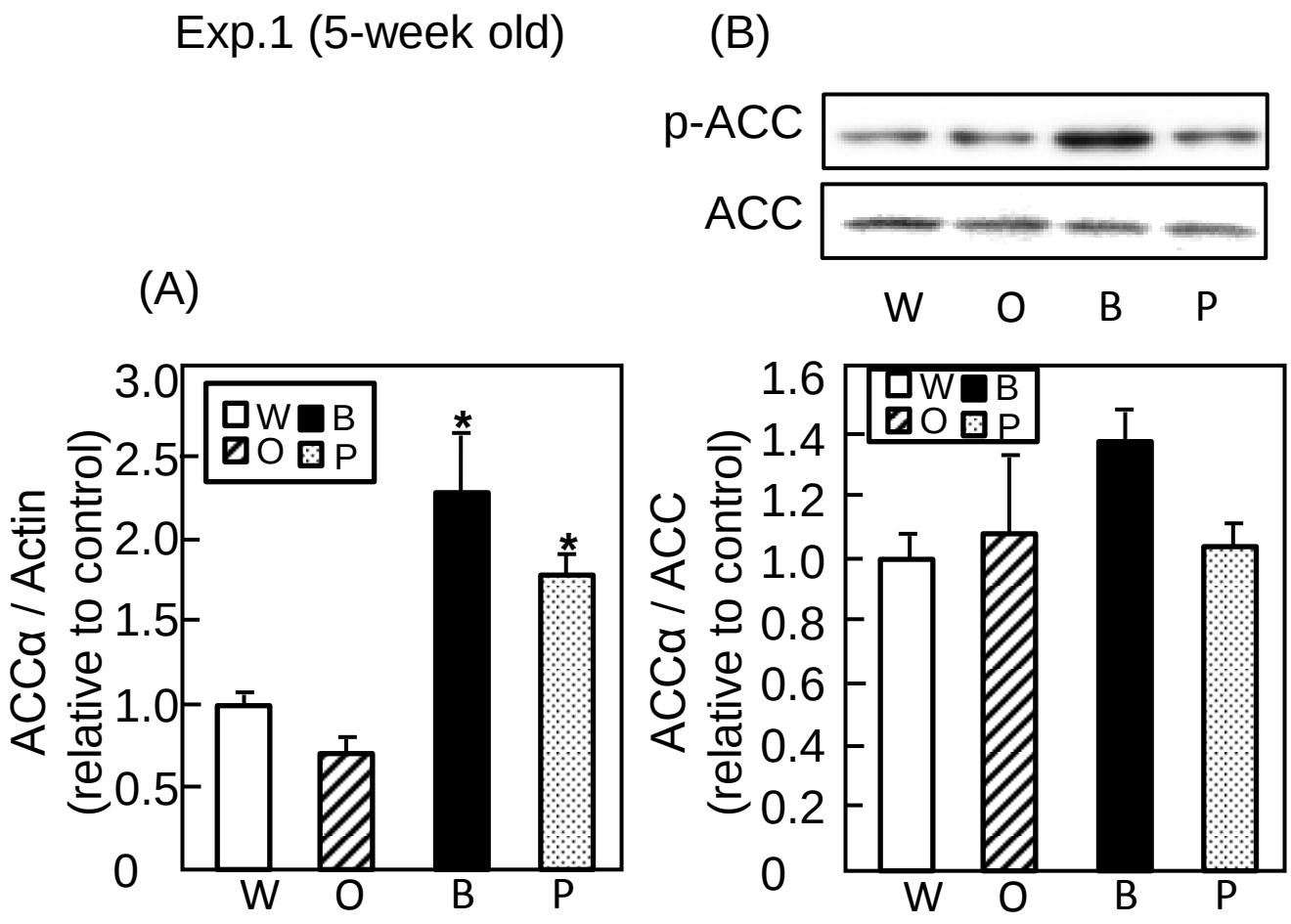
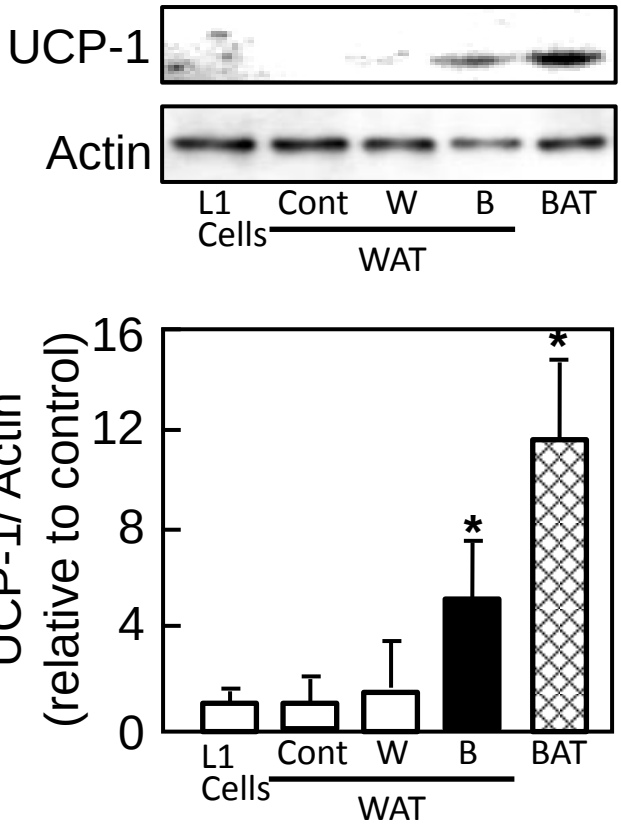


Figure 3

(A) Exp.1 (5-week old)



(B)

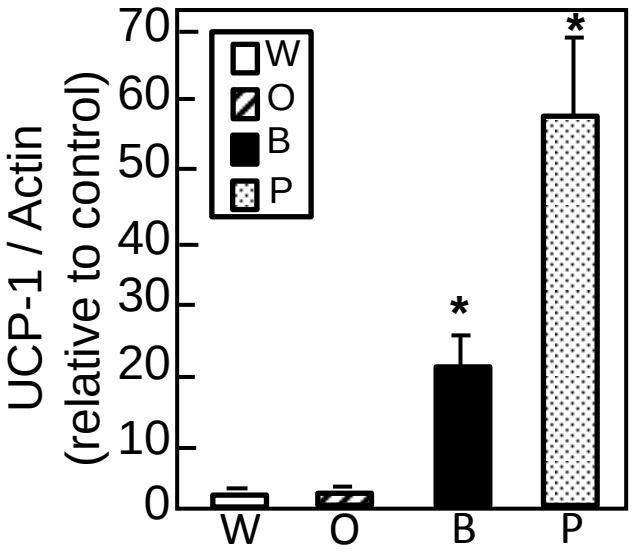
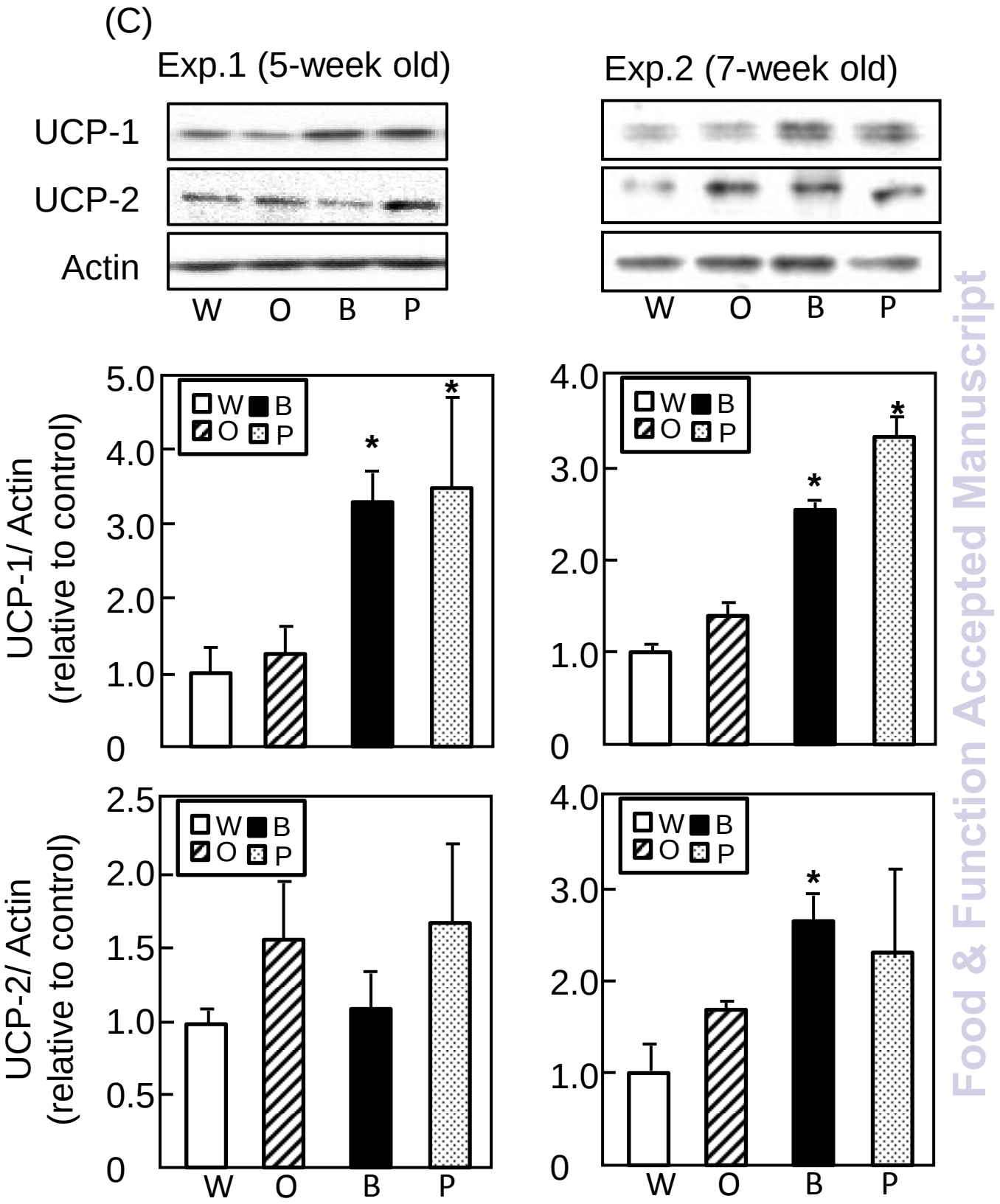


Figure 3



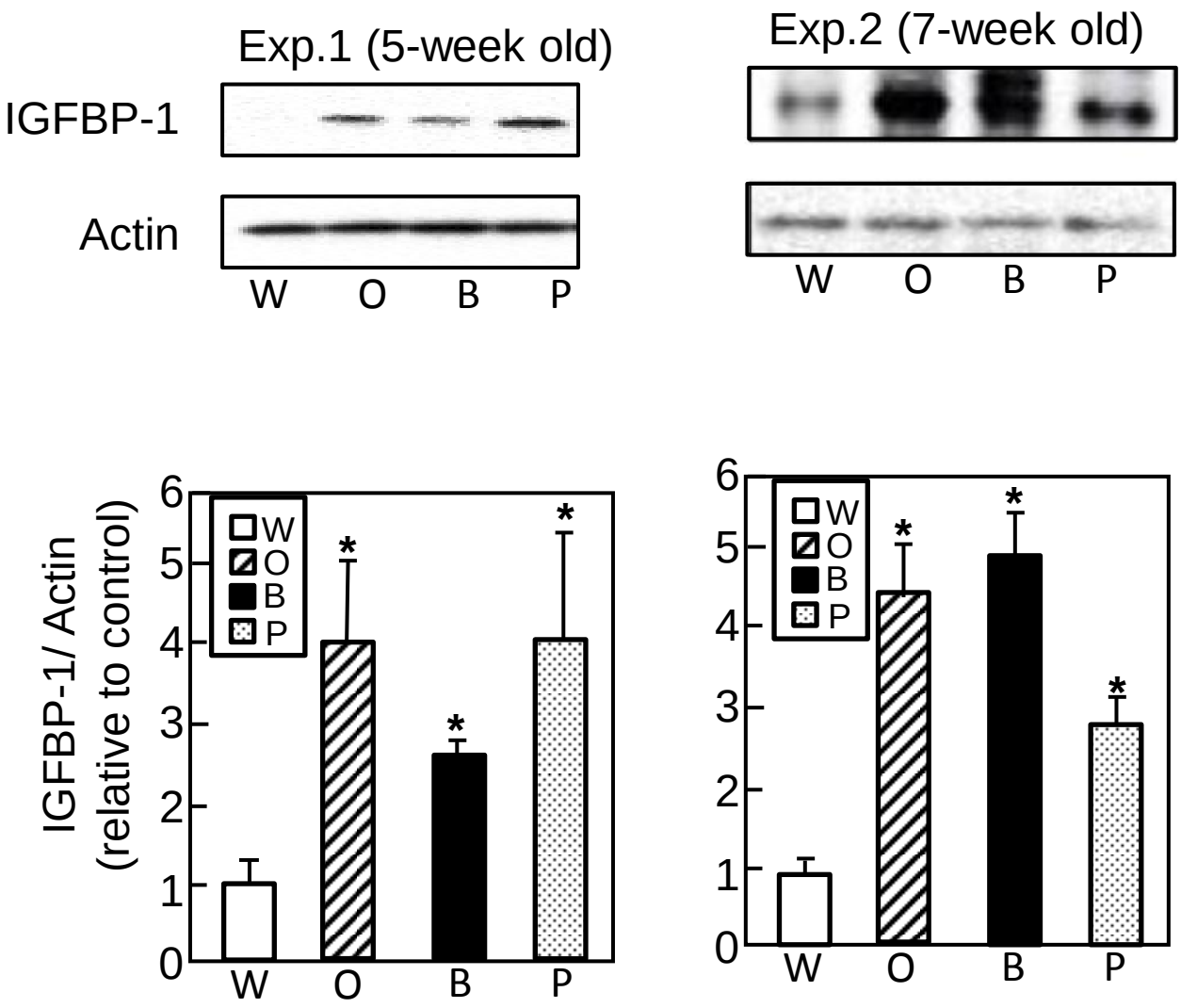


Figure 5