



# Dietary Apigenin promotes lipid catabolism, thermogenesis, and browning in adipose tissues of HFD-Fed mice

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## ABSTRACT

Dietary Apigenin (AP), a natural flavonoid from plants, could alleviate high-fat diet (HFD) induced obesity and its complication. Nonetheless, the direct correlation between dietary AP and their effects in adipose tissues remained unclear. In this study, male C57BL/6 mice were fed with low-fat diet, HFD with or without 0.04% (w/w) AP for 12 weeks. Dietary AP ameliorated HFD induced body weight gain, glucose intolerance, and insulin resistance. Energy expenditure was increased with no influence on energy intake, which indicated us that AP prevented obesity by enhancing energy export. Interestingly, AP activated lipolysis (ATGL/FOXO1/SIRT1) without higher cycling free fatty acids (FFAs). FFAs were consumed by the upregulation of fatty acid oxidation (AMPK/ACC), thermogenesis, and browning (UCP-1, PGC-1 $\alpha$ ). Additionally, adipose tissue metabolic inflammation (NF- $\kappa$ B, MAPK) was also reduced by AP. Our study proposed that dietary AP could be explored as a new dietary strategy to combat obesity and related insulin resistance.

## 1. Introduction

Obesity, a vital predictor of type 2 diabetes development, has become a worldwide epidemic. The World Health Organization (WHO) reported that over 1.9 billion adults and nearly 400 million children and adolescents were overweight or obese by 2016 (<https://www.who.int/en/news-room/fact-sheets/detail/obesity-and-overweight>).

Therefore, it presents a global concern to prevent obesity and its complication. During the development of HFD-induced obesity, the imbalance of lipid synthesis and catabolism causes excessive lipid accumulation and ultimately leading to adipocyte hypertrophy and hyperplasia (Wang et al., 2013). In rodents, adipocytes are classified into three distinct types: white adipocyte, brown adipocyte and beige adipocyte (Rosen and Spiegelman, 2014). White adipocyte store excess energy as triglyceride (TG), while brown adipocyte and beige adipocyte (two types of UCP-1-positive adipocyte) dissipate energy into the form of heat and increase whole-body energy expenditure (Wu et al., 2012). Thereunto, brown adipocyte and beige adipocyte originated from distinct lineages can be found in brown adipose tissue (BAT) and white adipose tissue (WAT) (Kajimura and Saito, 2014; Long et al., 2014; Sae-Tan et al., 2015) respectively. The more browning propensity in WAT causes the higher resistance to HFD-induced obesity (Seale et al., 2011). In addition, FFAs released by lipolysis serve both as activators of UCP-1

and fuel for non-shivering thermogenesis in brown and beige adipocytes (Bartelt et al., 2011; Fedorenko et al., 2012; Li et al., 2014). Once adipose tissue lipolysis was impaired, ATGL and HSL, the major lipolytic enzymes, could not transform TG into FFAs which ultimately interfered with the thermogenesis and browning (Osuga et al., 2000; Schweiger et al., 2006). In diet-induced obesity, HFD diet not only accumulates much TG in adipocyte, but also reduces the ability of lipolysis, thermogenesis, and browning. So, the pharmacologic or nutritional enhancement of this progress might be regarded as possible strategies to protect organisms from HFD-induced obesity and related insulin resistance.

Nowadays, many weight-reducing pills have serious side effects. Hence, naturally occurring bioactive compounds attract a lot attention for replacing them. AP is known as 4', 5, 7-trihydroxyflavone and abundantly presents in common fruits and vegetables. Many investigators have focused on AP due to its high chemopreventive efficiency in mice, anti-obesity (Gentile et al., 2018), anti-inflammation (Feng et al., 2016), anti-steatohepatitis (Feng et al., 2017), and the remission of insulin resistance (Feng et al., 2017). Moreover, some studies also showed that AP could improve lipid accumulation and insulin resistance in 3T3-L1 adipocyte (Ono and Fujimori, 2011; Kim et al., 2014; Guo et al., 2016; Gomez-Zorita et al., 2017) and HepG2 (Bumke-Vogt et al., 2014). Nevertheless, the regulatory effects of

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## Abbreviations

<b>ACC</b>	1-aminocyclopropane-1-carboxylic acid
<b>AKT</b>	protein kinase B
<b>AMPK</b>	AMP-activated protein kinase
<b>AP</b>	apigenin
<b>ATGL</b>	adipose triglyceride lipase
<b>ATM</b>	adipose tissue macrophage
<b>BAT</b>	brown adipose tissue
<b>EAT</b>	epididymis adipose tissue
<b>FFAs</b>	free fatty acids
<b>FOXO1</b>	forkhead box O1
<b>GTT</b>	glucose tolerance test
<b>HFD</b>	high-fat diet;
<b>0.02 HAPHFD</b>	+ 0.02% (w/w) AP
<b>0.04 HAPHFD</b>	+ 0.04% (w/w) AP
<b>HSL</b>	hormone-sensitive lipase

<b>IKK<math>\alpha</math>/<math>\beta</math></b>	I $\kappa$ B kinase $\alpha$ / $\beta$
<b>I<math>\kappa</math>B<math>\alpha</math></b>	NF- $\kappa$ B inhibitor $\alpha$
<b>ITT</b>	insulin tolerance test
<b>JNK</b>	c-Jun N-terminal kinase
<b>LFD</b>	low fat diet;
<b>MAPK</b>	mitogen-activated protein kinase
<b>NF-<math>\kappa</math>B</b>	nuclear factor- $\kappa$ B
<b>P38 MAPK</b>	P38-mitogen activated protein kinase
<b>PGC-1<math>\alpha</math></b>	Peroxisome proliferator-activated receptor gamma coactivator-1 $\alpha$
<b>RER</b>	respiratory exchange ratio
<b>SAT</b>	subcutaneous adipose tissue
<b>SIRT1</b>	silent information regulator 2 homologae 1
<b>SVF</b>	stromal vascular fraction
<b>TG</b>	triglyceride;
<b>UCP-1</b>	uncoupling protein 1
<b>WAT</b>	white adipose tissue

dietary AP on adipocyte lipolysis, thermogenesis and browning in mice still remain unclear.

On the basis of aforementioned facts, we did systematically exploration in mice. After the successful establishment of mice model, we firstly assessed the effects of dietary AP on energy intake and expenditure. Subsequently, further studies were performed on the mechanisms of reducing fat. Investigation results demonstrate that AP could enhance lipolysis (ATGL/FOXO1/SIRT1) providing much FFAs for fatty acid oxidation (AMPK/ACC), thermogenesis and browning (PGC1- $\alpha$ , UCP-1). Altogether, these results indicate that dietary AP ameliorates obesity and related insulin resistance through strengthening lipolysis, thermogenesis, and browning in adipose tissues.

## 2. Materials and methods

### 2.1. Chemicals

Apigenin ( $\geq 99\%$ ) was obtained from Supring & Automn Biological Engineering (Nanjing, China). D-glucose and collagenase were purchased from Sigma-Aldrich company (Saint Louis, MO, USA). ELISA kits were purchased from BD Bioscience company (San Diego, USA). RNAiso Plus reagent and SYBR green mix were purchased from TaKaRa (Dalian, China). BCA assay kit was purchased from Sango company (Shanghai, China).

### 2.2. Animals and treatments

Male 3-week-old C57BL/6 mice were provided by Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All the mice were constantly housed in standard conditions (temperature 24–25 °C, humidity 70–75%, lighting maintained in 12L/12D cycle). All the protocols were performed as per the regulations of National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by Institutional Animal Care and Use committee of Hefei University of Technology, China. After 2 weeks of adaption, mice were randomly divided into 3 groups (12 mice each) based on diet: LFD (Low fat diet) based on research diet D12450B containing 10% of energy from fat; HFD based on research diet D12451 containing 45% of energy from fat; 0.04% (w/w) AP ( $\geq 99\%$ , Nanjing, China) supplement is the major difference between HFD and 0.04 HAP group. The body weight and food intake were calculated each alternative week. After 12 weeks, all the mice were euthanized, and their blood samples, epididymal adipose tissue (EAT), subdermal adipose tissue (SAT), BAT and liver were harvested immediately and stored at  $-80$  °C. In addition, to detect the relationship between dose and effect of AP, another group HFD + 0.02% (w/w) AP (0.02 HAP) was set up in pre-experiment. After

feeding for 16 weeks, they were harvested as mentioned earlier.

### 2.3. Glucose tolerance tests (GTT) and insulin tolerance tests (ITT)

After 12 weeks, GTT and ITT were conducted on 17-weeks-old mice as previously described (Xiao et al., 2018). For GTT, mice were intraperitoneally (*i.p.*) injected with D-glucose (1 g/kg body weight) (Sigma-Aldrich, Saint Louis, MO, USA) after 12 h of fasting. For ITT, mice were *i.p.* injected with insulin (1.5 IU/kg body weight, WanBang BioPharma, Xuzhou, China) after 6 h of fasting. Blood samples were taken from tail veins at 0, 15, 30, 45, 60, 90, and 120 min right after *i.p.* injection. A blood glucose meter (Omnitest Plus, B. BRAUN, Melsungen, Germany) was used to measure blood glucose levels at indicated time intervals.

### 2.4. Metabolic parameter measures

After 12 weeks of treatment, mice were transferred to metabolic cages. Each metabolic cage housed 1 animal with free access to food and water. After 24 h of acclimatization, oxygen consumption ( $VO_2$ ), carbon dioxide production ( $VCO_2$ ) were constantly checked at 30-min intervals during the next 24 h using a combined indirect calorimetry system (TSE Systems GmbH, Bad Homburg, Germany) and the system calculated respiratory exchange ratio (RER) with the formula  $RER = VCO_2/VO_2$  at the same time. All the data were collected under ambient temperature at 25 °C during the dark period (19:00 to 7:00) and light period (7:00 to 19:00). After the metabolic measures, mice and their tissues were harvested for further experimentation.

### 2.5. Stromal vaescular fraction (SVF) isolation and flow cytometry

EAT was minced in PBS and incubated in collagenase (Sigma-Aldrich, Saint Louis, MO, USA) at 37 °C for about 20 min with shaking. Adipocytes were removed using a 75  $\mu$ m filter. The SVF pellet was harvested by centrifuging the suspension at 500 g for 5 min. Then after, the SVF pellet was resuspended and incubated with Red Blood Cell Lysis Buffer for 3 min. After washing with PBS, the rest cells were incubated with Fc Block (BD Biosciences, San Diego, USA) for 15 min and antibodies against F4/80-APC, CD11c-PE, and CD206-FITC (Bioscience, San Diego, USA) for 1 h. Finally, these cells were monitored by MoFlo XDP (Beckman Coulter, USA) (Zhang et al., 2016).

### 2.6. Histology and immunohistochemistry

Adipose tissues and liver samples were fixed in 4% formalin, embedded in paraffin and sectioned at 7  $\mu$ m thickness. The adipose tissue

and liver sections were stained with haematoxylin-eosin staining on regular basis. Anti-Mac-2 monoclonal antibody (1:800, Bio Legend, CA, USA) was used to label adipose tissue macrophage (ATM) in EAT sections. The five random fields from every part were targeted and visualized using Image-Pro Plus Version 6.0 (Media Cybernetics, Bethesda, MD, USA) to measure the adipocyte average diameters and macrophage positive area (Liu et al., 2013).

## 2.7. ELISA

After a spin at 5000 g for 30 min, serum was separated from blood. The levels of TG, FFAs, Leptin, Adiponectin, TNF- $\alpha$ , MCP-1, and IL-6 in serum samples were detected by corresponding mouse ELISA kits (BD Bioscience, San Diego, USA) (Dong et al., 2014).

## 2.8. RNA extraction and real-time PCR

Total RNA of adipose tissues was extracted by RNAiso Plus reagent (TaKaRa, Dalian, China). The extracted total RNA was used for the synthesis of first-stand cDNA. The real-time PCR amplifications were carried out by the power SYBR green mix (TaKaRa, Dalian, China) (Xu

et al., 2014). The primer sequences of genes used were listed in Table S1.

## 2.9. Protein extraction and western blotting

Frozen mice adipose tissues were lysed in RIPA. Cell homogenates were centrifuged at 12,000 g for 30 min at 4 °C. BCA assay kit (Sango, Shanghai, China) was used for measuring the protein concentration. Equal amounts of cell protein were separated by SDS-PAGE gels and electrophoretically transferred to a PVDF membrane (Millipore, Bedford, MA, USA). Skim milk was used to block the non-specific binding sites and the immunoblot were incubated with primary antibody at 4 °C overnight. After that, appropriate secondary antibody was used, and the reactive bands were detected by Electro-Chemi-Luminescence kit (Thermo, Beijing, China) (Sahin et al., 2017). The immunoblots were imaged and analyzed by using ImageQuant LAS 4000 mini (GE Healthcare, Shanghai, China).

## 2.10. Statistical analysis

All data were presented as the mean  $\pm$  SEM. Due to small sample

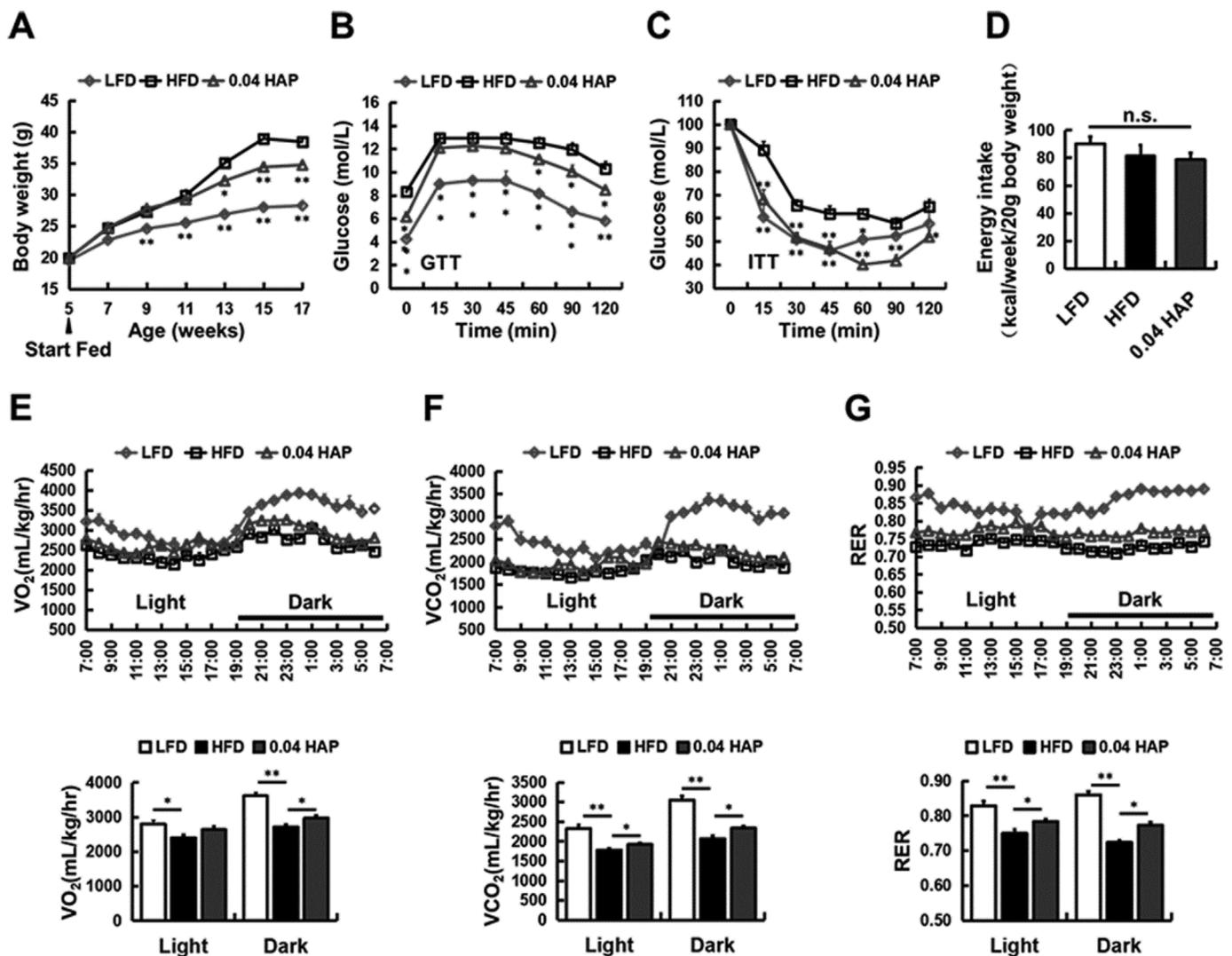


Fig. 1. Dietary AP reduces body weight gain, improves insulin resistance and enhances energy expenditure in HFD-induced obese mice. Five-week old male mice were treated as described previously. (A) body weight (g) was tested every other week. At the end of 12th week, Mice were injected with D-glucose for (B) glucose tolerance test and insulin for (C) insulin resistance test. (D) The average of energy intake (kcal) per 20 g body weight of the 12 weeks. (E) Oxidation consumption (VO<sub>2</sub>), (F) carbon dioxide production (VCO<sub>2</sub>) and (G) respiratory exchange ratio (RER) were tested. Data in (A)–(G) were presented as the mean  $\pm$  SEM (n = 12 per group). \*p < 0.05 and \*\*p < 0.01 versus HFD group.

sizes and abnormal data distribution, the statistical differences among animal groups were assessed by using nonparametric Mann-Whitney *U* test at statistical significance of  $P < 0.05$ .

### 3. Results

#### 3.1. Dietary AP reduced body weight by increasing energy expenditure in HFD-Fed mice

In order to investigate the effect of dietary AP on HFD-induced obesity, mice were fed with LFD, HFD or 0.04 HAP for 12 weeks which is a typical scheme for HFD-induced obesity management (Chen et al., 2017). Dietary AP markedly attenuated the impact of HFD on body-weight gain since 6th week after diet intervention and this trend became increasingly apparent as mice aged (Fig. 1A). Moreover, GTT and ITT assays showed that AP can improve the obesity-related insulin resistance and glucose intolerance (Fig. 1B and C) markedly at the end of the experimental period. For this reason, the imbalance between energy input and energy output leads to obesity (Rosen and Spiegelman, 2014). Fig. 1D revealed dietary AP has no impact on appetite. The total energy expenditure indicated mice fed with LFD and 0.04 HAP had more  $O_2$  consumption (Fig. 1E) and  $CO_2$  production (Fig. 1F) than those in HFD-feeding mice. Furthermore, we also examined the RER, which serves as

marker for substrates (glucose, lipids and protein) of energy expenditure (Valente et al., 2015). As shown in Fig. 1G, dietary AP increased RER, implying that mice prefer to use carbohydrate substrates. These results proved that the effect of dietary AP on energy expenditure elevation contributes for the weight losing to a great extent.

#### 3.2. Dietary AP decreased lipid accumulation in SAT, EAT, and BAT

As known adipose tissue is the main organ for lipid storage, the fat weights: SAT, EAT and BAT, were studied. Dietary AP could decrease the three kinds of fat weight, respectively (Fig. 2A). Adipocyte size was also reduced in 0.04 HAP group (Fig. 2B), which indicated that AP diminished the adipocyte lipid content. In order to investigate the mechanism of dietary AP on lipid metabolism in adipose tissue, we targeted the adipogenesis firstly. Some mark genes of adipocyte differentiation (*PPAR $\gamma$* , *LPL* and *AP2*) and lipogenesis (*FASN*, *SCD1* and *LPIN1*) were down-regulated in AP supplement group of SAT and EAT (Fig. 2C and D), except in BAT (Fig. 2E). The differences between WAT and BAT might be due to their distinct precursor cells. These results clearly indicated that AP alleviates lipid accumulation in adipocyte partly through inhibiting adipogenesis.

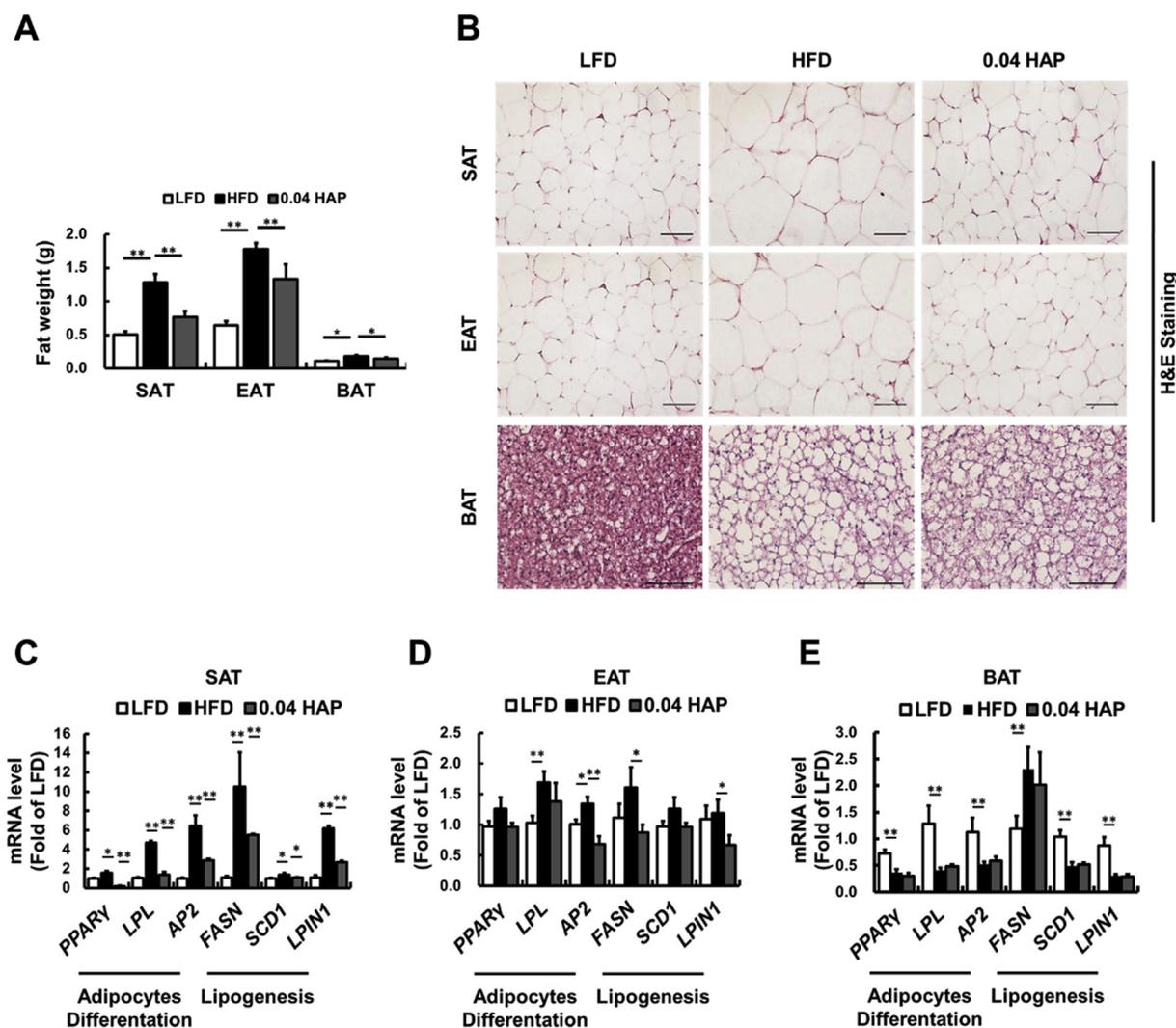


Fig. 2. Weight of three kinds of adipose tissues (g). (B) H&E staining results of adipocyte diameters in SAT, EAT and BAT, the scale bars in (B) is 100  $\mu$ m in length (200 $\times$ ). Real-time PCR was used to detect the mRNA expression of adipogenesis genes: *PPAR $\gamma$* , *LPL*, *AP2*, *FASN*, *SCD1* and *LPIN1* in (C) SAT, (D) EAT and (E) BAT ( *$\beta$ -actin* was control). Data in (A) and (C)–(E) was presented as the mean  $\pm$  SME ( $n = 12$  per group). \* $p < 0.05$  and \*\* $p < 0.01$  versus HFD group.

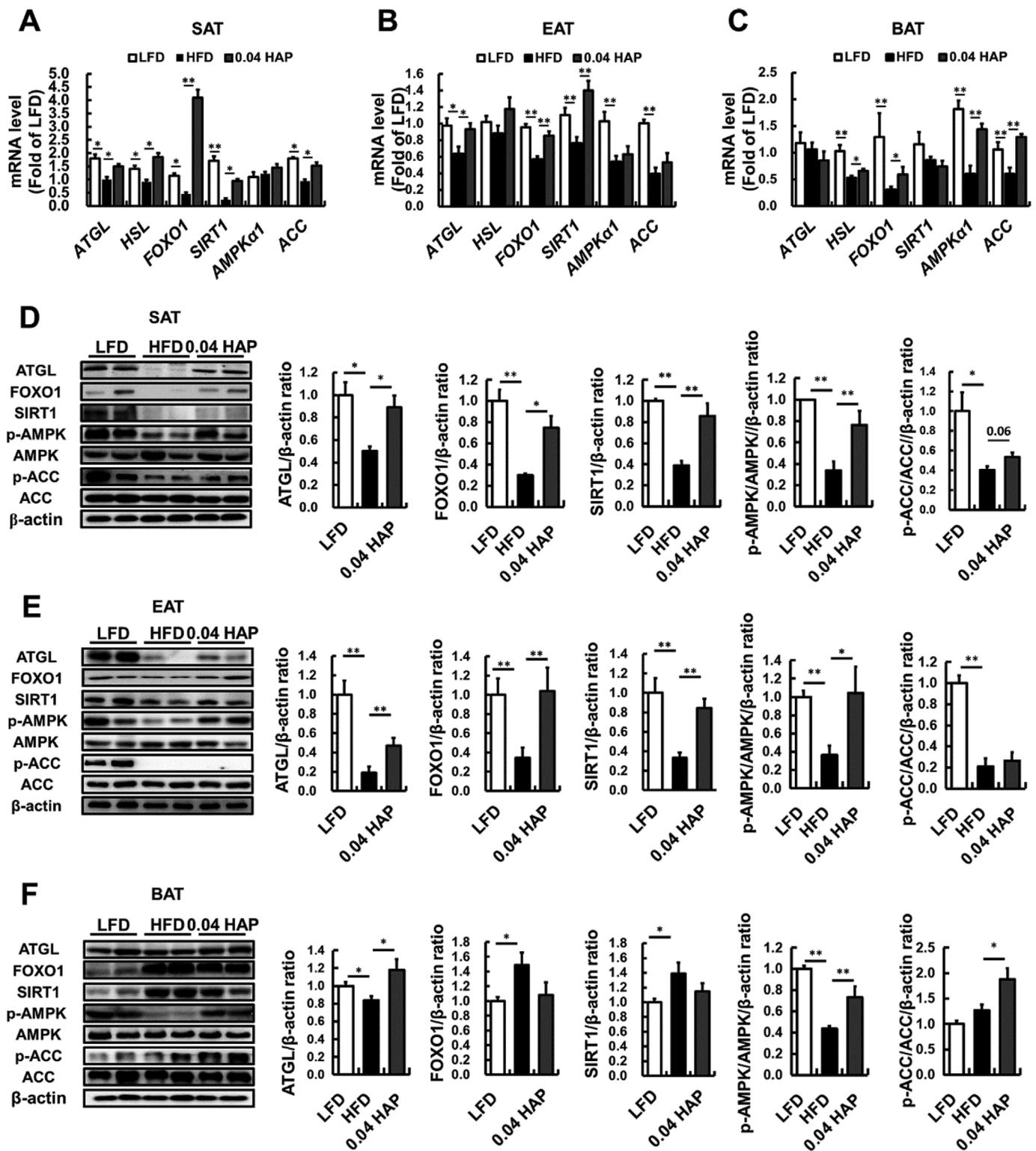


Fig. 3. Dietary AP enhances lipolysis and fatty acid oxidation in adipose tissues. The mRNA expression of lipolysis and fatty acid oxidation genes: *ATGL*, *HSL*, *FOXO1*, *SIRT1*, *AMPKα1* and *ACC* in (A) SAT, (B) EAT and (C) BAT ( $\beta$ -actin was control). (D)–(F) showed protein expression and quantification of *ATGL*, *FOXO1*, *SIRT1*, *p-AMPK* and *AMPK*, *p-ACC* and *ACC* in the three kinds of adipose tissues respectively.  $\beta$ -actin was used as reference protein in western blot analysis. Data in (A)–(F) were presented as the mean  $\pm$  SME ( $n = 12$  per group). \* $p < 0.05$  and \*\* $p < 0.01$  versus HFD group.

3.3. Dietary AP mainly promoted lipolysis in WAT and strengthened the fatty acid oxidation in BAT

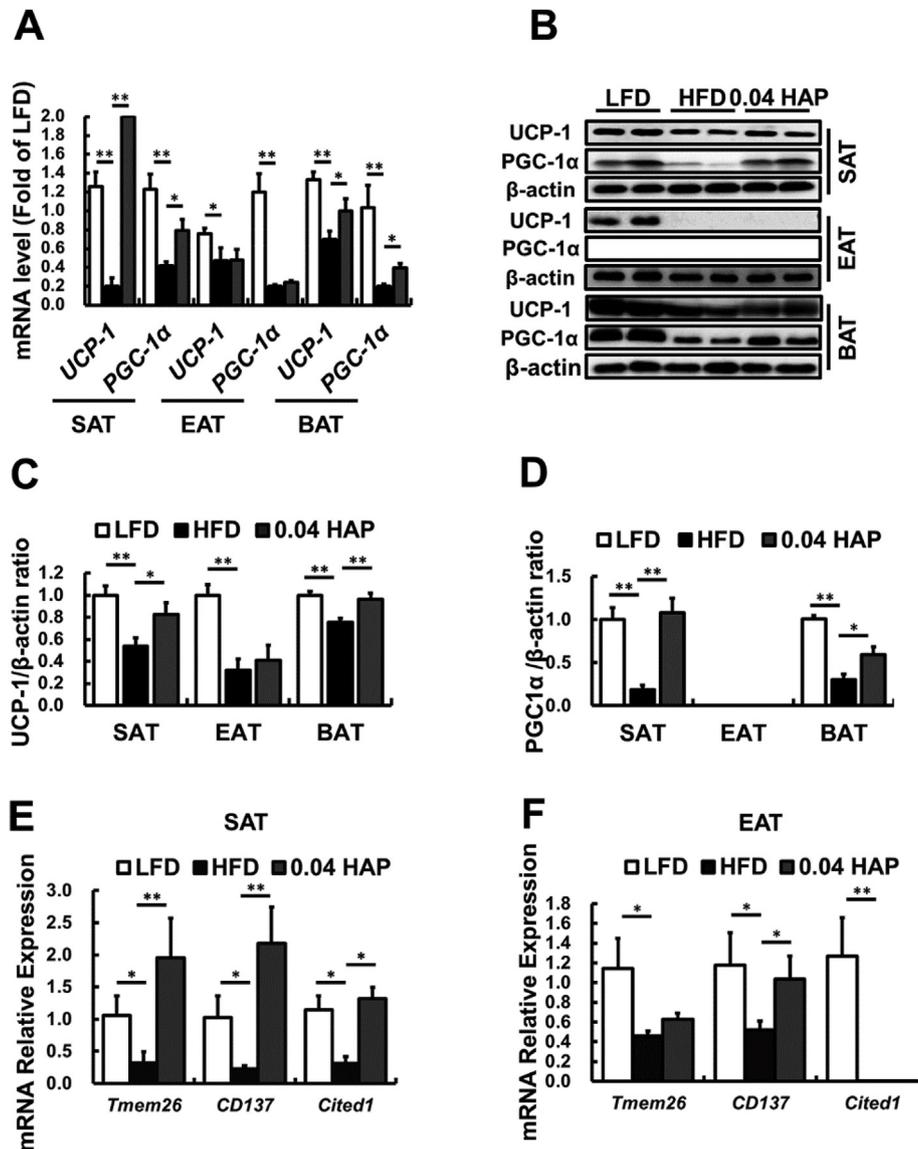
According to the fact that TG catabolism controls adipose tissue mass and lipolysis is catalyzed by *ATGL* and *HSL* (Zechner et al., 2012).

And, *SIRT1* controls the adipocyte lipolysis via *FOXO1*-mediated *ATGL* expression (Chakrabarti and Kandror, 2009; Chakrabarti et al., 2011). It was speculated that dietary AP promotes lipolysis via *SIRT1*/*FOXO1*/*ATGL* signal pathway. Our results showed that dietary AP increased *ATGL*, *HSL*, *FOXO1*, and *SIRT1* mRNA expression compared with HFD

**Table 1**  
Biological parameters in mouse serum.

Parameter	LFD	HFD	HFD+0.04% AP
FFAs ( $\mu\text{mol/mL}$ )	$0.57 \pm 0.08^{**}$	$0.94 \pm 0.09$	$0.64 \pm 0.05^{**}$
TG ( $\mu\text{mol/mL}$ )	$0.69 \pm 0.03^*$	$0.9 \pm 0.09$	$0.10 \pm 0.04^{**}$
Leptin (pg/mL)	$2866.75 \pm 695.46^{**}$	$41327.42 \pm 8124.35$	$11182.67 \pm 3912.43^{**}$
Adiponectin (pg/mL)	$23655.27 \pm 1933.13$	$18071.29 \pm 1390.52$	$22815.33 \pm 1421.91$
TNF- $\alpha$ (pg/mL)	$16.65 \pm 2.43^{**}$	$73.09 \pm 16.20$	$34.32 \pm 6.94^{**}$
MCP-1 (pg/mL)	$28.31 \pm 2.99^{**}$	$65.38 \pm 8.33$	$41.33 \pm 4.14^{**}$
IL-6 (pg/mL)	$1.02 \pm 0.26^{**}$	$2.42 \pm 0.31$	$1.14 \pm 0.21^*$

C57BL/6 mice were divided into three groups fed with LFD, HFD, 0.04 HAP for 12 weeks. After the feeding, the serum was collected to test these parameters. All data were presented as the mean  $\pm$  SME (n = 12 per group). \* $p < 0.05$  and \*\* $p < 0.01$  versus HFD group.

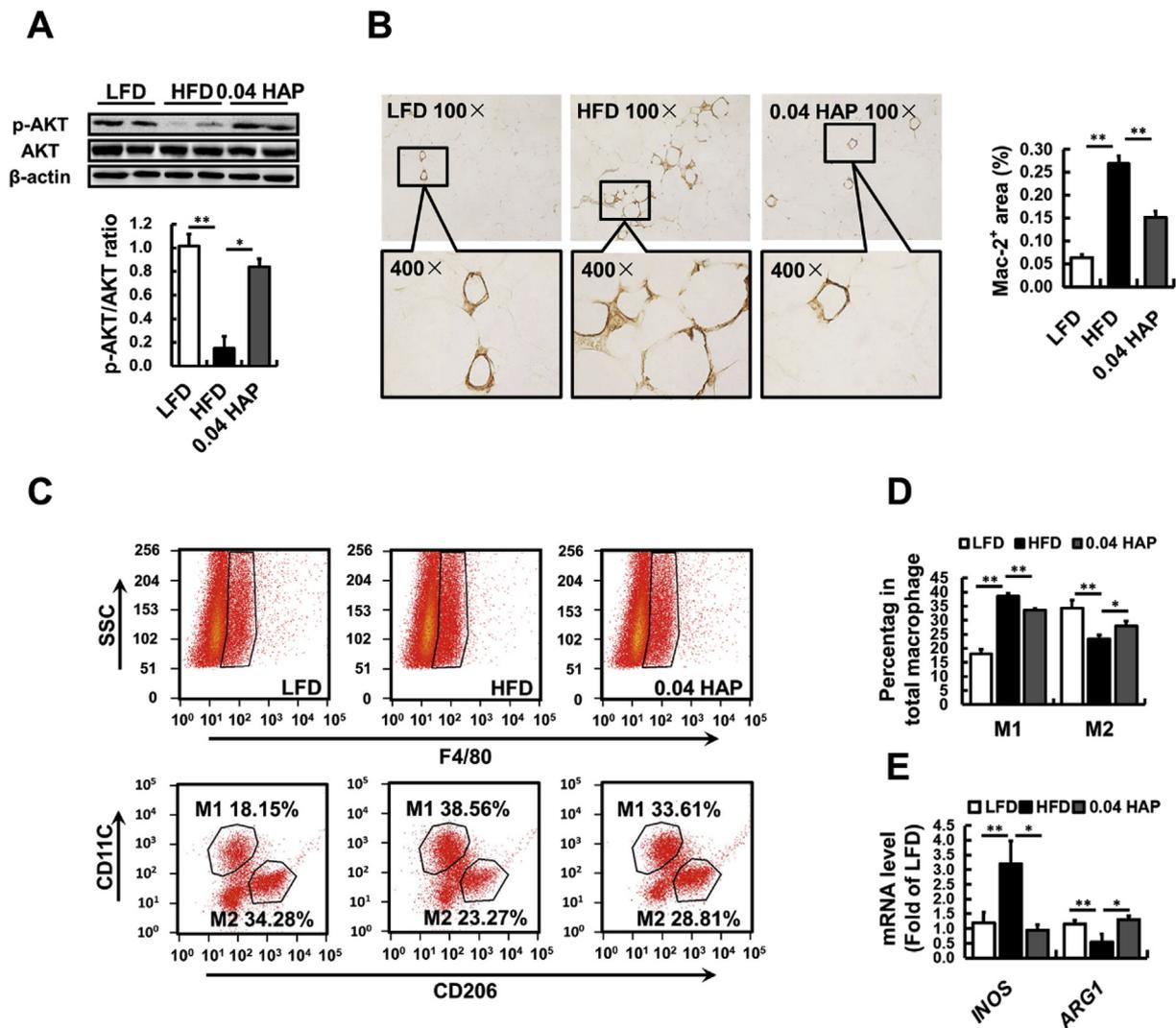


**Fig. 4.** Dietary AP enhances thermogenesis capability in BAT and browning in SAT. (A) The mRNA expression of *UCP-1* and *PGC-1 $\alpha$*  ( $\beta$ -actin was control). (B)–(D) Protein expression and quantification of *UCP-1* and *PGC-1 $\alpha$*  in SAT, EAT and BAT were also detected. (E) and (F) showed the mRNA level of browning markers in SAT and EAT respectively. Data in (A), and (C)–(D) were presented as the mean  $\pm$  SME (n = 12 per group). \* $p < 0.05$  and \*\* $p < 0.01$  versus HFD group.

group in three kinds of adipose tissues (Fig. 3A–C) and reversed the HFD-induced ATGL, SIRT1, and FOXO1 protein decline significantly in WAT, particularly in SAT. These results indicated that dietary AP mainly promotes WAT lipolysis partly through ATGL/SIRT1/FOXO1.

Excess lipolysis could lead to high concentrations in circulation FFAs and result in accumulating as TG in non-adipose tissue, such as liver (Chen et al., 2017; Schweiger et al., 2017). In this study, serum

levels of FFAs and TG were not increased in 0.04 HAP group (Table 1). Furthermore, lipid accumulation in liver was not elevated after AP administration either (Fig. S2). In order to investigate how FFAs produced by lipolysis was consumed, fatty acid oxidation (the most important catabolic pathway for FFAs degradation) was targeted. Most importantly, dietary AP increased the p-AMPK in the three kinds of adipose tissues (Fig. 3D–F), while increased the p-ACC slightly in SAT



**Fig. 5.** Dietary AP decreases macrophages infiltration and M1 polarization in EAT. (A) Protein and phosphorylation levels of AKT in EAT were detected by immunoblots and the quantification of AKT was described as p-AKT to total AKT.  $\beta$ -actin was used as reference protein in western blot analysis. (B) Mac-2 immunostaining were detected in EAT. (C) The F4/80<sup>+</sup> cells in EAT were further analyzed by coating anti-CD11c and anti-CD206 antibodies. M1 macrophages were identified as F4/80<sup>+</sup> CD11c<sup>-</sup> CD206<sup>-</sup> cells and M2 macrophages were identified as F4/80<sup>+</sup> CD11c<sup>+</sup> CD206<sup>+</sup> cells. (D) The percentage of M1 and M2 macrophages of the whole macrophages. (E) M1 macrophages mark gene *INOS*, M2 macrophages mark gene *ARG1* expression ( $\beta$ -actin is control). Data in (A), (B), (D) and (E) were presented as the mean  $\pm$  SEM (n = 12 per group). \*p < 0.05 and \*\*p < 0.01 versus HFD group.

(Fig. 3D) and significantly in BAT (Fig. 3F). Among these results, dietary AP mainly increased lipolysis in WAT and enhanced the fatty acid oxidation in BAT, which inspired us that dietary AP promotes WAT fuelling FFAs for BAT fatty acid oxidation.

### 3.4. Dietary AP induced BAT and SAT thermogenic program in HFD-Fed mice

BAT could use the actions of UCP-1 to transform stored chemical energy into heat (Kajimura and Saito, 2014), while WAT could also use UCP-1 positive beige adipocyte to drive the same thermogenic program (Wu et al., 2012). According to this, we further investigated the effects of dietary AP on thermogenic program in WAT and BAT. Compared with mice fed only with HFD, dietary AP increased UCP-1 and PGC-1 $\alpha$  transcript (Fig. 4A) and protein (Fig. 4B–D) levels in SAT and BAT. However, these alterations did not occur in EAT fed 0.04 HAP (Fig. 4A–D). Consistent with the results in WAT, the beige cell-selective markers *Tmem26*, *CD137*, and *Cited1* were also increased in SAT (Fig. 4E), but not in EAT (Fig. 4F). Therefore, we conclude that dietary AP activates BAT thermogenesis and SAT browning.

### 3.5. Dietary AP reduced obesity-associated ATM infiltration and M1 polarization in EAT

Among the three kinds of adipose tissues, EAT is represented by inflammation (Cancello et al., 2006). And, chronic low grade inflammation could reduce lipolysis (Mowers et al., 2013). So, we speculated that dietary AP may promote EAT lipolysis by inhibiting inflammation. First of all, it was revealed that AP increased the p-AKT (Fig. 5A). Then after, the effect of AP on ATM infiltration in EAT was detected by immunohistochemical analysis. The results showed that 0.04 HAP group had smaller macrophage positive corona-areas (Fig. 5B), correlating with the flow cytometry analysis of ATM amounts (Fig. 5C). Finally, it was further clarified whether AP induced ATM phenotypic switch. Fig. 5C and D showed that 0.04 HAP group contained fewer M1 ATM and more M2 ATM than those fed with HFD. Meanwhile, M1 ATM mark gene *INOS* was down regulated, while M2 ATM mark gene *ARG1* was up regulated (Fig. 5E). These results suggested that dietary AP reduces ATM accumulation in EAT and inhibits the polarization of ATM toward M1 state.

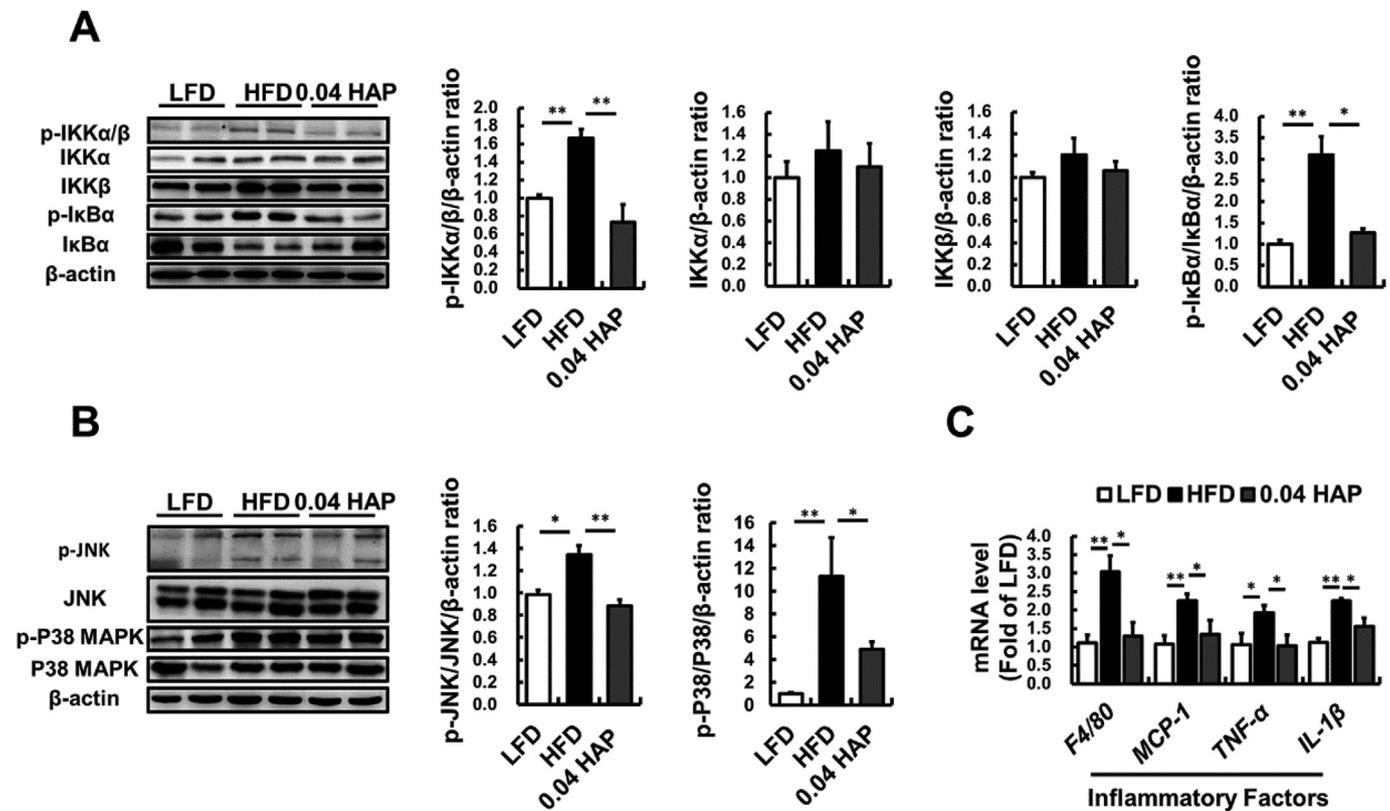


Fig. 6. Dietary AP inhibits chronic low-grade inflammation in EAT partly through NF- $\kappa$ B and MAPK signaling pathways. Western blot was used to detect (A) p-IKK $\alpha$ / $\beta$ , IKK $\alpha$ , IKK $\beta$ , p-I $\kappa$ B $\alpha$ , and I $\kappa$ B $\alpha$  of NF- $\kappa$ B pathway, (B) p-JNK, JNK, p-P38 MAPK and P38 MAPK of MAPK pathway protein expression.  $\beta$ -actin was used as reference protein in western blot analysis. (C) Inflammatory genes (*F4/80*, *MCP-1*, *TNF- $\alpha$*  and *IL-1 $\beta$* ) expression in EAT ( $\beta$ -actin is control). Data in (A)–(C) was presented as the mean  $\pm$  SEM (n = 12 per group). \**p* < 0.05 and \*\**p* < 0.01 versus HFD group.

### 3.6. Dietary AP inhibited EAT inflammation through MAPK and NF- $\kappa$ B signaling pathway

NF- $\kappa$ B and MAPK pathways are two classical inflammatory pathways (Lumeng et al., 2007). Herein, it was studied whether NF- $\kappa$ B and MAPK pathways were involved in AP induced decrease adipose tissue inflammation. As shown in Fig. 6A, p-IKK $\alpha$ / $\beta$  was restrained by AP. AP also inhibited the degradation and the phosphorylation of I $\kappa$ B $\alpha$ . In addition, AP reduced p-JNK and p-P38 MAPK in EAT (Fig. 6B). In addition, it was shown that AP decreased the F4/80, MCP-1, TNF- $\alpha$  and IL-1 $\beta$  inflammatory factors both in serum (Table 1) and EAT (Fig. 6C), which were consistent with the previous results. Taken together, these findings collectively suggested that AP inhibits the activation of NF- $\kappa$ B and MAPK pathways in EAT, and finally promotes lipolysis.

## 4. Discussion

The balance between fat synthesis and fat catabolism mainly determines the adipose tissue mass (Arner et al., 2011). In mice, TG lipolysis liberates amounts of FFAs into serum (Schweiger et al., 2006). Fatty acid oxidation degrades FFAs fuelling for UCP-1 mediated thermogenesis in both classic brown adipocyte and beige adipocyte (Harms and Seale, 2013). Thus, the effective pharmacologic or nutritional strategies for promoting lipolysis and adipocyte thermogenesis can help for improving obesity and its complication.

In this study, the model of HFD-induced obesity in C57BL/6 mice was used to assess the effects of dietary AP on energy intake and expenditure. Consistence with Daniela Gentile et al. (2018) and Carlos Escande et al. (2013), our results showed AP suppressed HFD-induced body weight gain and related insulin resistance. It was found that AP didn't affect the food intake but enhanced energy expenditure. Then,

the mechanisms of dietary AP on weight lose were studied. Histological analysis showed that AP significantly reduced lipid accumulation in adipose tissue. And, Dietary AP inhibited adipogenesis and enhanced lipolysis progress in SAT and EAT. Correspondingly, the fatty acid oxidation and thermogenesis related progresses improved in SAT and BAT after AP supplementation. Considering EAT is the representative of inflammation, the role of AP in EAT lipid metabolism mechanism (representative of adipose tissue inflammation) was investigated. Present results showed that dietary AP supplement not only antagonized the ATM infiltration and M1 polarization, but also normalized the activation of NF- $\kappa$ B and MAPK pathways.

From previous studies, the dose of AP supplement was not clear. 0.05% AP significantly reduced the body weight along with inhibiting food intake (Myoung et al., 2010). However, diet with lower level of 0.005% AP could not reduce obesity (Jung et al., 2016). Some studies had reported different experiments with several approaches, such as *i.p.* injection (Escande et al., 2013; Feng et al., 2017; Nicholas et al., 2007; Rithidech et al., 2012; Wang et al., 2014) and oral gavage (Lee et al., 2015; Ren et al., 2016; Shukla et al., 2014; Thaiss et al., 2016). Then after, pre-experiments were performed to ensure an effective supplement that didn't influence energy intake (Fig. S1). Both 0.02 HAP and 0.04 HAP could reduce body weight significantly without reducing energy intake and 0.04 HAP showed lower body weight compared with 0.02 HAP. These results proved that AP could reduce body weight in a dose-dependent manner, and 0.04% supplement is a good choice.

For the reason that lipid mobilization has a close relationship with obesity, we detected the effect of dietary AP on lipid synthesis and degradation from aspects of adipogenesis, lipolysis, fatty acid oxidation, thermogenesis and browning. As shown in Figs. 2 and 3, dietary AP decreased adipogenesis and increased lipolysis in WAT. At the same time, AP enlarged thermogenesis in BAT and browning in SAT. AP acts

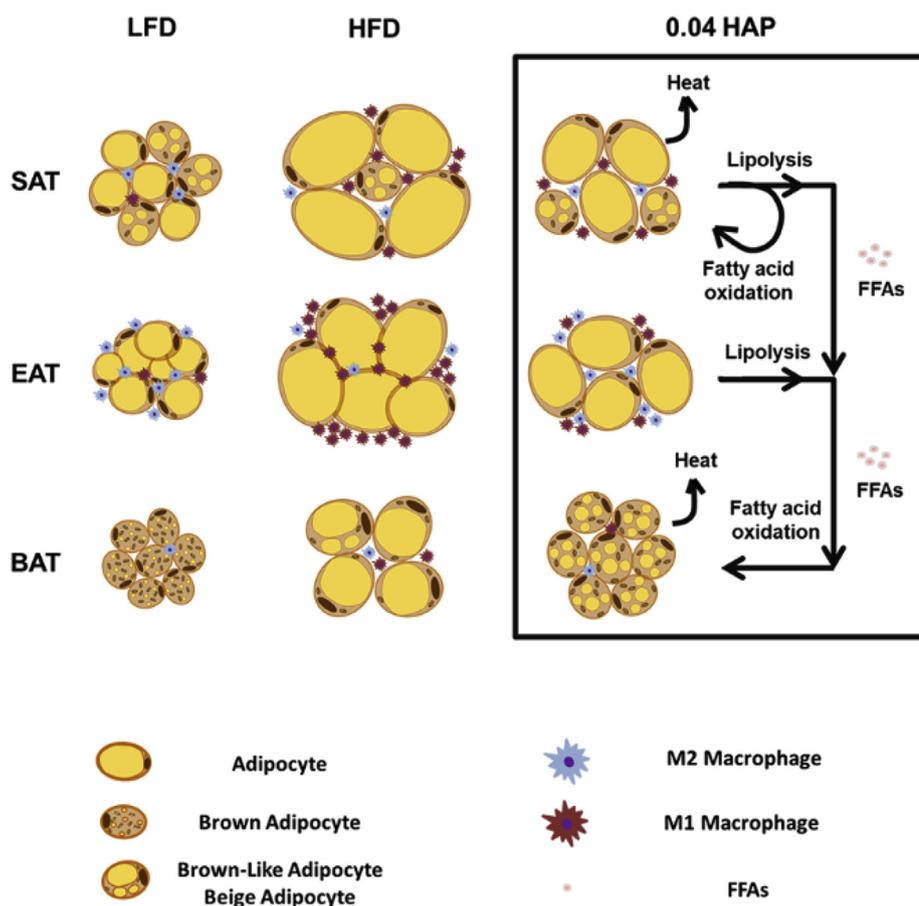


Fig. 7. Dietary AP promotes lipolysis, thermogenesis and browning resulting in ameliorating obesity. Dietary AP enhanced lipolysis and fatty acid oxidation which is responsible for lipid catabolism in adipocytes. Meanwhile, AP strengthened BAT thermogenesis capacity and SAT browning to transform the chemical energy from fatty acid oxidation into heat. Furthermore, in 0.04 HAP group, macrophage accumulation and M1 polarization were improved. The ameliorative inflammation status further promoted EAT lipolysis.

differently in distinct adipose tissues. This phenomenon could be explained in the following aspects: (i) It has been reported that FFAs is mainly released from SAT (Morelli et al., 2013), which means SAT are more likely to lipolysis compared with EAT. According with this, dietary AP was more likely to promoted ATGL/FOXO1/SIRT1 activation in SAT than in EAT. Not only that, AP enhanced SAT fatty acid oxidation and browning. (ii) Dietary AP reduced serum FFAs which were contradictory to the fact that lipolysis increases FFAs. For the reason that abnormal lipolysis leads to ectopic fat deposition, such as liver (Fuchs et al., 2014; Morigny et al., 2016). Similar with previous results (Feng et al., 2017; Jung et al., 2016), AP reduced liver weight, lipid accumulation and TG content (Fig. S2). Account for this, we studied the effect of AP on AMPK/ACC in adipose tissue, which consumed FFAs. p-ACC was increased in BAT and SAT of 0.04 HAP group, except in EAT. (iii) BAT is specialized cells that have high capacities for uncoupled oxidative metabolism, while WAT stores excess energy as TG with low oxidative abilities (Rosen and Spiegelman, 2014). And, it has been certified that WAT emerges white-to-brown transition under certain stimulations. In this study, the browning markers were increased significantly in SAT. However, AP had not shown potent abilities to influence these proteins and genes expression in EAT. Taken together, we confirmed that dietary AP decreased TG accumulation in WAT, providing FFAs for SAT and BAT thermogenesis progress without ectopic lipid accumulation.

Among the three kinds of adipose tissues, EAT is the representative of adipose tissue inflammation (Cancello et al., 2006; Chen et al., 2017). And, there is a close interaction between lipolysis, chronic inflammation and beige adipogenesis (Babaei et al., 2018). Hence, we speculated that the inflammation status may inhibit the lipolysis and browning progress in a part. Firstly, dietary AP could reduce inflammatory factors in serum. Then after, dietary AP reduced the ATM infiltration and M1/M2 ratio in EAT. Lastly, two control points of pro-

inflammatory gene expression and macrophage polarization were targeted: NF- $\kappa$ B and MAPK (Akira and Takeda, 2004). Similar with Feng et al. (Feng et al., 2016), AP reduced the p-IKK $\alpha/\beta$  and p-I $\kappa$ B $\alpha$  of NF- $\kappa$ B pathway in EAT. Additionally, we found dietary AP significantly suppressed the p-JNK and p-P38 MAPK in EAT. We hypothesized that AP had no significant effect on lipolysis and browning progress in EAT comparing with SAT is owing to the presence of inflammation. And these results suggested that AP promoted the lipolysis partly by inhibiting inflammation in EAT.

## 5. Conclusions

In summary, our study described for the first time, that low-dose AP ameliorated HFD-induced obesity via enhancing lipid catabolism, thermogenesis, and browning *in vivo*. And the inhibition effect of dietary AP on chronic low-grade inflammation further promoted the lipolysis progress (Fig. 7). On the basis of these results, it could be stated that lipid disorder and inflammation played important roles on insulin resistance. The present study indicated that AP could be used as a natural food additive in the management of HFD-induced obesity and related syndrome.

## Conflicts of interest

There is no conflict of interest to declare.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fct.2019.110780>.

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