Biochemistry and Biophysics Reports 4 (2015) 417–424

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Tea polyphenols ameliorate fat storage induced by high-fat diet in *Drosophila melanogaster*

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ARTICLE INFO

Article history:
Received 10 June 2015
Received in revised form
27 September 2015
Accepted 28 October 2015
Available online 30 October 2015

Keywords:
Tea polyphenol
Catechin
Drosophila
Fat accumulation-suppressing effects
Fat body
Functional food

ABSTRACT

Background: Polyphenols in tea are considered beneficial to human health. However, many such claims of their bioactivity still require *in vitro* and *in vivo* evidence.
Results: Using *Drosophila melanogaster* as a model multicellular organism, we assess the fat accumulation-suppressing effects of theaflavin (TF), a tea polyphenol; epitheaflagallin (ETG), which has an unknown function; and epigallocatechin gallate (EGCg), a prominent component of green tea. Dietary TF reduced the malondialdehyde accumulation related to a high-fat diet in adult flies. Other physiological and genetic responses induced by the high-fat diet, such as lipid accumulation in the fat body and expression of lipid metabolism-related genes, were ameliorated by the addition of TF, ETG, and EGCg, in some cases approaching respective levels without high-fat diet exposure. Continuous ingestion of the three polyphenols resulted in a shortened lifespan.
Conclusion: We provide evidence in *Drosophila* that tea polyphenols have a fat accumulation-suppressing effect that has received recent attention. We also suggest that tea polyphenols can provide different desirable biological activities depending on their composition and the presence or absence of other chemical components.

1. Introduction

Throughout history, tea has been present in diverse parts of the world such as Asia, the Middle East, the Americas, and Europe, and in each region it is inextricably linked with various social customs. There are many types of tea, which are distinguished by their relative degree of fermentation: fully fermented, semi-fermented or minimally fermented [1]. In China, teas are categorized into six types according to degree of fermentation and manufacturing method. The best-known types of tea worldwide are black and green tea, which are representative fully fermented and minimally fermented teas, respectively. Although tea is normally consumed for its thirst-quenching ability and relaxing aroma, it has recently been receiving attention as a health-promoting beverage. These possible health-promoting effects are suggested to be due to tea's polyphenol content [2].

Green tea contains mainly catechins, especially epigallocatechin gallate (EGCg), whereas black tea contains theaflavins (TFs) and thearubigins in addition to catechins. [3]. Among the polyphenols contained in tea, EGCg has the best-known biological activity. EGCg is reported to have various potential activities such as an antioxidant effect *in vitro* [4] and anticancer [5], antimicrobial [6,7], and anti-inflammatory activity [8]. However, there is insufficient *in vivo* evidence of such activities although many studies using rodent models have been performed where polyphenols activated fatty acid oxidation. Yet, there are several disadvantages in using higher organism models such as rodents, such as the long-term observations and large number of animals needed to assess effects on lifespan and survival rates. The complexity of higher organisms also makes assessment of biological effects

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http://dx.doi.org/10.1016/j.bbrep.2015.10.013

difficult. Therefore, more extensive studies are required to elucidate the mechanism of each reported biological activity using lower organisms. As for TF, its acclaimed biological activities are not firmly established, neither *in vitro* nor *in vivo*. In a previous study of the production process of Fu tea, a dark post-fermented tea, we investigated how EGCg in tea leaves is derived and found that a large quantity of a specific catechin, epitheaflagallin (ETG), was produced *in vitro* from tannase and laccase enzyme treatments (in preparation). ETG, which has a molecular weight of 400.34, is possibly derived from EGCg as a result of a structural change induced by the loss of its galloyl group (Supplementary Fig. S1). Furthermore, it is likely to be a prominent component of fermented teas such as black tea. However, there are currently no reports on the possible bioactivity of ETG.

D. melanogaster is a classic model organism in genetics and in developmental biology but more recently has become the model organism of choice for investigating pathophysiology and disease prevention in humans, as the similarity of its genome, organs, and tissues to those of humans has become apparent. It is also used presently as a model organism for nutrigenomics, a method for investigating the effects of food composition on the body [9]. The best-known example of nutrigenomic studies of *Drosophila* is that of the lifespan-extending effect of resveratrol, a sirtuin gene-related molecule [10]. Other examples include studies assessing the effects on superoxide dismutase activity and mean lifespan extension of the intake of curcumin, which is the main compound contributing to the color of *Curcuma longa* [11], and of lutein, which is a major carotenoid present in most fruits and vegetables [12]. Our previous studies on adult *Drosophila* have established the effect of ingestion of freeze-dried royal jelly on enhancement of the number of eggs laid [13], and the effect of dipeptide-enriched diet on free amino acid profiles [14].

There are several studies on the biological activities of tea polyphenols in *Drosophila*, which have reported mean lifespan extension and antioxidant effects after ingestion, as seen in studies on curcumin and lutein [15,16]. However, these studies do not clearly specify which of the polyphenols in tea is partially or completely responsible for the reported biological activities, nor is it clear whether the activities represent the compound effects of multiple constituents. Furthermore, the independent *in vivo* biological activities of each of the four TFs (TF, theaflavin-3-galate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate) and of the novel polyphenol ETG have yet to be determined. The verification of a fat accumulation-suppressing effect for EGCg, and whether TF and ETG also possess similar properties must be performed in *Drosophila* as well. By using *Drosophila* in this study to assess the fat accumulation-suppressing effects of TF, ETG, and EGCg purified from tea extracts, we have provided insight to the systemic benefit of tea polyphenols on multicellular organisms and found indications that the beneficial bioactivity of these polyphenols can be selectively induced by varying their composition or by consuming them alongside other nutrients.

In this study, we investigated the possible biological activities of these three tea polyphenols—TF, ETG, and EGCg—on a systemic level. More specifically, we used *D. melanogaster* to assess whether these tea polyphenols have fat accumulation-suppressing effects, as the use of *Drosophila* allowed for the relatively simple investigation of the specific mechanism of TF, ETG, and EGCg's effects at the organismal level, compared to more complex animal models.

2. Materials and methods

2.1. Fly stocks, maintenance, and experiments

A w^{1118} line (stock number: 108479; identical to Iso31, isogenic w^{1118} stock in Bloomington Drosophila stock center #5905) was obtained from the Drosophila Genetic Resource Center (Kyoto, Japan) and used as the wild-type experimental animal. We previously established a transgenic fly line, called pplGG4 (w^{1118}; P{$w^{+mW.hs}$=GawB}ppl^{NP5440} P{w^{+mC}=UAS-EGFP}34 / TM6, P{w-=UAS-lacZ.UW23-1}UW23-1), by crossing the ppl-Gal4 (*y* w*; P{$w^{+mW.hs}$=GawB}ppl^{NP5440}/TM6, P{w^{-}=UAS-lacZ.UW23-1}UW23-1*) and UAS-GFP lines (*w^{1118}; P{w^{+mC}=UAS-EGFP}34/TM3, Sb^{1}*). Using meiotic recombination, the offspring containing ppl-Gal4 and UAS-GFP on the same 2nd chromosome were picked out for the establishment of the lineage. This line specifically expresses GFP in the fat body and so was used in experiments requiring precise dissection of the organ. Flies were raised under a 12 h light–dark cycle at 25 °C and 50% humidity on standard culture medium (standard food; SF) consisting of 10% (w/v) glucose, 7% (w/v) corn meal, 4% (w/v) yeast extract, and 0.55% (w/v) agar medium containing 0.3% (v/v) propionic acid and 0.35% (v/v) butyl *p*-hydroxybenzoate as antifungal agents, in keeping with our previous studies [13,17]. The fat-enriched diet was prepared by dissolving 5 or 15% (w/v) lard in SF. To take circadian rhythm into account, we performed all studies involving repeated observation and measurements at the same time each day whenever possible.

2.2. Measurement of dietary intake in adult Drosophila

A modified version of the CAFE assay described by Ja et al. [18] was used to quantify feed consumption. Liquid feed (5SY) containing 5% (w/v) sucrose, 5% (w/v) yeast extract, 0.01% (w/v) Brilliant Blue FCF, and 10% (v/v) EtOH was loaded on a 5 μl glass capillary (Hirschmann Laborgeräte, Eberstadt, Germany) and inserted into a 50 ml conical tube. The tube contained 5–17 adult male flies, which were allowed to feed for a fixed time (approximately 20 h). The quantity that was consumed was measured and evaporation was corrected for by subtracting the difference in starting and ending volume found in a similar setup that did not contain any flies. This corrected quantity was defined as the adult food consumption, and was divided by the number of flies and feeding time to determine the food consumption per h per fly for each feed. The tea polyphenols were dissolved in EtOH to create the liquid feed. As the 5SY contained Brilliant Blue FCF, we were able to verify intake of food from a blue coloration in the abdomen and feces of flies (data not shown).

2.3. Tea polyphenol preparation and supplementation

Feed containing TF, ETG, and EGCg was prepared. For TF, the enzyme-catalyzed product of catechins from China containing a mixture of TFs (TF, theaflavin-3-galate, theaflavin-3′-gallate and theaflavin-3,3′-digallate) was treated with tannase, then subjected to medium pressure liquid chromatography to separate the most TF-rich fraction. This fraction comprised more than 78% of the four types of TFs mentioned above, and did not contain any catechins or caffeine (Supplementary Fig. S2). For ETG, EGCg treated with tannase and laccase was adsorbed onto Diaion™ HP20 (Mitsubishi Chemical, Tokyo, Japan) that had been washed with 15% EtOH, and was then eluted with 35% EtOH. The collected fraction contained 31.1% ETG and no caffeine, epigallocatechin, or EGCg (Supplementary Fig. S1). For EGCg, commercially acquired purified (−)-epigallocatechin-3-gallate (Mitsui Norin, Tokyo, Japan) was used.

The tea polyphenol powders were dissolved in 100% EtOH to final concentrations of 0.01% (w/v), 0.1% (w/v) (for TF and ETG), 1% (w/v) (for ETG), and 1, 5, or 10 mM (for EGCg).

The polyphenols were confirmed to be stable in SF for at least 3 days (Supplementary Fig. S3). The SF mixtures containing TF, ETG, or EGCg were prepared freshly when needed and were refreshed after a maximum of 3 days.

2.4. MDA measurement

Fourteen-day-old adult flies from each experimental condition were collected and 10 individuals were pooled as one sample. The extraction and measurement of MDA were carried out using a malondialdehyde assay kit (KMD-008W; NIKKEN SEIL, Tokyo, Japan), according to the manufacturer's instructions. For each sample, the values were corrected for the total protein content and subsequently the relative amount was determined with control (0%, no TF) set as 1.

2.5. Oil red O staining

After hatching, the first instar larvae of the ppl-GG4 line were kept on SF with or without tea polyphenols until they became mid-third instar larvae (60 h after hatching). The resulting larvae were fixed in 4% (w/v) paraformaldehyde and the fat bodies were stained according to Gutierrez et al. [19].

2.6. RNA preparation, cDNA synthesis, and Quantitative RT-PCR

Fat bodies of third instar larvae (60 h after hatching) in each treated group were collected and the RNA of each treated group was extracted with RNAzol RT (Molecular Research Center, Cincinnati, OH). First-strand cDNA was synthesized using ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) as a second PCR template. Relative quantification of lipid metabolism-related gene expression was performed using a set of primers against the *Acox57D-d* (5′-CCACGAGATACTCGGCAGTG-3′, 5′-CCGTAGCGATCTCAGGGAAG-3′), *Acsl* (5′-AATTCTGCTTCCGGCAAGTG-3′, 5′-AACGAAGCCGATTCTGTTGC-3′), *FABP* (5′-TGTAGACGCGCACGCACTTA-3′, 5′-AGCATCATCACCCTGGATGG-3′), and *CCt1* (5′-ATGAACGGCTTCGAGCGATA-3′, 5′-AGAGTCCATGGCGCATTCTG-3′) transcripts as well as *rp49* [20]. Quantitative RT-PCR was performed on a ROTOR-GENE 6000 (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with SYBR green–based detection of PCR products. Relative quantification of each target gene expression was done against *rp49*.

2.7. Adult longevity assay

A longevity assay was performed as described in our previous study [17]. Briefly, in each lifespan experiment, newly eclosed adult flies (within 8 h of eclosion) of each treated group were collected, anesthetized with diethyl ether, and separated into virgin males and females. Flies ($n \leq 20$) were placed in a single vial (diameter 28 mm, length 100 mm) containing SF and transferred every 3–4 days to a fresh vial. The number of dead flies was recorded until no living flies remained.

2.8. Statistical analysis

Statistical analysis was performed using the Tukey–Kramer *post hoc* test. In survival, the Kaplan–Meir method was used to estimate this curve from the observed survival time without the assumption of an underlying probability distribution. Statistical significance was set considered at $p < 0.05$.

3. Results

3.1. Dietary tea polyphenols in Drosophila adults

To investigate the possible biological activities of tea polyphenols in adult *Drosophila*, it was first necessary to verify the intake and the possible adverse effects of the tea polyphenol samples. We used a modified version of the capillary feeding (CAFE) assay [18], which measures adult *Drosophila* food intake. The addition of TF, ETG, and EGCg to the feed did not influence the amount consumed by the 5-day posteclosion adult *Drosophila* (Supplementary Fig. S4). There were no significantly differences for survival rates between the groups receiving tea polyphenols during the 20 h administration period (data not shown). This indicates that TF, ETG, and EGCg consumption by adult *Drosophila* in not problematic.

3.2. TF function for high fat diet-induced rapid accumulation of lipid hydroperoxide and early death

High-fat diet (HFD) intake enhances lipid hydroperoxidase (LPO) accumulation and reduces the survival of adult *Drosophila* [21]. The increase of LPO leads to the increase of malondialdehyde (MDA), a product of lipid peroxidation and an important indicator of LPO. We made use of this effect and assessed the change in the amount of MDA and the rate of survival when TF was added to the HFD. The lifespan of *Drosophila* adults is influenced by temperature; and at high temperature, lifespan decreases [22,23]. We therefore assumed that high-temperature conditions place additional stress on *Drosophila* adults placed on a HFD, and thus performed the experiment at 27 °C. As reported by Li et al. [21], the median survival of *Drosophila* adults on the HFD decreased with higher lard concentration in the feed. Median survival of the group receiving standard food (SF) with an addition of 15% lard as an HFD (20) was 33.4% lower than that of the group receiving SF alone (30) ($p < 0.01$, Supplementary Fig. S5A and S5B). There was no difference in survival rate between groups when TF was included in the HFD as compared to the groups receiving no lard (Supplementary Fig. S5A and S5B). However, analysis of MDA accumulation in the body of 14-day-old *Drosophila* adults revealed that the addition of 0.01% TF to a 15% lard HFD resulted in MDA accumulation 1.3 times that of the SF group, whereas in the group given a 15% lard HFD with no TF, MDA accumulation was 2.5 times that of the SF group. This represented a 46.6% reduction of MDA accumulation between the two 15% lard HFD groups (Fig. 1). These results indicate that although TF does not ameliorate the HFD-induced reduction in lifespan, it can reduce the early-stage accumulation of LPO.

3.3. Effect on lipid metabolism and inflammation

The lipid accumulation-suppressing effect of tea polyphenols at the cellular and tissue levels was investigated. We focused on the fat body, which is the equivalent structure in *Drosophila* of the liver and white adipose tissue in humans, and some previous studies have reported that lipid accumulates in the fat body during ingestion [19,24]. We previously established the pplGG4 *Drosophila* lineage, which specifically expresses green fluorescent protein (GFP) in the fat body (see Experimental procedures). The GFP-labeled fat bodies of the mid-third instar larvae were excised and stained with Oil Red O to determine lipid accumulation. The whole fat body of the mid-third instar larvae bred on SF was stained red, indicating lipid accumulation, except for the marginal region, which was unstained and presumably does not accumulate lipids (Fig. 2A). However, the fat body of the same stage of larvae bred on an HFD was stained almost completely (Fig. 2B), indicating enhancement of lipid accumulation in the fat body. Using this simple

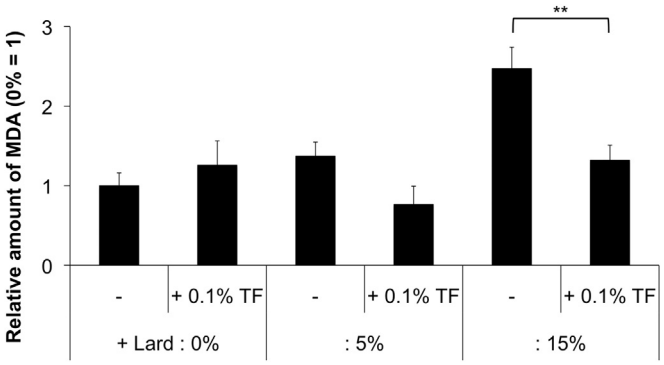

Fig. 1. TF inhibits high fat diet-induced rapid accumulation of lipid hydroperoxide Animals were reared on: 0%, standard food (SF) control; 0%+TF, SF containing 0.01% (w/v) TF mixture (TF, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate); 5%, SF containing 5% (w/v) lard; 5%+TF, SF containing 5% (w/v) lard and 0.01% (w/v) TF; 15%, SF containing 15% (w/v) lard; or 15%+TF, SF containing 15% (w/v) lard and 0.01% (w/v) TF. Malondialdehyde (MDA) accumulation in 14-day-old adults in each treated group. n=9 (0%), 12 (0%+TF), 11 (5%), 10 (5%+TF), 4 (15%), and 7 (15%+TF) in groups of 10 animals each. Data are represented as mean ± SE. $^{**}p < 0.01$.

phenomenon, we assessed the influence of TF, ETG, and EGCg on lipid accumulation. The intake of any of the three polyphenols together with the HFD led to a reduction of Oil Red O staining compared to HFD intake alone, revealing some clusters of unstained cells that were similar to those seen in the SF fat body (Fig. 2C–E). This effect was especially strong for ETG, where numerous unstained cells were present throughout the marginal region (Fig. 2D). This result indicates that tea polyphenol supplementation moderates the lipid accumulation caused by HFD, either due to an enhanced lipid metabolism or the suppression of lipid accumulation.

3.4. Effects of TF, ETG, and EGCg on fat metabolism-related gene expression

Lipid metabolism-related gene activation is regulated by its own expression. To further clarify the involvement of TF, ETG, and EGCg in lipid metabolism, we used quantitative reverse-transcription polymerase chain reaction (RT-PCR) to investigate the effect of adding TF, ETG, and EGCg to the HFD on the expression of lipid metabolism-related genes in the fat body. Differences between gene expression levels in *Drosophila* fed a HFD and those fed SF were observed for all investigated genes. Among the genes involved in lipid catabolism, the expression of *Acyl-coenzyme A oxidase at 57D distal (Acox57D-d)*, *Acsl*, *FABP*, and *CCt1* tended to be decreased on the HFD. These changes in gene expression were moderated when TF, ETG, or EGCg was added to the HFD (Fig. 3). The moderating effects were strongest with EGCg for *Acox57D-d* (Fig. 3A), TF for *Acsl*, *FABP* (Fig. 3B and C respectively), and TF or ETG for *CCt1* (Fig. 3D). These results suggest that TF, ETG, and EGCg can prevent the changes of expression in lipid metabolism-related genes induced by an HFD and thereby improve lipid metabolism.

3.5. Effects of TF, ETG, and EGCg on adult longevity

We investigated the effect of TF, ETG, and EGCg ingestion on adult lifespan. The median lifespan ± SD of *Drosophila* kept on SF without addition of tea polyphenols was 55 ± 16.2 days for females and 49 ± 12.8 days for males. No extension was observed in the median lifespans of groups receiving SF with tea polyphenols compared to those receiving SF alone. On the contrary, the ingestion of the polyphenols led to significant decreases in lifespans (Fig. 4). The median lifespan of females kept on SF containing TF was 55 ± 15.2 days, which did not differ from that of its counterpart on SF alone; however, the lifespan of males kept on TF was 46 ± 13.6 days, which was 6% lower than that of the SF group ($p < 0.01$). For the group kept on SF containing ETG, median

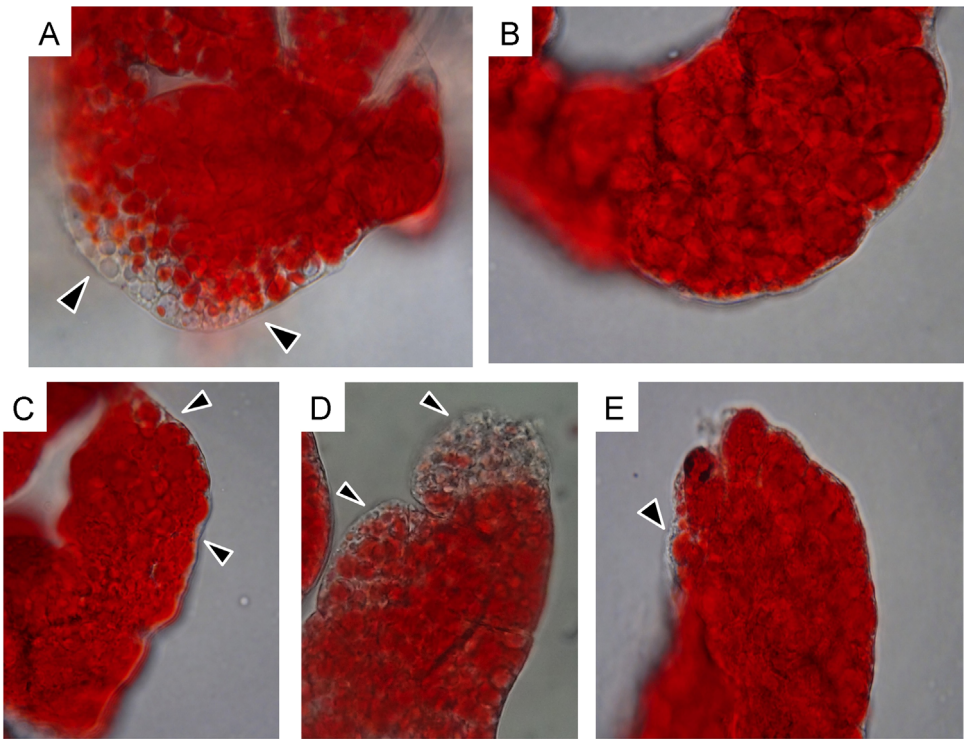

Fig. 2. TF, ETG, and EGCg ameliorate high fat diet-induced fat storage in fat body of third instar larvae Fat body of mid third instar larvae reared on (A) SF, (B) SF+15% lard (HFD), (C) HFD+0.1% TF, (D) HFD+0.1% ETG, or (E) HFD+10 mM EGCg. All presentations are of close to the same region (anterior section of fat body, near central nervous system). Arrowhead represents fat cells where no Oil Red O staining was detected.

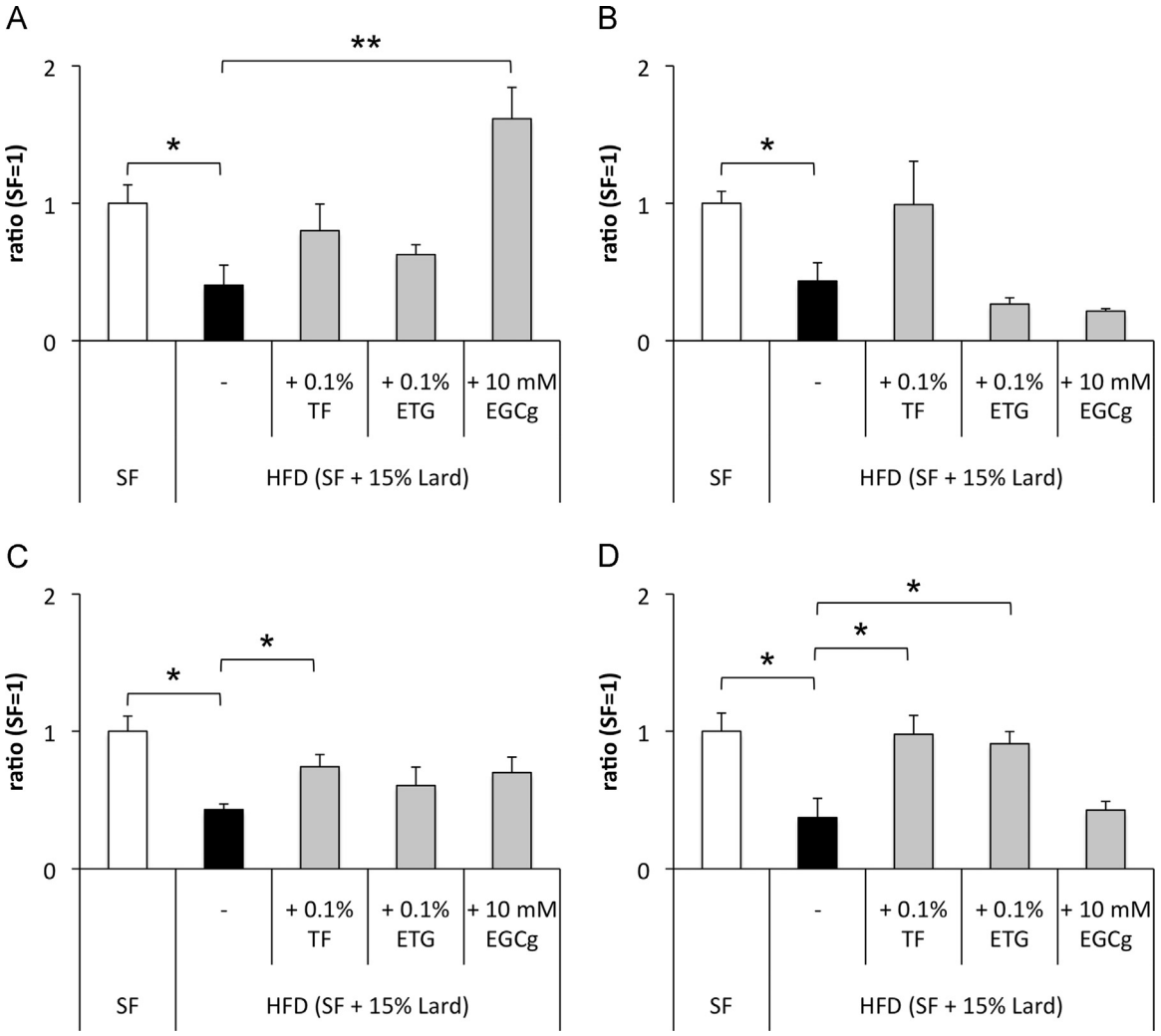

Fig. 3. Dietary TF, ETG, and EGCg ameliorates high fat diet-induced alteration of lipid metabolism-related genes expression in fat body Relative gene expression in fat bodies of (A) Acox57D-d (acyl-Coenzyme A oxidase at 57D distal, CG9709), (B) Acsl (Acyl-CoA synthetase long-chain, CG8732), (C) fabp (fatty acid binding protein, CG6783), (D) CCt1 (CTP: phosphocholine cytidylyltransferase 1, CG1049). The rp49 gene was used as the reference in calculations of relative transcript levels of the genes tested. $n=3$–5 groups of 2–3 fat bodies. Data are represented as mean ± SE.

lifespan was 49.5 ± 17.7 days for females and 48 ± 11.5 days for males, which was 10% and 2% lower, respectively ($p < 0.05$), than that of their counterparts on SF alone. Females kept on 1 mM EGCg appeared to have a 5% increased median lifespan compared to females on SF (58 ± 16.9 days) but this change was not significant ($p=0.06$, log-rank test). The median lifespan of males on 1 mM EGCg was 47 ± 11.3 days, 4% lower than that of males kept on SF ($p < 0.01$). The most prominent lifespan-shortening effect was observed for 10 mM EGCg; the median lifespan for females was 48 ± 15.4, 12.7% lower than that of the SF group, and 43 ± 11.8 for males, 12.2% lower than that of the SF group ($p < 0.01$). Contrary to the reports that have shown lifespan-extending effects in tea extracts [15,16], our results indicate that TF, ETG, and EGCg decrease lifespan.

4. Discussion

4.1. Drosophila does not avoid consuming TF, ETG, and ECGg

Quantitative analysis of food intake revealed that TF, ETG, and EGCg were ingested by adult *Drosophila* without incidence. The reason why plants produce catechins is not fully understood. The most plausible explanation is that catechins are produced for the removal of the reactive oxygen species that accumulate as a result of photosynthesis [25,26]. Another possible explanation is for plants to protect themselves from insects and bacteria that attempt to consume or inhabit them [27,28]. Indeed, some compounds produced by plants are toxic to insects, such as pyrethrin produced by *Tanacetum cinerariifolium* [29] and phrymarolin produced by *Phryma leptostachya* subsp. *asiatica* [30], and are therefore utilized in insecticides [31]. Of the observed polyphenols, EGCg is reported to have antibacterial, antifungal, and antiviral effects [6,7]. Because the bitter and astringent flavor that humans can detect in tea is possibly caused by the activation of transient receptor potential channel A1 by polyphenols such as EGCg [32], it is possible that these compounds are produced by plants to discourage consumption by mammals as well, including humans. However, as *Drosophila* did not avoid consuming TF, ETG, and ECGg, and there were no deaths resulting from the consumption, it seems that *D. melanogaster* (which is not herbivorous) is an appropriate model organism for the investigation of the biological activities of these polyphenols.

4.2. Effect of tea polyphenols on fat metabolism

The intake of TF did not ameliorate the HFD-induced reduction

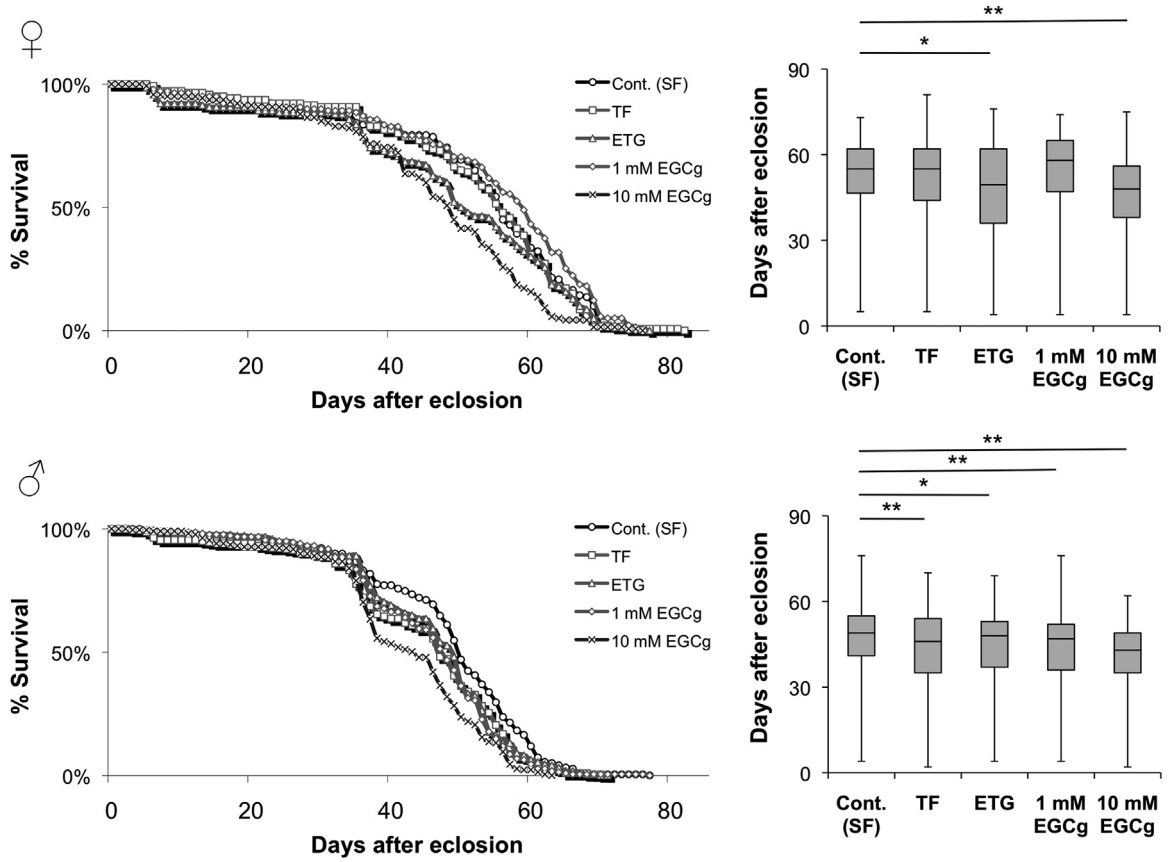

Fig. 4. Dietary TF, ETG, and EGCg induce a short-lived phenotype Survival curves by sex (female, upper left; male, lower left) of w^{1118} flies reared on TF, ETG, 1 mM EGCg, 10 mM EGCg, and SF as a control. Profiles for lifespan of the female flies (upper right) and the male flies (bottom right) are shown as box plots. Boxes and median lines represent inter-quartile range and median values of data, vertical lines represent minimum and maximum values of data. Sample sizes: female, $n=140$ (SF, TF, ETG, 10 mM EGCg), 139 (1 mM EGCg); male, $n=220$ (TF, ETG, 1 mM EGCg), 219 (SF, 10 mM EGCg). * $p<0.05$, ** $p<0.01$, Wilcoxon signed-rank test.

of survival rate (Supplementary Fig. S5). It has been reported that the diminishing of early stage survival caused by 10% dietary lard was rescued by simultaneous intake of 10 mg BTE [16]. The TF used in the present study is different from BTE as it does not contain catechins other than TFs, such as EGCg. It is possible that the effect on survival differed from that of BTE for this reason, even though TF is also derived from tea polyphenols. Taken together with previous reports, we suggest from the results of the present study that the effect of ameliorating the diminished survival caused by an HFD is due to the compound effects of TFs and other catechins or constituents when ingested together. Indeed, in mouse liver, catechin-free fractions derived from green tea affect the gene expression of enzymes related to lipid metabolism [33]. Among tea polyphenols, the absorption of green tea polyphenols in particular are improved in the presence of low-molecular weight compounds such as ascorbic acid [34] and quercetin [35], as these compounds bind to and stabilize the polyphenols. Regarding the lifespan-extension effect of resveratrol, studies have reported that it is influenced by factors such as strain, sex, and resveratrol concentration [36,37]. The chemical composition of black tea consumed by humans is closer to BTE than to TF, that is to say in black tea, other catechins besides TFs are present. Humans may benefit from a protective effect of black tea on the negative effects of excessive fat intake, and this protective effect most likely relies on both TFs and other catechins. Our study indicates that intake of TF has a moderating effect on the early LPO accumulation caused by an HFD. Similar effects have been observed for BTE and GTC [16,21]. It seems that both TFs and GTCs have this effect of reducing HFD-induced LPO accumulation. Taking in consideration the tea polyphenol content of TF, BTE, and GTC, it seems that either of the two types of compounds alone is sufficient to achieve this effect.

The excessive fat accumulation in *Drosophila* larvae caused by an HFD was moderated by the addition of TF, ETG, and EGCg to the diet. Reduction of fat accumulation due to fasting has been previously reported [19], but our study is the first to report the alteration of the degree of lipid accumulation in the fat body due to a difference in nutritional components. In some areas of the fat body, the difference in Oil Red O staining was not apparent between SF and HFD conditions. However, our assay system, which focused on the area with the most prominent difference, may be applied to assessing the fat accumulation-suppressing effects of not only tea polyphenols but also of various other food components. As obesity increases the risks of developing various cancers, for example colorectal, prostate, breast, and endometrial cancer [38], identification and utilization of anti-obesity nutritional components are much desired [39]. There are many candidates for obesity-preventing nutritional components, and applying our assay system, which utilizes *Drosophila* fat bodies for the preliminary screening of *in vivo* effectiveness, would greatly enhance the efficiency of researching these nutritional components.

The changes induced by the HFD in the expression of lipid metabolism-related genes were moderated by the intake of TF, ETG, and EGCg. *Acox57D-d* is a gene related to the function of acyl-CoA oxidase, and is involved in fatty acid beta-oxidation in the peroxisomes [40]. The expression of *ACO*, which has a similar Acyl-CoA oxidase-related function in mammals, was increased by 40% in the liver of an obese animal model that was given highly concentrated tea catechins (0.5% w/v) [41]. In mice, the expression of *ACO* was increased by an HFD, which is contrary to our finding that

Acox57D-d expression in the *Drosophila* fat body was decreased by an HFD. However, *ACO* expression was increased by polyphenol ingestion, which is consistent with the previous report. It is therefore likely that tea polyphenols universally increase the expression of *Acyl-CoA oxidase*. As for the other genes influenced by HDF, their expression was reduced for the following two fatty acid beta-oxidation-related genes: *acsl* (acyl-CoA synthetase long-chain), which catalyzes the pre-step reaction for beta-oxidation of fatty acids [42]; and *fabp* (fatty acid binding protein), which is considered to be a main component of fatty-acid beta-oxidation [43]. A similar decrease in expression level by an HFD was observed for *CCt1,* the gene encoding the rate-limiting enzyme in phosphatidylcholine synthesis [44]. Our results seem to contradict the expected results, in which the excessive fat intake from the HFD would lead to the catabolism of fatty acids in the body by fatty acid beta-oxidation, thereby suppressing their anabolism. From our results, it would seem that the HFD influences the molecular mechanisms involved in the fatty acid metabolism of the fat body, leading to a further enhancement of fat accumulation. The tea polyphenols seem to have a lipid metabolism-enhancing effect, as TF, ETG, and EGCg had moderating effects on the HFD-induced changes in expression of lipid metabolism-related genes. In addition, our results suggest that the mechanisms of fat accumulation-suppressing effect differ between TF, ETG and EGCg; it seems that TF and ETG activate fatty acid oxidation in mitochondria, whereas EGCg activates fatty acid oxidation in peroxisomes. The ratio of the polyphenols contained in black and green tea differs, with fermented (black) tea having high TF content and minimally fermented (green) having high EGCg content. It is plausible that the difference in biologically beneficial actions exhibited by each type of tea is due to these differences in polyphenol compositions [45]. Such moderation of the expression of lipid metabolism-related genes by tea polyphenols has been reported *in vitro* [46], in mice [47] and in chickens [48], and we have now demonstrated it in *Drosophila*.

4.3. Adult longevity caused by fat metabolism

Adult lifespan can diminish as a result of continuous intake of TF, ETG, and EGCg under normal condition without excessive lipid intake. This finding is contrary to previous reports on the administration of BTE [16] and epicatechin [49]. Well-known factors that alter lifespan modulators include diet restriction and sirtuin activation [10,50], but mutation in insulin/insulin-like signaling (IIS)-related genes also results in an altered lifespan [51]. Functional analysis of IIS-related genes indicates that inner fat storage and metabolism are regulated by IIS, contributing to lifespan extension [52,53]. The ligands of IIS, which are insulin-like peptides, may also contribute to the determination of lifespan by regulating lipid metabolism [54]. More direct examples of lifespan-affecting factors include the improved starvation resistance and lifespan extension caused by the mutation of the lipid metabolism-related gene *brummer* [55] and lifespan extension due to the overexpression of the fly homologs of *apolipoprotein D* (*ApoD*) and *Glial Lazarillo* (*GLaz*) [56]. These reports illustrate the close relationship between lipid metabolism and lifespan, and indicate a direct correlation between the volume of accumulated body fat and lifespan. As shown in Figs. 2 and 3, TF, ETG, and EGCg seemingly suppress lipid accumulation in the fat body by regulating the expression of lipid metabolism-related genes. Taken together, it seems that continuous intake of TF, ETG, and EGCg's has shortened the adult lifespan by strongly suppressing fat accumulation, leading to the exhaustion of stored caloric energy. This provides yet another indication that TF, ETG, and EGCg are powerful suppressors of fat accumulation.

5. Conclusion

Our study demonstrated that *Drosophila* is an ideal tool for assessing the bioactivities of tea polyphenols. Combining past knowledge and the findings from this study, we suggest that although tea polyphenols may have various biological activities, such as antioxidant activity and suppression of fat accumulation, it is possible to selectively enhance their desired beneficial effects by altering their combination and concentration. *Drosophila* provides us with various possibilities for genetic analysis such as mutation and gene expression control by the GAL4/UAS system. We plan to continue our investigation by using *Drosophila* to elucidate which genes and metabolic pathways are influenced by tea polyphenols. Until now, few studies had explored the bioactivity of ETG. Ours is the first to report the fat accumulation-suppressing effect of ETG in *Drosophila* larval fat body, which was even more effective than that of TF or EGCg. Experiments using more complex animal species such as mouse and clinical trials have yet to prove that these tea polyphenols are effective in humans and that tea can be called a functional food.

Acknowledgments

We thank the members of our laboratory for fruitful discussions during the course of the study. This work was supported by the research program “From Shizuoka to the world: Research and development of next-generation bottled tea drinks and tea extracts,” Shizuoka Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence, JST, and a Grant-in-aid for Young Scientists (B) (24700837) (to Y.K.). There are no known conflicts of interest associated with this publication.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.10.013.

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