L-Theanine Activates the Browning of White Adipose Tissue Through the AMPK/α-Ketoglutarate/Prdm16 Axis and Ameliorates Diet-Induced Obesity in Mice

Wan-Qiu Peng,1 Gang Xiao,1 Bai-Yu Li,1 Ying-Ying Guo,1 Liang Guo,1,2 and Qi-Qun Tang1

Diabetes 2021;70:1458–1472 | https://doi.org/10.2337/db20-1210

L-Theanine is a nonprotein amino acid with much beneficial efficacy. We found that intraperitoneal treatment of the mice with L-theanine (100 mg/kg/day) enhanced adaptive thermogenesis and induced the browning of inguinal white adipose tissue (iWAT) with elevated expression of Prdm16, Ucp1, and other thermogenic genes. Meanwhile, administration of the mice with L-theanine increased energy expenditure. In vitro studies indicated that L-theanine induced the development of brown-like features in adipocytes. The shRNA-mediated depletion of Prdm16 blunted the role of L-theanine in promoting the brown-like phenotypes in adipocytes and in the iWAT of mice. L-theanine treatment enhanced AMPKα phosphorylation both in adipocytes and iWAT. Knockdown of AMPKα abolished L-theanine–induced upregulation of Prdm16 and adipocyte browning. L-Theanine increased the α-ketoglutarate (α-KG) level in adipocytes, which may increase the transcription of Prdm16 by inducing active DNA demethylation on its promoter. AMPK activation was required for L-theanine–induced increase of α-KG and DNA demethylation on the Prdm16 promoter. Moreover, intraperitoneal administration with L-theanine ameliorated obesity, improved glucose tolerance and insulin sensitivity, and reduced plasma triglyceride, total cholesterol, and free fatty acids in the high-fat diet–fed mice. Our results suggest a potential role of L-theanine in combating diet-induced obesity in mice, which may involve L-theanine–induced browning of WAT.

Obesity has become a global epidemic and is a major risk factor associated with several metabolic syndromes, such as type 2 diabetes, insulin resistance, heart disease, stroke, hyperglycemia, hypertension, and cancer (1,2). Adipose tissue is a key metabolic organ. Adipose tissue composition is always in a dynamic process of change. Adipocytes play critical roles in whole-body energy metabolism and homeostasis, which can be classified into three types. White adipocytes store excess energy in lipid droplets (3), while beige and classical brown adipocytes are characterized by their unique ability to transform mitochondrial energy into heat via uncoupling protein 1 (Ucp1) (4).

Mounting evidence suggests that browning of white adipose tissue (WAT) is of great benefit to human metabolic health. It can not only burn fat, but also enhance the insulin sensitivity of the body and reduce the fibrosis and inflammatory response of WAT, which plays an important role in maintaining the metabolic homeostasis. Compound supplementation that promotes browning of white adipocytes provides a therapeutic option to ameliorate obesity.

Natural products have been used for medical purposes for a long time. Many kinds of teas are consumed widely for health care needs. Growing studies suggest that drinking green tea reduces the risk of obesity and related disorders (5). Green tea is enriched with many secondary

1Key Laboratory of Metabolism and Molecular Medicine of the Ministry of Education, Department of Biochemistry and Molecular Biology of School of Basic Medical Sciences and Department of Endocrinology and Metabolism of Zhongshan Hospital, Fudan University, Shanghai, China
2School of Kinesiology, Shanghai University of Sport, Shanghai, China

Corresponding authors: Qi-Qun Tang, qqtang@shmu.edu.cn, and Liang Guo, guoliang@sus.edu.cn

Received 30 November 2020 and accepted 12 April 2021

This article contains supplementary material online at https://doi.org/10.2337/figshare.14414216

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metabolites, such as catechins, caffeine, and l-theanine (6). For a long time, attention was significantly focused on catechins and caffeine for their metabolic benefits. However, in recent years, there is increasing attention on the health benefits of l-theanine. l-Theanine accounts for 1–3% of the dry weight of tea, which varies according to growing environment, variety, collection, and production process of the tea (7). l-Theanine was reported to improve sleep quality, emotional state, and cognitive performance and to combat cancer, cardiovascular diseases, and the common cold (8). However, its role in adipose tissue metabolism and antiobesity effects are poorly understood.

In this study, we showed that an l-theanine intervention significantly induced the browning of subcutaneous WAT in mice. The molecular mechanism underlying the above role of l-theanine was dissected both in vitro and in vivo. We have demonstrated that l-theanine could regulate a thermogenic program in WAT, and these effects are AMPK/α-ketoglutarate (α-KG)/Prdm16 axis–dependent. Our data clarified a previously unknown role of l-theanine in increasing energy expenditure, which might have a prospective application in improving metabolism and combating obesity-related metabolic diseases.

**RESEARCH DESIGN AND METHODS**

**Cell Culture and Reagents**

C3H10T1/2 cells were obtained from the ATCC (Manassas, VA). For the ex vivo system, iWAT from wild-type C57BL/6 mice was fractionated with digestion buffer. Primary iWAT stromal vascular fractions (SVFs) were fractionated by collagenase digestion and plated in culture according to the methods described previously (9). C3H10T1/2 cells and primary SVFs were cultured in complete DMEM with 10% FCS at 37 degrees and 5% CO2. Adipocytes were differentiated with an adipogenic cocktail (0.5 mmol/L isobutylmethylxanthine, 1 μmol/L rosiglitazone, 1 μg/mL insulin, and 1 μmol/L dexamethasone) in medium containing 10% (v/v) FBS (Invitrogen). After 2 days, the medium was exchanged for DMEM supplemented with 10% (v/v) FBS, 1 μg/mL insulin, and 1 μmol/L rosiglitazone for another 2 days. Media was changed every other day starting from the 4th day postdifferentiation with DMEM containing 10% (v/v) FBS. Cells were treated with l-theanine and infected with adenoviruses from the 6th day, and cell cultures were harvested 48 h later. Insulin, dexamethasone, and isobutylmethylxanthine were purchased from Sigma-Aldrich. Rosiglitazone was purchased from Cayman Chemical. The primary antibodies used in this study are as follows: anti-Ucp1 (ab209483; Abcam); anti-PGC1α, (ab54481; Abcam); anti-Prdm16 (ab106410; Abcam); anti-AMPK (detecting AMPKα1, 5832; Cell Signaling Technology); anti–phospho-AMPK (Thr-172 of AMPK, 2535; Cell Signaling Technology); HSP-90 (4874; Cell Signaling Technology); S-hydroxymethylcysteine (5-hmc) (A4001; Zymo Research); 5-methylcytosine antibody (5-mc) (A3001; Zymo Research); and anti–β-actin (sc-47778; Santa Cruz Biotechnology). l-Theanine was purchased from Shandong Topscience Biotech Co., Ltd. (T2800) and dissolved in saline. Hoechst was from US Everbright Inc. (H4047), and MitoTracker Green (MTG) was from Beyotime Biotechnology (C1048). Oil Red O stock solution (0.25 g of Oil Red O [Sigma-Aldrich] in 50 mL of isopropanol) was diluted with water (3:2 v/v), followed by filtration. After staining, samples were washed three times in water.

**Animals**

Our animal protocol has been reviewed and approved by the Animal Care and Use Committee of the Fudan University Shanghai Medical College (20180302–010). Mice were maintained under a 12:12-h light/dark cycle at room temperature (25°C) with free access to food and water. For the cold tolerance test, mice were placed in a temperature-controlled chamber (Friciell; MMM Group, München, Germany). Body temperature was measured using an animal electronic thermometer (ALT-ET03; Shanghai Alcott Biotech Co. Ltd.). For high-fat feeding studies, male C57BL/6J mice were put on the diet beginning at 6 weeks of age and continuing for up to 16 weeks. Blood and various tissue samples were collected. Animals were given l-theanine or saline by intraperitoneal (i.p.) injection for 7 days at room temperature during chow diet or for 10 consecutive weeks during high-fat diet (HFD). For metabolic studies, mice were housed and monitored individually in metabolic cages (Columbia Instruments International) with free access to regular chow and drinking water for 48 h. The animals were acclimated to the system for 24 h, and measurement of VO2 and VCO2 was performed during the next 24 h. Each cage was monitored for metabolic parameters (including oxygen consumption and carbon dioxide production) at 25-min intervals throughout the 48-h period. Parameters of oxygen consumption (in milliliters per kilogram per hour), carbon dioxide production (in milliliters per kilogram per hour), heat (in kilocalories per hour), and respiratory exchange ratio (RER) (as VCO2/VO2) were calculated for each mouse divided by its body weight. Body fat and lean mass were measured using an NMR analyzer (Minispec LF9011; Bruker Optics). The serum total triglyceride (TG) and total cholesterol (TC) were measured using a Fully Automatic Biochemical Analyzer (Roche) according to the manufacturer’s protocol. The plasma free fatty acid (FFA) levels of the mice were measured by using the FFA assay kit from Abcam.

**Glucose Tolerance Test and Insulin Tolerance Test**

For the glucose tolerance test (GTT), mice were subjected to fasting at 6:00 P.M. On the next day at 10:00 A.M., GTT was conducted through i.p. injection of glucose at 2 g/kg body weight. For the insulin tolerance test (ITT), mice were subjected to fasting for 5 h from 7:00 A.M. to 12:00 P.M., after which ITT was performed by i.p. injection of human insulin (0.5 units/kg body weight). Glucose level was measured in tail blood at the indicated time after glucose or insulin administration using a glucometer (Accu-Chek; Roche).
Hematoxylin and Eosin Staining and Immunohistochemistry
For histology, adipose tissues were fixed with neutral-buffered formalin and embedded in paraffin, and sections were stained with hematoxylin and eosin (H&E). Immunohistochemistry (IHC) was performed as described previously (10). The anti-Ucp1 (ab209483; Abcam) antibody was used for IHC. Images were captured using a charge-coupled device camera, and representative images are shown.

RNA Extraction and Quantitative PCR
Total RNA from adipose and adipocyte samples was isolated using TRIzol (Invitrogen) reagent according to the manufacturer’s instructions and reverse-transcribed with SuperScript III Reverse Transcriptase (Invitrogen). cDNA was reverse-transcribed from 1 μg of RNA. Quantitative PCR (QPCR) was performed with SYBR Green QPCR Master Mix (Applied Biosystems, Carlsbad, CA) using a Prism 7500 instrument (Applied Biosystems), with 18S rRNA or 36b4 as an endogenous control. All primer sequences are listed in Supplementary Table 1. Expression levels were calculated according to the relative 2-ΔΔCt method (11).

Western Blotting Analysis
Whole-cell protein lysates were prepared using lysis buffer containing 1% SDS, 50 mmol/L Tris-HCl (pH 6.8), 10 mmol/L dithiothreitol, 10% glycerol, 0.002% bromophenol blue, 1 mmol/L sodium fluoride (Sigma-Aldrich), 1 mmol/L sodium orthovanadate (Sigma-Aldrich), and protease inhibitor cocktail (Complete Mini EDTA-free; Roche Applied Science). Protein was resolved using Tris-glycine gels and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with 5% nonfat dried milk in Tris-buffered saline with Tween, the membranes were incubated with the primary antibodies and secondary antibodies and visualized with horseradish peroxidase-coupled secondary antibodies.

Oxygen Consumption Assays
Cultured adipocytes were detached from plates by trypsin. Cells or minced tissues were then resuspended in 1 mL Dulbecco’s PBS (25 mmol/L glucose, 1 mmol/L pyruvate, and 2% BSA). Cellular respiration was measured with a Clark-type oxygen electrode (Oxygraph+ system; Hansa-tech Instruments Ltd.). Data were normalized by total protein content or tissue weight.

MTG Staining
Cultured cells were incubated in DMEM with MTG (final concentration 200 mmol/L; C1048; Beyotime Biotechnology) for 20 min in a 37°C incubator and then washed twice in PBS. The cells were observed and photographed under an Axioskop 2 microscope (Carl Zeiss) with a DP70 charged-coupled device system (Olympus). Cell nuclei were labeled with Hoechst.

Lactate Dehydrogenase Activity
Cytotoxicity was determined in a colorimetric assay based on the measurement of lactate dehydrogenase (LDH) released from the C3H10T1/2 cells into the supernatant according to the method (12). The LDH release activity was determined using an LDH cytotoxicity assay kit (Beyotime Biotechnology) according to the manufacturer’s instructions.

Analysis of mtDNA Content by Quantitative Real-time PCR
Cells and tissues were homogenized and digested with proteinase K in a lysis buffer for DNA extraction by using the DNeasy Blood and Tissue Kit (Qiagen). The ratio of mtDNA to nuclear DNA reflects the concentration of mitochondria per cell (13). The Prism 7500 instrument (Applied Biosystems) was used to amplify nuclear DNA (forward, 5’-GCCAGCTTCTCTGATTTTAGTGT-3’ and reverse, 5’-GGGACAAAAAAGACCTTCTTG-G-3’) and mtDNA (forward, 5’-CCGCAAGGAAAAGATGAAGA-3’ and reverse, 5’-TCGTTTGGTITTCGAGGTTTC-3’). The results were calculated from the difference in the threshold cycle (ΔCT) values for mtDNA and nuclear-specific genes.

Construction of Adenoviral Expression Vectors and Infection
Adenoviral expression vector pBLOCK-iT (Invitrogen) encoding shRNA against LacZ, Prdm16, and AMPKα was constructed according to the manufacturer’s protocol. The targets of shRNA were listed as: shRNA against LacZ (shLacZ), 5’-CTACAAACATCGCGATT-3’; shRNA against Prdm16 (shPrdm16), 5’-AGTGACTTTGAGGATATCA-3’; shRNA against AMPKα1 (shAMPKα1), 5’-GCACACCCCTGATGAATTTAATA-3’; and shRNA against AMPKα2 (shAMPKα2), 5’-GCTGAGAAACACTCTCTTCTTCT-3’. Adenovirus vectors were amplified and purified with Sartorius Adenovirus Purification kits. Viral titers were determined by the 50% tissue culture infectious dose method using 293A cells. For in vivo studies, 10^9 plaque-forming units/mouse of adenoviruses harboring shLacZ or shPrdm16 were diluted to 200 μL with PBS, and then 100 μL of adenovirus solution was injected to the subcutaneous fat pads at each side, twice a week as indicated. For in vitro studies, adenoviruses harboring shLacZ, shPrdm16, or shAMPKα at a multiplicity of infection of 70 were added to the cell culture medium. Then, cells were cultured with the viruses for 48 h and then replaced with fresh culture medium. Experiments were performed 48 h after adenovirus infection.

α-KG Assay
The α-KG contents in iWAT and the adipocytes derived from C3H10T1/2 cells were analyzed using liquid chromatography-tandem mass spectrometry. C3H10T1/2 cells were cultured in 2 mL medium on 3.5-cm dishes and induced into mature adipocyte. Cells were collected on day 8 of differentiation and then lysed with precooled 800 μL of 80% methanol/water (v/v) for 20 min at 4°C. Half of the cell lysis solution was vacuum freeze dried and resol-ved in 200 μL of H2O. For measuring the α-KG in
iWAT, up to 50 mg adipose tissue was weighted and homogenized in 1 mL precooled 80% methanol/water (v/v). The supernatants were collected by centrifugation at 12,000 rpm 4°C for 10 min. The samples were firstly separated by a UPLC system using a Waters Acquity UPLC LC-20AB (ZIC-HILIC; Merck KGaA) (2.1 x 150 mm, 5 μm) with a two-solvent system (A: 5 mL 1 mol/L ammonium acetate and 2 mL ammonia in 1 L double-distilled H2O; and B: 100% acetonitrile) and a flow rate of 0.2 mL/min and then analyzed by API 4000 Qtrap Mass Spectrograph (AB Sciex API 4000 Qtrap; Thermo Fisher Scientific).

**Ten-Eleven Translocation Hydroxylase Activity Assay**
C3H10T1/2 cells nuclei were isolated by using Nuclear and Cytoplasmic Extraction Reagents (P0027; Beyotime Biotechnology). Ten-eleven translocation hydroxylase (TET) activity was determined by using the TET Hydroxylase Activity Quantification Kit (ab156913, Fluorometric; Abcam) according to the manufacturer’s instructions.

**Chromatin Immunoprecipitation**
Chromatin immunoprecipitation (ChIP) was performed as previously described (14). Briefly, C3H10T1/2 cells were fixed, lysed, and sonicated to obtain 200–1,000-bp DNA fragments. Soluble chromatin was precleared by using 30 μL of Protein G Plus/Protein A-Agarose suspension (Calbiochem) saturated with salmon sperm (1 mg/mL) and then incubated with 1 μg anti-Prdm16 or PCG1α antibody. Bound DNA–protein complexes were eluted, cross-links were reversed, and samples were phenol-chloroform extracted, ethanol precipitated, and used for QPCR with the primers listed in Supplementary Table 2.

**Hydroxymethylated and Methylated DNA Immunoprecipitation**
According to the method described previously (15), genomic DNA of C3H10T1/2 cells at day 8 of differentiation was extracted by phenol-chloroform method. Genomic DNA (10 μg) was diluted in 300 μL TE buffer and sonicated by 4°C Waterbath Sonicator for 12–16 cycles 30-s on/30-s off, until the size of DNA was 300–1,000 bp. The sonicated DNA was purified, and 10% of the sonicated DNA was used as input sample. The sonicated DNA (3 μg) was diluted in 450 μL TE buffer, 10 min at 95°C, and then immediately cooled on ice along with adding 50 μL 10 times IP buffer (100 mmol/L Na-phosphate, pH 7.0, 1.4 mol/L NaCl, and 0.5% Triton X-100). Then, 5-hmc (A4001; Zymo Research) or 5-mc antibody (A3001; Zymo Research) was added to the DNA solution with rotation overnight at 4°C. The DNA–antibody mixture was further incubated with preblocked Pierce magnetic protein A/G (88802; Thermo Scientific) for 2 h. The beads–antibody–DNA complex was washed three times with one time IP buffer and responded in 250 μL digestion buffer (50 mmol/L Tris HCl, pH 8, 10 mmol/L EDTA, and 0.5% SDS) and then digested with 7 μL proteinase K (10 mg/mL) by incubating at 50°C for 3 h. The purified DNA and input samples were quantized by QPCR analysis using the primers listed in Supplementary Table 2. Relative enrichment folds of indicated DNA regions were determined after normalization to input, and 36b4 promoter was used as a negative control.

**Statistical Analyses**
GraphPad Prism 8 (GraphPad Software) was used for statistical analysis. Only wounded animals were excluded from the analyses (n = 6 in this study). Data are presented as mean ± SD. Comparisons between groups were made using unpaired two-tailed Student t tests. For comparison of three or more independent groups with only one variable, one-way analyses of variance plus Bonferroni post hoc tests were performed. For comparison of two or more independent groups with two variables, two-way ANOVA plus Bonferroni post hoc tests were carried out. The statistical analyses were also indicated in the legends of each figure, and P < 0.05 was considered significant. All experiments were repeated at least three times, and representative data are shown.

**RESULTS**

**l-Theanine Promotes the Browning of Subcutaneous WAT in Mice**
The metabolic effects of l-theanine were evaluated by i.p. administration of l-theanine into normal chow diet (NCD)–fed C57BL6 male mice. To examine the thermogenic activity, the 8-week-old mice were treated with l-theanine or saline as a control once every day for 7 days.

After cold exposure (4°C) for 6 h, l-theanine–treated mice had a better capacity to maintain body temperature compared with controls (Fig. 1A). Mice treated with l-theanine showed less body weight gains as compared with saline-treated control mice (Supplementary Fig. 1A). Adipose tissue oxygen consumption rate (OCR) is usually associated with mitochondrial energy metabolism. l-Theanine significantly increased the OCR of WAT compared with the control group (Fig. 1B), which means enhanced mitochondrial function.

As is demonstrated in Fig. 1C, the abundance of mitochondria in WAT was quantified by mtDNA copy number, which was increased by l-theanine treatment. Histological examination of the subcutaneous (iWAT) and epididymal WAT (eWAT) revealed smaller adipocytes, which presented brown-like transformation of the WAT (Fig. 1D). We next isolated RNA from the three different fat pads—eWAT, iWAT, and brown adipose tissue (BAT)—and compared mRNA expression levels between l-theanine–treated mice
and saline-treated mice. Expression levels of genes involved in controlling energy expenditure and the thermogenic program, including Prdm16, PGC1α, Ucp1, mtTFA, Dio2, and Cpt1b, were examined in iWAT, eWAT, and BAT. Although to different extents, L-theanine induced the expression of the above genes in iWAT (Fig. 1E), eWAT (Supplementary Fig. 1C), and iWAT in mice after 6-h cold exposure. Data were normalized to the saline group. D: Representative H&E staining images of BAT, eWAT, and iWAT of the mice after 6-h cold exposure. Scale bars, 40 μm. E: The mRNA expression of the indicated genes in iWAT after 6 h of cold exposure. n = 6. F: Immunoblotting for indicated proteins in iWAT after 6-h cold exposure. For statistical analysis, two-way ANOVA and Bonferroni post hoc tests were performed in A, and unpaired two-tailed Student t tests were performed in B, C, and E. All groups were compared with the saline group. All values are represented as means with error bars representing SD. *P < 0.05, **P < 0.01, ***P < 0.001 as compared with saline group. n = 6 for each group. ATP syn, ATP synthase; P-AMPK, phosphorylated AMPK.

**Figure 1**—L-Theanine treatment promotes subcutaneous fat browning and adaptive thermogenesis in NCD-fed mice. The 8-week-old C57BL6 NCD-fed male mice were administered L-theanine i.p. (100 mg/kg/mouse, once a day) or saline as a control for 7 days. A: Rectal temperature of the mice was recorded at the indicated time points after cold exposure (4°C). B: Basal OCR of iWAT and eWAT tissues in mice after 6-h cold exposure was measured by Clark-type electrode oxygraph and shown. C: Relative mtDNA copy number of iWAT and eWAT in mice after 6-h cold exposure. D: Representative H&E staining images of BAT, eWAT, and iWAT of the mice after 6-h cold exposure. Scale bars, 40 μm. E: The mRNA expression of the indicated genes in iWAT after 6 h of cold exposure. n = 6. F: Immunoblotting for indicated proteins in iWAT after 6-h cold exposure. For statistical analysis, two-way ANOVA and Bonferroni post hoc tests were performed in A, and unpaired two-tailed Student t tests were performed in B, C, and E. All groups were compared with the saline group. All values are represented as means with error bars representing SD. *P < 0.05, **P < 0.01, ***P < 0.001 as compared with saline group. n = 6 for each group. ATP syn, ATP synthase; P-AMPK, phosphorylated AMPK.

L-Theanine Increases Energy Consumption in NCD-Fed Mice
As the above results show, L-theanine promotes the browning of WAT during cold exposure. With this in mind, we next investigated the metabolic effect of L-theanine in mice using a comprehensive laboratory animal monitoring system. As shown in Fig. 2A–D, basal oxygen consumption and carbon dioxide release rates were significantly higher in mice administered L-theanine compared with control subjects through a 12-h light/dark cycle. As shown in Fig. 2E, the L-theanine–treated mice showed a higher consumption in the whole-body energy through a 12-h light/dark cycle. The RER was calculated from VCO2 and VO2. Higher RER values suggest higher carbohydrate and fat oxidation.

**Figure 2**—L-Theanine increases energy expenditure in mice. Mice were treated as indicated in Fig. 1. Energy expenditure was evaluated by measurement of oxygen consumption (VO2) as shown in A and B, carbon dioxide release (VCO2) as shown in C and D, and heat production of the mice as shown in E. The above parameters were measured in mice placed in a metabolic cage during a 12:12-h light/dark cycle. Energy expenditure expressed as kcal/h per animal. F: RER was calculated using equations described in Research Design and Methods. The adjacent bar graphs represent the average values for each group (B for A and D for C) (n = 6). Error bars represent SD, and significant differences compared with saline groups are indicated by *P < 0.05, **P < 0.01 (Student t test).
consumption, and lower RER values suggest more fat is used as the source of energy (16). As shown in Fig. 2F, L-theanine treatment decreased RER of the mice, demonstrating a tendency to fatty acid oxidation (17). Taken together, these results indicate that L-theanine promotes the whole-body energy expenditure and increase fatty acid combustion in NCD-fed mice.

**L-Theanine Induces a Brown Adipocyte-like Feature in Adipocytes In Vitro**

Because the browning of subcutaneous WAT in vivo is complicated, we examined whether L-theanine induces adipocyte browning directly. To better investigate the role of L-theanine in the browning of adipocytes and its underlying mechanism, we used the C3H10T1/2 cell line, which is a mesenchymal stem cell line and can be induced to become mature adipocytes in vitro. We treated the adipocytes derived from C3H10T1/2 cells with L-theanine at different concentrations of 0 μmol/L, 10 μmol/L, 50 μmol/L, and 100 μmol/L. To evaluate the possible cytotoxicity of L-theanine, we assessed LDH leakage after 24 h of exposure to L-theanine. Exposure of the adipocytes to 10 μmol/L, 50 μmol/L, and 100 μmol/L of L-theanine did not influence the leakage rate of LDH (Fig. 3A), which
suggests that \( \alpha \)-theanine was not toxic to C3H10T1/2 at the above three concentrations. We then measured C3H10T1/2 OCR using a Clark-type electrode oxygraph (Fig. 3B). The OCR of 50 \( \mu \)mol/L and 100 \( \mu \)mol/L \( \alpha \)-theanine–treated cells was significantly greater than that of controls. Increase of mitochondrial contents was also observed in \( \alpha \)-theanine–treated cells (Fig. 3C). MTG staining showed that \( \alpha \)-theanine increased mitochondrial abundance (Fig. 3D), which was consistent with the results of the above mtDNA content analysis. \( \alpha \)-Theanine treatment of the adipocytes upregulated the mRNA levels of the genes involved in the activation of energy expenditure and thermogenesis, including Prdm16, PGC1\( \alpha \), Ucp1, mtTFA, and Cpt1b (Fig. 3E) in the \( \alpha \)-theanine dose-dependent manner. Western blot analysis further confirmed that Prdm16 and Ucp1 protein were significantly upregulated by \( \alpha \)-theanine at the concentrations of 50 \( \mu \)mol/L and 100 \( \mu \)mol/L (Fig. 3F and G). Similar effects of \( \alpha \)-theanine were also observed in primary iWAT adipocytes (Supplementary Fig. 2). The lipid droplets in adipocytes were stained by Oil Red O, and the result was shown in Fig. 3H. The adipocytes derived from C3H10T1/2 cells treated with \( \alpha \)-theanine manifested diminishing tendency of lipid droplet size, as indicated by Oil Red O staining, which is consistent with the greater energy expenditure caused by \( \alpha \)-theanine treatment (Fig. 3H). All of the above phenotypes present enhanced acquisition of brown-like adipocyte features. These results demonstrate that \( \alpha \)-theanine could directly activate the browning of adipocytes in vitro.

Knockdown of Prdm16 Blunts the Role of \( \alpha \)-Theanine in Promoting the Brown Adipocyte-like Features in the Adipocytes Derived From C3H10T1/2 Cells
Prdm16 transcriptional complex is a dominant activator of brown/beige adipocyte development (18). The above results show that Prdm16 expression was obviously induced by \( \alpha \)-theanine treatment both in vivo and in vitro. The binding of Prdm16 to the enhancer and transcription start site region of Ucp1 and Dio2 was significantly enhanced by \( \alpha \)-theanine treatment (Fig. 4A). PGC1\( \alpha \) is a key regulator of adipose tissue energy metabolism, especially when involved in mitochondrial biosynthesis and thermogenesis (19). As shown in Fig. 4B, \( \alpha \)-theanine–induced binding of PGC1\( \alpha \) to the above regions is less obvious. Next, we constructed adenovirus harboring shLacZ or shPrdm16 to infect the adipocytes. The \( \alpha \)-theanine–mediated upregulation of browning gene expression was blunted upon the knockdown of Prdm16 (Fig. 4C). Consistently, the induction of Ucp1 protein expression by \( \alpha \)-theanine was abolished in shPrdm16-treated cells (Fig. 4D and Supplementary Fig. 3). Oxygen electrode experiments and MTG staining showed that the effects of \( \alpha \)-theanine treatment on promoting mitochondria respiration and increasing mitochondrial abundance were eliminated by the knockdown of Prdm16 (Fig. 4E and F, respectively). These results indicate that Prdm16 plays an essential role in \( \alpha \)-theanine–mediated browning of adipocytes.

Adenovirus-Mediated Specific Knockdown of Prdm16 in iWAT Eliminates \( \alpha \)-Theanine–Induced Browning of iWAT
Prdm16 promotes browning of iWAT by activating a brown fat-like gene program (20). Given the importance of Prdm16 in \( \alpha \)-theanine–mediated browning of C3H10T1/2 and in order to clarify the role of Prdm16 in \( \alpha \)-theanine effects on browning of WAT and energy expenditure in vivo, mice were subjected to subcutaneous injection at the iWAT with adenovirus harboring shLacZ or shPrdm16 and then were administered \( \alpha \)-theanine or saline for 1 week (Fig. 5A). We isolated the SVF and mature adipocyte fraction from iWAT and performed RT-qPCR to examine the expression level of Prdm16. As shown in Supplementary Fig. 4, the expression level of Prdm16 in the mature adipocyte fraction was significantly downregulated by shPrdm16. It should be noted that Prdm16 expression level was also knocked down by Prdm16 in SVF, which indicates that the adenovirus can infect not only mature adipocytes, but also other cell types in iWAT. We measured the rectal temperature of mice every hour during the 6 h of cold exposure. The results show that mice administered shPrdm16 adenovirus at the iWAT displayed impaired cold tolerance compared with the mice injected with shLacZ adenovirus (Fig. 5B). \( \alpha \)-Theanine treatment significantly elevated rectal temperature of shLacZ-treated mice at cold temperatures, whereas it did not obviously impact that of shPrdm16-treated mice. Moreover, \( \alpha \)-theanine treatment increased the mRNA levels of browning markers such as Prdm16, Ucp1, Cidea, and Dio2 in iWAT of control mice, whereas it had no obvious effect on the above genes’ expression in the iWAT of shPrdm16-treated mice (Fig. 5C). Gene expression levels were also examined in eWAT (Supplementary Fig. 5A). Knockdown of Prdm16 in iWAT did not affect the expression level of Prdm16 in eWAT (shLacZ + Saline vs. shPrdm16 + Saline). In the control shRNA group (shLacZ group), treatment of the mice with \( \alpha \)-theanine increased the expression of browning marker genes in eWAT, such as Prdm16, UCP1, Cidea, and Cox8b (shLacZ + Saline vs. shLacZ + \( \alpha \)-theanine). However, knockdown of Prdm16 in iWAT blunted the ability of \( \alpha \)-theanine to promote the expression of browning marker genes in eWAT (shPrdm16 + Saline vs. shPrdm16 + \( \alpha \)-theanine). There may be an \( \alpha \)-theanine–triggered signal from iWAT to eWAT through some endocrine factors to enhance the browning gene expression in eWAT, which could be impaired by the knockdown of Prdm16 in iWAT. Further studies are needed to investigate the above hypothesis. Meanwhile, \( \alpha \)-theanine failed to promote adipose tissue oxygen consumption in the iWAT of shPrdm16-treated mice (Fig. 5D). Similarly, the Prdm16 and Ucp1 protein expression levels were upregulated upon \( \alpha \)-theanine treatment in shLacZ-treated mice, whereas they stayed unchanged in the iWAT of shPrdm16-treated mice (Fig. 5E and Supplementary Fig. 5B). H&E staining of the inguinal WATs showed that \( \alpha \)-theanine decreased adipocyte size and increased Ucp1 IHC-positive areas in shLacZ control groups. However, these effects were abolished in shPrdm16 groups (Fig. 5F). Therefore, we conclude that Prdm16 expression is essential for the \( \alpha \)-theanine–mediated browning effect on the iWAT and proadipocyte thermogenesis in mice.
Figure 4—Prdm16 plays an indispensable role in L-theanine–induced browning in the adipocytes derived from C3H10T1/2 cells. A and B: ChIP QPCR analysis for Prdm16 and PGC1α binding to the enhancer or promoter regions of the indicated genes in cultured adipocytes. The adipocytes were treated with saline or 50 μmol/L L-theanine for 48 h. Then, Prdm16 and PGC1α were processed by ChIP from C3H10T1/2 and analyzed for their binding at the indicated regions. Data were normalized to saline group. Shown is a representative ChIP QPCR.

C–F: C3H10T1/2 cells were infected with shLacZ or shPrdm16 adenoviruses as indicated, and medium was changed 48 h later. Then, the cells infected with adenovirus were treated with saline or 50 μmol/L L-theanine for 48 h. The following experiments were then performed. C: The mRNA levels of the indicated genes. D: Representative Western blot images of the indicated proteins. E: OCR in cells. F: Cells were stained with MTG and Hoechst, and representative images are shown. Scale bar, 100 μm. Data are expressed as mean ± SD. *P < 0.05, **P < 0.01 as compared with saline group in A and B or compared with shLacZ saline group in C and E by Student t test.

TSS, transcription start site.
AMPK Activation Is Indispensable for L-Theanine–Induced Browning of Adipocytes

AMPK is a crucial cellular energy sensor that has recently been demonstrated to be important in regulating the metabolic activity of brown-like adipocytes (21). It was found that the phosphorylation of AMPK was activated by L-theanine in mice livers (22). We therefore investigated the role of L-theanine in the activation of AMPK in adipocytes. Our results demonstrated that L-theanine induced the phosphorylation of AMPK both in the adipocytes (Fig. 3F) and in iWAT (Fig. 1F) of the mice. Therefore, we sought to explore if L-theanine promotes the browning of adipocytes via activating the

Figure 5—Prdm16 plays an indispensable role in L-theanine–induced iWAT browning in mice. Eight-week-old male WT mice were injected subcutaneously (i.h.) into iWAT with adenovirus of shLacZ or shPrdm16 twice a week for 2 weeks. The mice were i.p. injected with saline or L-theanine in the 2nd week for 7 days (d). Then, the following experiments were performed. A: Schematic description of animal experiments. B: Rectal temperature of the mice was recorded at the indicated time points after cold exposure (4°C). C: The mRNA levels of the indicated genes. n = 6. D: OCR of iWAT was measured by Clark-type electrode oxygraph and shown. E: Western blot analysis of the protein levels in iWAT by using the indicated antibodies. Hsp90 serves as an internal control. F: H&E staining (HE) and IHC for UCP1 protein in iWAT sections of the mice. Scale bars, 50 μm. n = 6. Data are expressed as mean ± SD. *P < 0.05, **P < 0.01 compared with shLacZ + saline group by Student t test.
AMPK signaling pathway. The shRNA-mediated knockdown of the AMPKα subunit was performed in the adipocytes. As shown in Fig. 6A, knocking down of AMPK blunted the role of L-theanine in inducing the mRNA expression of Prdm16 and Ucp1. Western blotting showed that knockdown of AMPK reduced the expression of Ucp1 and Prdm16 promoted by L-theanine (Fig. 6B and C and Supplementary Fig. 6). As shown in Fig. 6D, L-theanine promoted a significant increase in oxygen consumption in C3H10T1/2, and this increase was completely abrogated by AMPK knockdown in C3H10T1/2. Enhanced mitochondrial abundance by L-theanine was also abolished in C3H10T1/2 infected with shAMPK adenovirus (Fig. 6E). As downregulation of AMPK signaling blunted the role of L-theanine in inducing the expression of Prdm16, it is suggested that AMPK and Prdm16 were both involved in L-theanine–mediated browning of adipocytes, and Prdm16 could be a downstream target of AMPK. These data demonstrate a potential L-theanine/AMPK/Prdm16 pathway activation in L-theanine–mediated browning of adipocytes.

L-Theanine Increases the Cellular Content of Tricarboxylic Acid Metabolite α-KG and Decreases the DNA Methylation Level of Prdm16 Promoter in C3H10T1/2

As Prdm16 plays an important role in L-theanine–mediated browning of adipocytes, the mechanism of how Prdm16 expression is regulated was then investigated. DNA methylation

Figure 6—AMPK activation is necessary for L-theanine to promote adipocyte browning. C3H10T1/2 cells were infected with shLacZ or shAMPKα adenoviruses as indicated, and medium was changed 48 h later. Then, the cells infected with adenovirus were treated with saline or 50 μmol/L L-theanine for 48 h. The following experiments were then performed. A: The mRNA levels of the indicated genes. B: Cell lysates were then subjected to Western blotting by using the indicated antibodies. Hsp90 serves as an internal control. C: Statistical analysis of Western blotting gray-level results for B. D: OCR in cells was measured. E: Cells were stained with MTG and Hoechst, and representative images are shown. Scale bar, 100 μm. Data are expressed as mean ± SD. *P < 0.05, **P < 0.01 compared with shLacZ saline group by Student t test.
is a complicated process and plays essential roles in regulating gene expression. Based on a methylated and hydroxymethylated DNA immunoprecipitation procedure, we further studied DNA methylation (5-mc) and hydroxymethylation (5-hmc) in the Prdm16 promoter in three different regions of Prdm16 promoter. As shown in Fig. 7A and B, l-theanine significantly decreased 5-mc level and increased 5-hmc level in Prdm16 promoter. The tricarboxylic acid cycle (TCA) metabolite α-KG is known to facilitate DNA demethylation. The α-KG concentration was determined by liquid chromatography-tandem mass spectrometry and showed a significant elevation in l-theanine–treated adipocytes (Fig. 7C). Moreover, the content of α-KG was increased in the iWAT of l-theanine–treated mice (Fig. 7D).

Given that the TETs, including TET1, 2, and 3, catalyze active DNA demethylation, the effect of l-theanine on TET activity was examined. It was found that l-theanine...
significantly enhanced TET activity in the adipocytes derived from C3H10T1/2 cells (Fig. 7E). This result suggests that t-theanine–induced DNA demethylation on the Prdm16 promoter may be mediated by enhanced TET activity. Further, we found that t-theanine–mediated elevation of α-KG concentration was significantly blunted in shAMPK-treated cells compared with the corresponding control group (Fig. 7F). In addition, knocking down of AMPK abolished the role of t-theanine in decreasing 5-mc level and increasing 5-hmc level on the Prdm16 promoter (Fig. 7G and H). This suggests that AMPK is an essential factor in α-KG/Prdm16 axis and is an upstream regulator of α-KG. AMPK signaling–mediated elevation of α-KG promotes Prdm16 promoter DNA demethylation, thereby stimulating Prdm16 expression. Overall, our data suggest that t-theanine inhibits the DNA methylation of the Prdm16 promoter and facilitates Prdm16 expression through the AMPK–α-KG pathway.

Antiobesity Effects of t-Theanine in HFD-Induced Obese Mice
Because t-theanine promotes browning of adipose tissue, we aimed to investigate the potential antiobesity effects of t-theanine. C57BL6 male mice were fed with HFD for 16 weeks. From the 7th week of HFD feeding, mice were injected i.p. with saline or t-theanine (100 mg/kg), once a day for the next 10 consecutive weeks (Fig. 8A). t-Theanine did not affect the amount of food intake in mice (Supplementary Fig. 7A). As shown in Fig. 8B, t-theanine reduced the weight gain of mice induced by HFD, which is associated with the reduced fat mass content (Fig. 8C). In addition, we weighed three types adipose tissue. t-Theanine significantly reduced tissue weight in iWAT and eWAT, while brown fat content did not statistically differ between the two groups (Fig. 8D). Obesity is closely associated with elevation of lipids in plasma. The mice plasma was collected and analyzed for TG fatty acid, TC, and FFA. Plasma TG, TC, and FFA levels were reduced in mice treated with t-theanine compared with saline-treated mice (Fig. 8E). HFD-induced obesity is also closely related to glucose intolerance and insulin resistance. We next evaluated whether t-theanine treatment ameliorates glucose intolerance and insulin resistance by conducting i.p. GTT and ITT (IP-GTT and IP-ITT, respectively). t-Theanine–treated mice exhibited an improved metabolic profile in glucose tolerance and insulin sensitivity (Fig. 8F–I). We isolated the iWAT from these mice. Proteins and mRNAs were extracted from tissues in the two groups for QPCR and Western blotting analysis. QPCR revealed significantly increased Prdm16, Ucp1, and other browning-related gene expression in the t-theanine–treated mice (Fig. 8J). Western blotting analysis demonstrated that the expression of Prdm16 and Ucp1 were significantly elevated in the t-theanine–treated group compared with the saline control group (Fig. 8K and Supplementary Fig. 7B).

There was also an upregulation of thermogenic genes in eWAT, as shown in Supplementary Fig. 7C. There was no expression level difference in most of the thermogenic genes except Dio2 in BAT (Supplementary Fig. 7D). H&E staining revealed that HFD-fed mice treated by t-theanine had smaller adipocytes in iWAT, and IHC staining of iWAT showed higher expression of Ucp1 (Fig. 8L). As shown in Supplementary Fig. 7E, visceral adipocyte sizes were reduced in mice administered t-theanine, and the adipocyte sizes in BAT were also slightly reduced. Furthermore, metabolic cage studies and an OCR test of iWAT were performed at the 9th week (9 weeks of HFD feeding with t-theanine treatment in the last 3 weeks). As shown in Supplementary Fig. 7F, 3 weeks of t-theanine treatment enhanced the OCR of iWAT in HFD-fed mice. In addition, metabolic cage studies indicated that 3 weeks of t-theanine treatment significantly promoted the basal oxygen consumption (Supplementary Fig. 7G) and energy expenditure (Supplementary Fig. 7H) of the whole body and mildly decreased RER (Supplementary Fig. 7I). These data suggest that t-theanine–mediated resistance to diet-induced obesity is driven by increased energy expenditure and increased iWAT thermogenesis. Overall, our results demonstrate that t-theanine activates the browning of WAT and contributes to ameliorating diet-induced obesity and related disorders in mice.

DISCUSSION

t-Theanine is a nonprotein amino acid mainly found in tea plants. t-Theanine is an important taste- and health-related component in tea and is used as an indicator to evaluate tea functions (23). t-Theanine has multifaceted health benefits (24) and is considered as an index of tea quality (25). However, the role and mechanism of t-theanine in treating obesity and metabolic diseases is still poorly investigated. In this study, we proposed that t-theanine promotes the browning of WAT, which contributes to the amelioration of obesity, the improvement of insulin sensitivity, and the reduction of plasma lipids in HFD-fed mice. In this article, we found that NCD-fed mice i.p. administered t-theanine showed an enhanced cold tolerance capacity compared with control mice. Our study further demonstrates that t-theanine activates the thermogenic program of adipose tissue in NCD-fed mice, with the most significant effects on iWAT (Fig. 1). t-Theanine significantly increases energy consumption in NCD-fed mice compared with the control group (Fig. 2). Moreover, using primary iWAT adipocytes and the adipocytes derived from C3H10T1/2 cells as an in vitro cell culture model, we found that t-theanine directly induced the browning of adipocytes in vitro (Fig. 3 and Supplementary Fig. 2). Prdm16 was a critical transcriptional regulator (26) and an essential modulator in the downstream of t-theanine because knockdown of Prdm16 in the adipocytes blunted the role of t-theanine in the induction of a brown-like
program in adipocytes (Fig. 4). Similarly, knockdown of Prdm16 in iWAT inhibited L-theanine–mediated brown-like fat formation in mice (Fig. 5). AMPKα phosphorylation played an important role in L-theanine–induced browning of adipocytes. We demonstrated that the L-theanine–induced browning of adipocytes, including elevated Prdm16 expression, was blocked by AMPKα knockdown (Fig. 6). We show a potential L-theanine/AMPK/Prdm16 pathway activation in L-theanine–induced browning of adipocytes (Fig. 7). We found that L-theanine significantly reduced the 5-mc level and increased the 5-hmC level in the Prdm16 promoter. Furthermore, L-theanine increased the concentration of CSH10T1/2 intracellular α-KG, which was blocked by the knockdown of AMPKα. α-KG is known to facilitate DNA demethylation. Our results suggest that L-theanine activates AMPK signaling to elevate α-KG concentration in adipocytes, which leads to DNA demethylation and decreases the DNA methylation level on the Prdm16 promoter, thereby facilitating Prdm16 expression and ultimately promoting adipocyte browning (Fig. 7). Furthermore, HFD-fed mice administered L-theanine exhibited improved glucose and insulin sensitivity and reduced

Figure 8—L-Theanine reduces obesity in diet-induced obese mice. A: Schematic description of animal experiments. C57BL6 male mice were fed with HFD for 16 weeks. From the 7th week of HFD feeding, mice were injected i.p. with saline or L-theanine (100 mg/kg) once a day for the next 10 consecutive weeks. B: Body weight of mice after treatment of saline or L-theanine. C: Body composition of the mice after 10 weeks of L-theanine or saline treatment. D: Tissue weight (normalized to body weight) of iWAT, eWAT, and BAT after 10 weeks of saline or L-theanine treatment. E: Plasma TG, TC, and FFA. F: Glucose concentrations during an IP-GTT. G: Area under the curve analysis of IP-GTT. H: Glucose concentrations during an IP-ITT. I: Area under the curve analysis of IP-ITT. J: The mRNA levels of the indicated genes in iWAT. K: Tissue lysates were subjected to Western blotting by using the indicated antibodies. Hsp90 serves as an internal control. L: H&E (HE) and Ucp1 IHC staining of iWAT. Scale bars, 40 μm. For statistical analysis in B–J, data were compared between the saline group and the L-theanine group. All values are represented as means with error bars representing SD. *P < 0.05; **P < 0.01; ***P < 0.001. n = 6 for each group. 6w, 6 weeks; ATP syn, ATP synthase; P-AMPK, phosphorylated AMPK.
plasma TG, TC, and FFA levels compared with saline--treated mice. (Fig. 8).

Browning of adipose tissue was inducible by exercise, cold exposure, and fasting in WAT, which has substantial metabolic benefits (27). Compared with classical brown adipocytes, beige adipocytes have a better inducibility toward thermogenic gene expression, especially Ucp1, and therefore a higher thermogenic capacity in response to stimulus (28). Accordingly, thermogenesis, which means dispersing energy in the form of heat, will contribute to maintaining metabolic homeostasis through enhancing energy expenditure. This process can be achieved by the activation of Ucp1, an uncoupling hallmark mitochondria inner membrane protein abundantly expressed in BAT. Ucp1 induces uncoupling respiration and mitochondrial ATP synthesis by reducing the proton gradient across the mitochondrial inner membrane (29). Ucp1 ablation is reported to induce obesity and abolish thermogenesis at thermoneutrality (30). Our data suggest a functional role of L-theanine in inducing the browning of white adipocytes, which help to improve thermogenic capacity and combat obesity in mice.

AMPK plays an important role in energy metabolism and mitochondrial biogenesis (31,32). However, the mechanisms by which AMPK regulates metabolism are not completely understood. Many studies have shown that activating AMPK can improve metabolic rate and help to resist obesity. For instance, metformin promotes browning of adipose tissue and prevents fatty liver (33). Taurine activates AMPK to promote browning of WAT and combat obesity in mice (34). Obesity and glucose metabolic disorder are closely associated with a decrease in AMPK activity (35). Our results identified that the master regulator AMPK is involved in the L-theanine-mediated WAT thermogenic program.

The structure of L-theanine is similar to that of L-glutamine. L-Glutamine is conditionally essential and the most abundant amino acid in the body. It acts as an important energy source and supplies carbons to the TCA cycle to produce ATP. Therefore, insufficiency of L-glutamine could cause an energy shortage and lead to AMPK activation (36). It has been reported that L-theanine could compete with L-glutamine in binding to the L-glutamine transporter on the cell surface, which is responsible for the incorporation of extracellular L-glutamine (37). In this way, L-theanine could inhibit the uptake of L-glutamine by the cells. Therefore, L-theanine treatment of the adipocytes may inhibit the uptake of L-glutamine and decrease the intracellular L-glutamine level, which would lead to an energy shortage and activate AMPK phosphorylation. Further studies are needed to investigate the above hypothesis.

Our data revealed a functional role of L-theanine in the activation of thermogenic program in adipocytes, which is shown to be involved in the increased energy expenditure and antiobesity function. Indeed, our results show that L-theanine could activate differentiated C3H10T1/2 and iWAT SVF adipocytes to express more thermogenic-related genes and exhibit brown adipocyte-like phenotypes. We demonstrated that transdifferentiation of adipocytes could be induced by L-theanine in vitro. L-Theanine could upregulate the expression of many browning-associated genes. The most prominent shift in this process was Prdm16. Notably, Prdm16 is a critical regulator of browning and the thermogenic gene expression program (20,38). Raising the Prdm16 level ameliorates fibrosis of aged mice and promotes beige adipocyte activation (39). Thus, Prdm16 plays an important role in L-theanine-mediated browning of white adipocytes.

C3H10T1/2 and iWAT exhibit a significant increase in α-KG concentration after L-theanine treatment, which could facilitate DNA demethylation of Prdm16. Beneficial effects of α-KG on extending the life span of adult worms have been reported (40). α-KG is a product of the TCA cycle and produced from isocitrate by isocitrate dehydrogenase, which can also be produced from glutamate by glutamate dehydrogenase. It functions as a cofactor of TETs to catalyze DNA demethylation. Inhibition of the AMPK/α-KG signaling pathway suppressed BAT development, which contributes to obesity (41). Our results reveal an L-theanine/AMPK/α-KG/Prdm16 axis that induces the brown-like features in white adipocytes.

In conclusion, our results show a previously unknown role for L-theanine in regulating adipose tissue and adipocyte metabolism. We identified that L-theanine promotes the browning of adipose tissue in NCD- and HFD-fed mice, which ameliorates obesity, improves glucose and insulin tolerance, and reduces levels of plasma lipids (Supplementary Fig. 8). Thus, L-theanine may provide an approach to help combat obesity and obesity-associated disorders based on its positive role in WAT browning.

Funding. This study was supported by the National Key R&D Program of China (2018YFA0800401 to Q.-Q.T.) and National Natural Science Foundation of China grants (32070751 and 31871435 to L.G. and 81730021 and 32070760 to Q.-Q.T.).

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. W.-Q.P. and L.G. were involved in study design, conducted the experiments, analyzed the data, and drafted the paper. G.X., B.-Y.L., and Y.-Y.G. performed the experiments. L.G. and Q.-Q.T. designed and supervised the study and wrote the paper. W.-Q.P. and L.G. are the guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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