Ellagic acid promotes browning of white adipose tissues in high-fat diet-induced obesity in rats through suppressing white adipocyte maintaining genes

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Abstract. Promoting brown adipose tissue (BAT) formation and function reduces obesity. Ellagic Acid (EA), located abundantly in plant extracts and fruits, has been shown to modulate formation and differentiation of adipocytes, although its role in the process of browning of white adipose tissue (WAT) has not been elucidated. In this study, fifty-six five-week old SD rats were randomly assigned to receive normal diet (ND, 10% lipids) or high-fat diet (HFD, 60% lipid) with or without various dosages of EA for 24 weeks. Our results showed that high fat diet intake triggered overweight, glucose intolerance and white adipocyte hypertrophy, the effects of which were mitigated by EA treatment. Meanwhile, EA supplementation reduced serum resistin levels, improved hepatic steatosis and serum lipid profile in DIO (high fat diet induced obesity) rats. Moreover, EA supplementation significantly decreased mRNA expression of Zfp423 and Aldh1a1, the key determinants of WAT plasticity. EA also increased mRNA expression of brown adipocyte markers including UCP1, PRDM16, Cidea, PGC1α, Ppar-α; beige markers including CD137 and TMEM26; mitochondrial biogenesis markers including TFAM in inguinal WAT (iWAT) when compared to their counterparts. EA treatment significantly improved mitochondrial function, as measured by citrate synthase activity. More importantly, EA markedly elevated the expression of UCP1 in iWAT, which is a specific protein of brown adipocyte. In conclusion, our results provided evidence that EA improved obesity-induced dyslipidemia and hepatic steatosis in DIO rats via browning of iWAT through suppressing white adipocyte maintaining genes and promoting expression of key thermogenic genes. These findings suggest that EA could be a promising therapeutic avenue to treat metabolic diseases.

Key words: Ellagic acid, Obesity, Uncoupling protein 1, Browning of white adipose tissue

OBESITY became the major independent risk factor for hypertension, dyslipidemia, type II diabetes and cardiovascular disease, and nonalcoholic fatty liver disease and has become a major global health concern [1-3]. Although excess caloric intake and decreased energy expenditure promote overall weight gain, visceral adiposity is particularly associated with metabolic diseases [1]. As such, unique factors may control the development and function of specific fat depots. WAT stores energy in the form of triglycerides. Brown and beige fat cells are specialized to dissipate chemical energy in the form of heat and likely evolved to protect mammals from hypothermia [4]. The strategies to induce BAT formation and/or browning of WAT are considered a potential and promising therapeutic strategy to combat obesity and the associated metabolic diseases [5]. This thermogenic function of brown and beige fat cells are mediated predominantly by the presence of uncou-
pling protein 1 (UCP1), a protein that catalyzes a proton leak across the inner mitochondrial membrane [6]. The key characteristic of beige cells in WAT is the ectopic expression of hallmark proteins for brown adipocytes such as UCP1, PR domain-containing 16 (PRDM16), Cell death-inducing DFFA-like effector A (CIDEa), and peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α) and peroxisome proliferator-activated receptor-α (PPAR-α) [7].

To date, there are no specific medical treatments approved for obesity. Chemical medicines possess the severe side effects [8]. Up-to-date, the best intervention for obesity is lifestyle modification, although compliance is challenging. Recent evidence indicates that browning of white fat tissue propose a promising strategy for prevention of obesity.

Dietary polyphenols have been extensively investigated for their effects on metabolic disease, such as obesity, type II diabetes, hyperglycemia, and insulin resistance in humans and other animals [9, 10]. In a rat experimental model of obesity, administration of epigallocatechin-galate alleviated the severity of obesity [11]. Moreover, edible plants, such as bitter gourd [12] and resveratrol [13], have been shown to ameliorate glucose and lipid metabolism disorders and improve high-fat diet-induced obesity.

Ellagic acid (EA) is the highest content of polyphenols in fruits, flowers and peels derived from a variety of plants [14]. In previous study, we found that EA could reduce adipogenesis through prevention of the induction of Rb phosphorylation to inhibit the differentiation in 3T3-L1 preadipocytes [15]. Panchal and colleagues showed that EA attenuates high-carbohydrate, high-fat diet-induced metabolic syndrome in rats [16]. Okla and coworkers showed that EA, the major polyphenols in the NAcy fraction of a muscadine grape phytochemical powder (MGP), attenuated new fat cell formation and fatty acid biosynthesis in adipose tissues and decreased visceral fat mass in mice [17, 18]. However, the role for EA in browning of WAT has not been evaluated.

Here in this study, we sought to delineate the role of EA in browning of WAT to explore the potential mechanism involved. Our data show that EA induces browning of white fat, a process probably mediated by altering the white adipocytes maintaining genes expression of Zpf423 and Aldh1a1.

Materials and Methods

Animal models

Animal procedures were approved by The Medical Experimental Animal Center of Xinjiang Medical University. Male SD rats (5 weeks old, 200 ± 20 g, n = 56) were purchased from The Medical Experimental Animal Center of Xinjiang Medical University. Given the estrogenic effects of the female, we used only male rats in our study to avoid potential obesogenous effects of estrogen on the body fat distribution in rats. After acclimatization for a week, rats were randomly divided into seven groups: normal diet-fed rats (ND; 10% kcal as fat; n = 8, Medical Experimental Animal Center, Xinjiang, China); high-fat diet rats (HFD; 60% kcal as fat; n = 8, Huafuwei; Beijing, China); high fat diet-fed rats supplemented with high dose ellagic acid (HF + HDEA; n = 8; 30 mg/kg/d; Sigma, USA); high fat diet-fed rats with low dose ellagic acid (HF + LDEA; n = 8; 10 mg/kg/d; Sigma, USA); High fat diet-fed rats treated with the following known anti-obesity drugs as positive control: Metformin Hydrochloride Extended-release Tablets (HF + DMBG; n = 8; 89.25 mg/kg/d food; SFDA approval number: H20023370; Shiguibao pharmaceutical co. LTD; Shanghai, China) and Orlistat Capsules (HF + Orl; n = 7–8; 37.8 mg/kg/d; SFDA approval number: H20143119; New time pharmaceutical co., LTD; Shandong, China) and Tiopronin enteric-coated tablets (HF + Tio; n = 7–8; 47.25 mg/kg/d; SFDA approval number: H41020799; Xinyi pharmaceutical co. LTD; Henan, China). All the above drugs were delivered to animals by oral gavage. Rats were housed in a 12-h light/12-h dark cycle and with controlled humidity (50 ± 10%) and temperature (21 ± 2°C) in pathogen-free cages with provided food and water ad libitum for 24 weeks. Body weight was measured at a fixed time weekly and food intake was monitored twice a week in the whole study.

Intraperitoneal glucosetolerance test (IPGTT) and insulin tolerance test (IPITT)

At the end of the intervention, the IPGTT and the IPITT were performed. For IPGTT, rats were fasted for 12 h and then injected intraperitoneally with glucose solution (1.5 g/kg body wt; C150623D; Celun pharmaceutical co. LTD; Hubei, China). For IPITT, rats were fasted for 4 h followed by intraperitoneal insulin injection (0.75 IU/kg body wt; 8026589, Novo nordisk (China) pharmaceutical co. LTD). Blood glucose levels were measured from tail vein at 0, 30, 60, 90, and 120 min with a glucometer (Roche, Basel, Switzerland). In addition, the area under the curve (AUC) of glucose concentration was calculated for both the IPGTT and the IPITT.

Blood and tissue collection and analysis

After the 24-week protocol, animals were fasted for 12 hrs and then deeply anesthetized (intraperitoneal sodium pentobarbital, 150 mg/g). Blood samples were collected by abdominal aortic artery. The plasma was separated by centrifugation at 4°C (3,000 r, 15 min) after placing 30
min at room temperature; then the plasma was stored at −20°C until further biochemical analysis for determination of serum resistin, adiponectin, total cholesterol (TC), triglycerides (TG), low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) by automatic biochemical analyzer (BS-320, Mindray, Shenzhen, China).

The subcutaneous inguinal fat pad, located in the mid-thigh and the lower part of the rib cage, was considered as the sWAT. All fat pads (epididymal, perirenal, retroperitoneal and inguinal) and liver were carefully dissected and weighed. The weight of all fat pads is used for calculating the adiposity index determined as the ratio between the sums of all fat pads masses divided by the body weight, represented as a percentage. sWAT (inguinal White adipose tissue, iWAT) was also prepared for routine histopathology or frozen to RT-qPCR analyses.

**Histological and immunostaining analysis**

Dissected liver and adipose tissue mass were fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin-embedded tissues were cut at 3 μm and the cutting and largest slides were stained using for hematoxylin-eosin (HE) or immunohistochemistry for UCP-1 (Ab10983; Abcam). We analyzed each slide using a digital image system (CX41; OLYMPUS, Japan).

**Quantitative real-time PCR**

Total RNA was isolated from iWAT and interscapular fat depots respectively using Trizol (1596-026, Invitrogen; China) according to the manufacturer’s instructions. The purity and concentration of RNA were detected by Nucleic acid quantitative analyzer (Thermo). cDNA was synthesized by reverse transcriptional PCR following the manufacturer’s instructions. Each cDNA sample triplicated and analyzed by quantitative real-time PCR that was accomplished on the Real-time instrument (ABI-7300, ABI, China) using SYBR Green PCR kit (#K0223; Thermo, USA) and gene-specific primers. The expression levels of target genes were normalized to the β-actin and Gapdh (see Table 1 for primer sequences).

**Western blot analysis**

Samples of iWAT and interscapular fat depots were homogenized and lysed in RIPA lysis buffer (Solarbio). The concentration of protein was assessed using BCA protein assay kit (Thermo). We mixed protein with Laemmli sample buffer according to the proportion of 1:3 and heated at 100°C for 10 min. Proteins (50 μg/ lane) was subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to Polyvinylidene Fluoride (PVDF) membranes (Millipore). Membranes were blocked with 10% skimmed milk powder in Tris-buffered saline Tween (TBST). Membranes were washed in TBST for 5 min and repeated 3 times and then incubated overnight at 4°C with rabbit polyclonal anti-uncoupling Protein-1 (anti-UCP1), rabbit polyclonal

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>UCP-1</td>
<td>TGGCATCCAGAGGCAAATC</td>
<td>GCATTGTAGGTCCCAAGTAG</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>AGGACACGAGGAAGGAAGAC</td>
<td>GGTAGCACTGGCTTGAATCTG</td>
</tr>
<tr>
<td>PPARγ</td>
<td>GTTGACACAGAGATGGCTTCACTTAC</td>
<td>GGAGAGCTGTCAGGGCGAACAG</td>
</tr>
<tr>
<td>β-actin</td>
<td>CTGAGGCGACAGTACAGAG</td>
<td>ATGGAGATGGCGACACACCTC</td>
</tr>
<tr>
<td>Zfp423</td>
<td>TCTACTCTTGCCCTTACTG</td>
<td>GTAAGCGCCTTAGATAG</td>
</tr>
<tr>
<td>Tnem26</td>
<td>AAGCAGGCACTAAGCAGAG</td>
<td>ATGAGATGGGCACACCTC</td>
</tr>
<tr>
<td>Tcfam</td>
<td>GGGATTGGGCAACAAGAG</td>
<td>GCATTGATGGCGCAAG</td>
</tr>
<tr>
<td>Prdm16</td>
<td>CCGAGTTCTCGGTGACTACAG</td>
<td>CTTCTCTGTCCTGGCTTAC</td>
</tr>
<tr>
<td>Ppar-α</td>
<td>TTGGACCTGGCATAGGACAGTGTTAC</td>
<td>ATGGCTTCTCAGGTCTTCTAG</td>
</tr>
<tr>
<td>Nrf-1</td>
<td>CTGTCAGGCTTCTGCGAGTAG</td>
<td>GGAGAGCTGTCAGGGCGAACAG</td>
</tr>
<tr>
<td>Cidea</td>
<td>GGTATGCGGGCGCTTATG</td>
<td>TTCTCTGGGGGGAATCCGT</td>
</tr>
<tr>
<td>CD137</td>
<td>AAGAGCCCTGCTCCCTCTACC</td>
<td>AAACCTGCTCCGTTAGTCTC</td>
</tr>
<tr>
<td>Aldh1a1</td>
<td>GGAAAGCAGTGCTGCTGAGAAG</td>
<td>CGCAGGGGGATGCAACAG</td>
</tr>
<tr>
<td>Gapdh</td>
<td>GGAGCTCTACTGGCGCTTCCAC</td>
<td>ATGAGCCCTTCCAGATGC</td>
</tr>
</tbody>
</table>
Adipose tissue citrate synthase activity

Anti-peroxisome proliferators activated receptor gamma co-activator-1 alpha (anti-PGC 1α), rabbit polyclonal anti-peroxisome proliferator-activated receptor gamma (anti-PPARγ), rabbit polyclonal anti-CCAAT/enhancer binding protein alpha (anti-C/EBP α), rabbit polyclonal CCAAT/enhancer binding protein beta (anti-C/EBPβ) (abcam, Cambridge, MA, USA), rabbit polyclonal anti-actin (Abcam, Cambridge, MA, USA). Bound antibodies were visualized with horseradish peroxidase (HRP) goat anti-rabbit IgG, goat anti-mouse IgG (Beyotime). Blots were developed with IMS image analysis system (JRDUN).

Statistical analysis

Data are presented as the means ± SD. Differences between the groups were analyzed using Student’s t-test or one way ANOVA followed by Dunnett’s multiple comparisons test by GraphPad Prism version 5.0 for Windows (GraphPad Software, La Jolla California USA). P < 0.05 was regarded as statistically significant.

Results

EA prevents body weight gain, WAT mass expansion, impaired glucose and insulin tolerance in HFD rats

At the beginning of the experiment, ND and HFD groups had no difference in their initial BW (body weight). After two week of dietary administration, the HFD group had a higher BW than the ND group, which continued to increase. As shown in Table 2, seven groups rats were fed either a ND or a HFD for 24 weeks, the weight gain in HFD group rats increased robustly compared with the ND groups (p < 0.05; Fig. 1A, Table 2). Treatment with HDEA, LDEA, DMBG, Orlistat (Orl) and Tiopronin (Tio) prevented the HFD-induced BW (p < 0.05 vs. HFD; Fig. 1A, Table 2). Average food intake was no difference in seven groups at any time point in the study (p > 0.05; Table 1B 2).

The HFD group had a higher adiposity index than the ND group. The treatments tackled this metabolic impairment as HDEA, LDEA, DMBG, Orlistat (Orl) and Tiopronin (Tio) had a reduced adiposity index when compared to the untreated HFD group (p < 0.05). These results are demonstrated in Table 2.

The HFD significantly uplifted fasting plasma levels of glucose (p < 0.001 vs. ND; Fig. 1C). Meanwhile, HFD also increased plasma glucose level 30 min after intra-peritoneal injection of glucose in the glucose tolerance test (p < 0.001; Fig. 1C), and same trend showed in the area under the curve (AUC) of plasma glucose (p < 0.001; Fig. 1D). Both high and low dosage of EA inhibited the HFD-induced increase in blood glucose in IPGTT (p < 0.001; Fig. 1C and D). Next, we performed IPITT to estimate relative insulin sensitivity. Results of IPITT showed HFD feeding caused impaired insulin sensitivity in mice. Treatment of EA maintained insulin sensitivity in mice fed a HFD for 24 weeks (p < 0.05 and p < 0.001, respectively; Fig. 1E and 1F).

Blood parameters were monitored by biochemistry test performed at the end of 24 weeks of the experiment. HFD + HDEA in our study did not significantly alter LDL-c (p = 0.3055) and TG (p = 0.076; Table 3). But TC increased in HF + HDEA and orlistat groups (p < 0.05; Table 3). And we observes a significant rise of HDLC in treatment of HF + HDEA, HF + LDEA at 24 weeks vs. HFD (p < 0.05; Table 3).

Table 2 Parameters of seven groups male SD rats fed a ND or HFD for 25 weeks

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ND</th>
<th>HFD</th>
<th>HFD + HDEA</th>
<th>HFD + LDEA</th>
<th>HFD + DMBG</th>
<th>HFD + Orl</th>
<th>HFD + Tio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>410.49 ± 22.33</td>
<td>559.45 ± 29.46*</td>
<td>417.06 ± 42.93*</td>
<td>395.61 ± 30.83*</td>
<td>412.07 ± 49.26*</td>
<td>408.09 ± 52.18*</td>
<td>395.93 ± 63.49*</td>
</tr>
<tr>
<td>Adiposity index</td>
<td>3.33 ± 0.06</td>
<td>6.26 ± 0.2*</td>
<td>3.96 ± 0.16*</td>
<td>4.17 ± 0.13*</td>
<td>4.89 ± 0.08*</td>
<td>4.31 ± 0.14*</td>
<td>3.82 ± 0.08*</td>
</tr>
<tr>
<td>The thickness of right perirenal fat (cm)</td>
<td>0.03 ± 0.01</td>
<td>0.22 ± 0.03**</td>
<td>0.05 ± 0.02**</td>
<td>0.06 ± 0.05*</td>
<td>0.05 ± 0.04*</td>
<td>0.01 ± 0.01***</td>
<td>0.08 ± 0.05</td>
</tr>
<tr>
<td>Average daily food intake (g)</td>
<td>37.27 ± 5.25</td>
<td>29.98 ± 4.97</td>
<td>27.13 ± 4.33</td>
<td>28.37 ± 4.90</td>
<td>30.26 ± 5.24</td>
<td>29.79 ± 4.16</td>
<td>30.59 ± 5.28</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>14.25 ± 2.22</td>
<td>16.73 ± 2.27*</td>
<td>12.28 ± 1.84**</td>
<td>13.67 ± 1.26*</td>
<td>14.23 ± 1.23*</td>
<td>14.26 ± 1.79*</td>
<td>14.04 ± 1.96*</td>
</tr>
</tbody>
</table>

* Values are expressed as Means ± SD.
** p < 0.05, *** p < 0.01 vs. ND group; ** p < 0.05, *** p < 0.01 vs. HFD group.
significantly increased \((p < 0.05)\) when fed HFD compared to the ND group, and high dose but not low dose EA treatment blocked the increase the serum resistin levels caused by DIO (Fig. 2A). As for adiponectin, HFD tended to decrease the level but no statistical significance was reached. Neither high nor low dose EA treatment altered the serum adiponectin levels (Fig. 2B).

**Treatment with EA ameliorated HFD-induced tissue lipid accumulation**

Tissue weight of liver was significantly increased in HFD. However, the treatments of HF + HDEA and HF + LDEA had a decreased liver weight when compared with HFD rats \((p < 0.01;\) Fig. 3A). Consistently, H&E staining showed that HFD induced lipid accumulation and cell enlargement in iWAT and BAT, which were ameliorated
in HF + HDEA (Fig. 3B–D). Meanwhile, H&E staining revealed greater steatosis (fat deposition in the pericentral areas) in livers of HFD rats. Interestingly, HFD-induced fat accumulation in the pericentral area was overtly attenuated by HDEA and LDEA (Fig. 3B, 3E).

Intervention with EA markedly increases browning of WAT depots and restrain key proteins of white adipogenic factors expression in iWAT

Reportedly, the browning of WAT can improve metabolic profile, exerting protection the rats from high-fat diet-induced obesity. Adipose tissue mass accumulation results from proliferation and differentiation of adipocytes, in which several key proteins of adipogenesis were included. To determine whether alterations in brown fat specific genes expression contributed to the protective effect of glucose tolerance and body composition after intervention with EA, we investigated the occurrence of browning in inguinal fat depots and key proteins of adipogenesis.

We firstly analyzed the expression of brown adipocyte marker genes in inguinal fat depot. The mRNA and protein level of UCP-1 \( (p < 0.001; \text{Fig. 4A, 5A–B}) \), PGC-1α \( (p < 0.001; \text{Fig. 4B, 5A, 5C}) \) were significantly reduced in HFD vs. ND rats. But PPARγ \( (p < 0.001; \text{Fig. 4C, 5D, 5E}) \), C/EBP-β \( (p < 0.001; \text{Fig. 4D, 5D, 5F}) \) and C/EBP-α \( (p < 0.001; \text{Fig. 4E, 5D, 5G}) \) were significantly elevated in HFD when compare with ND rats. The reducing expression levels of two brown fat markers, UCP-1 and PGC-1α, were recovered with high and low dosage treatment of EA compared with the HF group \( (p < 0.01 \text{ or } p < 0.001; \text{Fig. 4A–B, 5A–C}) \), and PPARγ, C/EBP-β and C/EBP-α were significantly reduced by the same treatment \( (p < 0.001; \text{Fig. 4C–E, 5D–G}) \).

We also examined the potential effect of EA on Zfp423 and Aldh1a1, the key determinants of WAT plasticity, in inguinal fat pads \([4, 19]\). Our data revealed that HFD increased Zfp423 \( (p < 0.05) \) and Aldh1a1 \( (p < 0.001) \) gene expression, the effects of which were attenuated significantly with the administration of high dose of EA \( (Zfp423: p < 0.01; \text{Fig. 4F–G}) \).

To further clarify the function of EA on WAT browning, we measured mRNA expression of known BAT markers including cell-death activator (Cidea), Ppar-α, PR domain–containing 16 (Prdm16) in the inguinal fat pad depot. HFD could not affect gene expression of Ppar-α and Prdm16, but HDEA could increase their expression \( (p < 0.001, \text{Fig. 4H; Prdm16: } p < 0.05; \text{Fig. 4I}) \).

### Table 3 The serum lipid levels of TG, TC, HDL-C and LDL-C on rats in seven groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>HDL-C (mmol·L⁻¹)</th>
<th>LDL-C (mmol·L⁻¹)</th>
<th>TC (mmol·L⁻¹)</th>
<th>TG (mmol·L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND</td>
<td>2.96 ± 0.37</td>
<td>0.38 ± 0.30</td>
<td>0.28 ± 0.16</td>
<td>6.59 ± 4.54</td>
</tr>
<tr>
<td>HFD</td>
<td>2.9 ± 0.59</td>
<td>0.81 ± 0.16*</td>
<td>1.37 ± 0.25*</td>
<td>9.86 ± 0.26*</td>
</tr>
<tr>
<td>HF + HDEA</td>
<td>3.97 ± 0.42**</td>
<td>0.92 ± 0.15</td>
<td>1.64 ± 0.15*</td>
<td>9.20 ± 0.42</td>
</tr>
<tr>
<td>HF + LDEA</td>
<td>3.51 ± 0.38**</td>
<td>1.00 ± 0.12</td>
<td>1.54 ± 0.1</td>
<td>9.07 ± 0.52</td>
</tr>
<tr>
<td>HF + DMBG</td>
<td>2.93 ± 0.42</td>
<td>0.67 ± 0.39</td>
<td>1.32 ± 0.23</td>
<td>9.55 ± 1.16</td>
</tr>
<tr>
<td>HF + Orl</td>
<td>3.43 ± 0.45**</td>
<td>0.86 ± 0.18</td>
<td>1.65 ± 0.26*</td>
<td>9.70 ± 0.97</td>
</tr>
<tr>
<td>HF + Tio</td>
<td>3.62 ± 0.65**</td>
<td>0.83 ± 0.15</td>
<td>1.57 ± 0.28</td>
<td>10.40 ± 2.59</td>
</tr>
</tbody>
</table>

Values are expressed as Means ± SD. * \( p < 0.05 \) vs. ND group; † \( p < 0.05 \) vs. HFD group.

**Fig. 2** The effect of EA on the serum resistin (A) and adiponectin (B). Data were expressed as mean ± S.D. \( n = 6 \) rats. * \( p < 0.05 \) vs. ND; † \( p < 0.05 \) vs. HFD.
Meanwhile, the increase of Cidea expression triggered by HFD were blocked by the HDEA (p < 0.01, Fig. 4J). For beige marker CD137 and transmembrane protein 26 (TMEM26), DIO failed to alter their expression although administration of HDEA significantly increased their levels (p < 0.01, Fig. 4K–L).

The mitochondrial biogenesis related genes were also measured. HFD decreased the levels of nuclear respiratory factor 1 (Nrf1) (Fig. 4M, p < 0.001), the effect of which was unaffected by HDEA. Meanwhile, administration of HFDA increased the gene expression of mito-
Fig. 4  EA promoted browning of iWAT

A–B). Relative mRNA expression of browning markers of iWAT from all groups (n = 7–8). UCP-1 (A), PGC1-α (B). C–E). Relative mRNA expression of white adipogenic factors in iWAT from all groups (n = 7–8). mRNA abundance was measured by qRT-PCR and expressed as fold increase compared with ND group. PPARγ (C), C/EBPβ (D), C/EBPα (E). F–G). Relative mRNA expression of the key determinants of iWAT plasticity, Zfp423 (F) and Aldh1a1 (G) in iWAT. H–I). Relative mRNA expression of known BAT markers: Ppar-α (H), Prdm16 (I), Cidea (J). K–L). Relative mRNA expression of beige marker CD137 (K) and TMEM26 (L). M–N). Relative mRNA expression of the mitochondrial biogenesis related genes: Nrf1 (M) and Tfam (N). Values are expressed as Means ± SD. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. ND group. † p < 0.05, †† p < 0.01, ††† p < 0.001 vs. HFD group.
Intervention with EA activated brown adipose tissue function in rats fed HF diet

Since intervention with EA increased browning of
WAT depots, we next assessed mRNA expression levels of genes related to proliferation and differentiation and energy expenditure in BAT. With the HFD, UCP-1 and PGC-1α were significantly downregulated in BAT. However, with the administration of HDEA mitigated the decreasing of the mRNA expression levels (\( p < 0.001 \), Fig. 6A–B).

**Intervention with EA activated a thermogenic program in WAT**

To test whether Intervention with EA altered iWAT function, we assayed mitochondrial activity using citrate synthase activity, the rate-limiting step of the tricarboxylic acid cycle [4]. Citrate synthase activity was significantly reduced in iWAT of HFD group but not in BAT as compared to those in WT mice. The impairment of citrate synthase activity was attenuated by administration of EA (Fig. 7A). The increase in UCP-1 expression in iWAT was confirmed by immunohistochemistry staining (Fig. 7B).

*Fig. 6* EA increased function of brown adipose tissue under high fat diet (HFD)-feeding

A–B). Relative mRNA expression levels of genes related to thermogenesis in BAT of all groups (\( n = 7–8 \)). Values are expressed as Means ± SD. *** \( p < 0.001 \) vs. ND group; **\( p < 0.01 \), *** \( p < 0.001 \) vs. HFD group.

*Fig. 7* EA activates a browning program in iWAT

A). Citrate synthase activity in iWAT and BAT (\( n = 6 \)). B). Representative UCP-1 staining of iWAT of the groups. Original magnification \( \times 400 \). Values are expressed as Means ± SD. * \( p < 0.05 \) vs. ND group; **\( p < 0.05 \) vs. HFD group.
Discussion

The salient findings from this study revealed that oral administration of EA for 24 weeks can prevent diet-induced obesity (DIO). These results have unraveled the potential therapeutic role of EA in obesity through apparent reduction in BW without influencing food intake, expansion of visceral fat mass, and sizes of adipocytes in DIO rats likely through a mechanism related to browning of WAT. In our hands, impaired glucose, insulin resistance and hepatic steatosis were improved with administration of EA in conjunction with reduced serum resistin levels, improved hepatic steatosis and serum lipid profile in DIO rats. Nevertheless, treatment with HDEA enhanced gene expression of markers from brown/beige adipocytes (UCP1, Ppara; PGC1-a, Cide a, PRDM16, CD137 and TEMEM26) and mitochondrial biogenesis related gene (Tfam) in the iWAT, suggesting browning in this site. HDEA treatment increased mitochondrial citrate synthase enzyme activity in iWAT but not BAT. Moreover, EA markedly elevated the expression of UCP1 in iWAT. These observations were in compliance with the presence of UCP1 positive beige adipocytes in iWAT, confirming the browning phenomenon with the HDEA treatment.

Reduced BW in EA treated groups is consistent with the reduced adiposity index, WAT cell size and the consequent improvement in insulin resistance. The enlarged adipocytes in HF group were in accordance with hyperinsulinemia found because of the disrupted adipinsular axis, caused by the chronic HF diet intake [20]. Hence, hyperinsulinemia impaired the pancreatic islet function with time, in addition to its stimulating effect on lipogenesis, resulting in adipocyte hypertrophy, along with, however, inhibition of adipocyte hyperplasia and white/brown plasticity [21].

It is known that liver is one of the target sites of resistance. In the current study, HDEA reduce plasma resistin levels induced by HFD. Gene ablation of resistin in leptin-deficient mice improved hyperlipidemia and hepatic steatosis [22]. Yoshimura and colleagues showed that EA improved hepatic steatosis and serum lipid composition through reduction of serum resistin levels [23]. Our result also showed that the supplementation of EA decreased the rise of serum resistin levels brought by HFD. However, EA fail to affect adiponectin level, which is consistent with the work from Yoshimura Y. and the colleague [23, 24]. So it is thus plausible to speculate that the HDEA may improve the hepatic steatosis through inhibition of resistin secretion.

Liu and coworkers displayed similar trend of elevated blood level of high-density lipoprotein with EA treatment, which may be responsible for restored cognitive performance related to age-related overweight participants [25]. This indicated that beneficial metabolic effects of EA were associated with improvement of lipid spectrum. In previous study, we found that EA treatment retards adipogenesis through inhibition of differentiation in 3T3-L1 adipocytes [15]. Gourineni V. et al. showed that the non-anticoagulant (NAc) fraction of muscadine grape phytochemicals (MGP) decreased visceral fat mass in mice [17]. EA, the major polyphenols in NAc fraction, dramatically suppressed lipid accumulation in a dose-dependent manner in vitro using the human adipogenic stem cell (hASCs) model. Meanwhile, EA attenuated new fat cell formation and FA biosynthesis in adipose tissues. It also decreased synthesis of TG and FA as well as FA oxidation in livers from HFD fed mice [18]. EA exerted distinct lipid-lowering properties to decrease biosynthesis of FA in both adipocytes and hepatocytes, although it augmented FA oxidation only in hepatocytes [18]. Selvi and associates recently revealed that EA inhibited adipogenesis through hindering coactivator-associated argininemethyltransferase 1 (CARM1) [26], which is required for adipogenesis [27], to negatively regulatehistone 3 arginine 17methylation (H3R17me2).

Adipocyte browning has drawn some recent attention to counter adverse metabolic consequences of obesity [28-31]. Transcriptional factor Zfp423 (C2H2 zinc-finger protein), a white adipocytes identity maintaining gene, is highly expressed in WAT than BAT. Zfp423, which functions in part through coactivation of Smad proteins in the bone morphogenetic protein (BMP) signaling cascade, regulates preadipocyte levels of Ppara and adipogenesis. Inducible genetic ablation of Zfp423 in white adipocytes of adult animals leads to a conversion of mature white adipocytes into functional beige adipocytes, fostering resistance and reversal of diet-induced obesity and impaired glucose homeostasis [19]. Mechanistically, Zfp423 acts in adipocytes to inhibit the activity of transcriptional regulators Ebf2 and suppress Prdm16 activation. These data have identified the role for Zfp423 as a molecular brake on adipocyte thermogenesis and suggested a therapeutic strategy to unlock the thermogenic potential of white adipocytes in obesity [19]. Retinaldehyde dehydrogenases 1a1 (Aldh1a1) was primarily expressed in visceral WAT, and its expression level was highly associated with obesity [4]. Florian W K. et al. showed that deficiency of the Aldh1a1 gene induced a BAT-like transcriptional program in WAT that drove uncoupled respiration and adaptive thermogenesis. WAT-selective Aldh1a1 knockout conferred this BAT program in obese mice, limiting weight gain and improving glucose homeostasis [4]. EA supplementation significantly decreased mRNA expression of Zfp423 and
Aldh1a1, which may decipher the possible mechanism(s) behind EA-induced promotion of browning of WAT.

Our current study also revealed that EA intervention effectively improved mRNA levels and protein expression of UCP-1 and PGC-1α in conjunction with decreased levels of PPARy, C/EBP-β and C/EBP-α in iWAT in the face of HFD intake. Meanwhile, the loss of UCP-1 and PGC-1α in DIO was significantly attenuated with browning of WAT [32, 33]. Recent studies [30]. PGC-1α serves as a target of UCP-1, closely associ‐

ated with the function of brown adipose tissues [30]. PGC-1α serves as a target of UCP-1, closely associ‐

ated with browning of WAT [32, 33]. Recent studies have unveiled a role of dietary polyphenols in prevention of obesity and obesity-related chronic diseases. Resvera‐

trol, a natural polyphenol present in the skin of grapes and other plants, significantly increased mRNA and/or protein expression of brown adipocyte markers including UCP1 and PGC-1α in differentiated iWAT stromal vascular cells (SVC), suggesting that resveratrol induced brown-like adipocyte formation in vitro. The UCP-1 protein content was 1.5-fold higher in the resveratrol group than that of the control group [34]. These observations of EA-promoted brown adipose tissue were consistent with earlier reports [32-34].

It is noteworthy that HFD treated animals did not show altered PRDM16, Ppar-α, TMEM26, CD137, Tfam genes levels. However, administration of HDEA signifi‐

antly increased their expression. PRDM16 is involved in the development and function of classical brown and beige adipocytes [7]. Paul Cohen, et al. reported that Adipo-PRDM16 KO mice showed reduced expression of a broad panel of thermogenic (Pgc1a, and Ucp1), brown adipose identity (Cidea and Ppar-α) [7]. Cide-a and UCP1 are both localized to the mitochondria, and CIDEA was identified as a likely inhibitor of UCP-1 activity by forming a complex with it [21], besides being positively related to the size of lipid [35]. Hence, HDEA treatment showed lowered Cide-a gene expression accompanied by an enhanced UCP1 gene expression.

TFAM and NRF-1 are crucial genes for mitochondrial biogenesis. The coupling between the nucleus and mitochondria is governed by Nrf-1, which then signals TFAM to activate mitochondrial DNA duplication [36]. The HDEA treatment was found to facilitate TFAM gene levels without affecting the level of Nrf-1. Citrate synthase is the rate-limiting enzyme in the tricarboxylic acid cycle [4]. The impaired enzyme activity in HFD was ameliorated by HDEA treatment. These observations were in consistence with the presence of UCP1 positive beige adipocytes in iWAT, which provide the evidence for the role of EA on increasing thermogenic capacity in white fat, confirming the effect of the HDEA treatment on browning.

Several experimental limitations prevail in our present study. First and foremost, there is emerging evidence for proven health benefits for urolithin A, EA-derived gut microbial metabolite of unabsorbed ellagic acid and ella‐

gitannins [37, 38]. It remains to be determined if it is urolithins rather than EA to account for a better strategy in the browning of iWAT. The absorbed EA is converted to methyl esters, dimethyl esters, and glucuronides, which are eliminated through urination within 1–5 h after ingestion [39, 40]. Neither tissue nor plasma EA level was monitored to correlate the dose of EA and browning of iWAT. Body temperature or energy expenditure and the mitochondria numbers should also be evaluated in white adipose tissue (WAT) to fully depict the function of dosed EA. Further studies need to be carried to clarify the direct mechanism of the effect of EA on insulin sensitiv‐

ity and reduced body weight or browning related gene expression profiles.

In conclusion, data from our study demonstrated that EA offers some promises for HFD-induced hyperglyce‐

mic, insulin resistance with altering the plasticity of WAT. These results suggest a role for EA in altered met‐

abolic profile through improving metabolic activity of adipose tissue, which is expected to have a significant impact on adiposity, glucose tolerance, and resistance to HFD through promoting the browning of WAT via altering the related gene profiles. EA reduces the expression of key determinants of WAT plasticity while increasing expression of brown/beige fat marker proteins, promoting browning of white adipose tissue to improve obesity‐

induced dyslipidemia and hepatic steatosis in DIO rats. Further studies are warranted to reveal the in-depth mechanisms underneath browning of iWAT. More impor‐

tantly, translational studies involving clinical trials and community intervention are needed to further consolidate the anti-obesity and/or anti-diabetic benefits of EA and other dietary resources with rich bioactive compounds.

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Ellagic acid promote browning of WAT

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