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Dietary kaempferol exerts anti-obesity effects by inducing the browing of white adipocytes via the AMPK/SIRT1/PGC-1α signaling pathway

Changyu Xu [a,1], Xiaoxi Zhang [a,b,1], Yihuan Wang [a,1], Yan Wang [a,1], Yixuan Zhou [a], Fenfen Li [a], Xiaoli Hou [b], Daozong Xia [a,*]

[a] Department of Food Science and Nutrition, School of Pharmaceutical Sciences, Zhejiang Chinese Medical University, Hangzhou, 310053, China
[b] Academy of Chinese Medical Sciences, Zhejiang Chinese Medical University, Hangzhou, 310053, China

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ABSTRACT

Browning of white adipose tissue is a novel approach for the management of obesity and obesity-related metabolic disorders. Kaempferol (KPF) is a common dietary nutrient found abundantly in many fruits and vegetables and has been shown to have the potential to regulate lipid metabolism. However, the detailed mechanism by which it affects the browning of white adipose tissue remains unclear. In the present study, we sought to determine how KPF induces adipocytes to undergo a browning transformation by establishing a primary adipocyte model and an obese mouse model. Our results showed that KPF-treated mice were rescued from diet-induced obesity, glucose tolerance and insulin resistance, associated with increased expression of adaptive thermogenesis-related proteins. KPF-promoted white adipose browning correlated with the AMPK/SIRT1/PGC-1α pathway, as the use of an AMPK inhibitor in preadipocytes partially reversed the observed browning phenotype of KPF-treated cells. Taken together, these data suggest that KPF promotes browning of white adipose tissue through activation of the AMPK/SIRT1/PGC-1α pathway. This study demonstrates that KPF is a promising natural product for the treatment of obesity by promoting white fat browning.

1. Introduction

Obesity stands as a global health challenge, with its incidence steadily increasing in recent decades. As a metabolic disorder resulting from a prolonged imbalance between the body's energy intake and expenditure, obesity clearly manifests itself as excess weight, abnormal fat accumulation, or an overall surplus (González-Muniesa et al., 2017; Lin and Li, 2021). This progression instigates an amplification in both the quantity and dimensions of adipocytes within the body, setting in motion a series of physiological and pathological alterations (Blüher, 2019; Pan et al., 2021; Lanigan, 2018). Obesity is a prominent risk factor for chronic ailments and exhibits a strong correlation with conditions such as hypertension, coronary heart disease, diabetes mellitus, fatty liver disease, cancer, among others (Blüher, 2019; Peters et al., 2018; Zhang et al., 2019). Consequently, the prevention and treatment of obesity have emerged as focal points of scholarly investigation.

Brown adipose tissue (BAT) plays a pivotal role in the body as it is rich in mitochondrial uncoupling protein 1 (UCP1), which co-ordinates non-shivering thermogenesis - the process of generating heat to expend energy in the form of calories, thereby fostering weight reduction (Wang et al., 2021; Tapia et al., 2018). Recent research has unveiled a remarkable transformation potential within white adipose tissue (WAT) to adopt a BAT-like thermogenic stance, recognized as beige adipose tissue, under external provocation (Wang et al., 2019). Increased UCP1 expression in beige adipocytes separates substrate oxidation from ATP synthesis by driving proton leak and dissipating energy as heat (Cheng et al., 2021; Lee et al., 2020; Singh et al., 2021). In order to recompense for the proton gradient loss, the maximum of mitochondrial respiration is triggered and the rate of respiration mostly determines heat production in mammals. AMP-activated protein kinase (AMPK) holds the reins in the regulation of energy expenditure within adipocytes and the control of beige adipocyte genesis and progression through the modulation of peroxisome proliferator-activated receptor γ coactivator α (PGC-1α), and silent information regulator of transcription 1 (SIRT1) (Day et al., 2017; Garcia and Shaw, 2017). Concurrently, evidence spotlights that AMPK activation correlates with lowered susceptibility to obesity, non-alcoholic fatty liver disease, type 2 diabetes, and cardiovascular

* Corresponding author.
E-mail address: xdz_zjtcm@hotmail.com (D. Xia).
[1] These authors contribute equally.

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Abbreviations :

KPF	Kaempferol
BAT	brown adipose tissue
WAT	white adipose tissue
AMPK	AMP-activated protein kinase
PGC-1α	peroxisome proliferator-activated receptor γ coactivator-1α
SIRT1	silent information regulator of transcription 1
DMSO	dimethyl sulfoxide
CMC-Na	sodium carboxymethylcellulose
FBS	fetal bovine serum
CC	Compound C
CCK-8	Cell Counting Kit-8
NCD	normal control diet group
NCD+KPF	normal diet KPF group
HFD	high fat diet group
HFD+KPF	high fat diet KPF group
UCP1	uncoupling protein 1
GTT	glucose tolerance test
ITT	insulin tolerance test
H&E	hematoxylin and eosin
TC	total cholesterol
LDL-C,	low-density lipoprotein Cholesterol
HDL-C,	high-density lipoprotein Cholesterol
TG	triglyceride
iWAT	inguinal white adipose tissue
eWAT	epididymal white adipose tissue
AUC	area under the curve
Hmsc	human mesenchymal stem cells
PPARγ	peroxisome proliferator-activated receptor γ
ATF2	activated transcription factor 2
THR	thyroid hormone receptors
PPARγ	peroxisome proliferator-activated receptor γ
PRDM16	PR domain containing 16

ailments (Desjardins and Steinberg, 2018; Carling, 2017). Hence, a more profound comprehension of AMPK's role in orchestrating WAT browning stands as a pivotal juncture in unearthing and advancing weight-loss pharmaceuticals.

Numerous flavonoids derived from vegetables and fruits exhibit notable efficacy in ameliorating obesity and metabolic syndrome (Heber, 2010). Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-1, KPF), a dietary flavonoid (Calderón-Montaño et al., 2011), is predominantly present in human diets and significantly contributes to daily consumption of flavonoids (Sampson et al., 2002). The biological activity of KPF mainly depends on the type of conjugate ingested and its bioavailability. Pharmacokinetic studies have shown that KPF absorption depends on the extent to which lipophilic aglycon passively diffuse from the intestinal lumen to enterocyte, and that they are either absorbed directly into the hepatic portal vein or treated prior to absorption (Williamson et al., 2018). KPF is metabolized in the liver and circulates in the blood as sulfate, methyl, and glucuronide conjugates (Dabeek and Marra, 2019). It was found that in 150 g of mature chrysanthemums (containing 9 mg KPF), the free KPF detected in plasma and urine accounted for 40% and 16% of the total kaempferol, respectively. The free KPF in urine was lower than that in blood, suggesting that this was due to the metabolism of some aglycon in the kidney before excretion (O'Leary et al., 2001). In addition, another study found that the absorption and digestion of black tea (containing KPF 27 mg) for three days, it was found that the excretion rate of KPF in the urine was 2.5% of the intake, which proved that the aglycon in the tea had a high bioavailability (De Vries et al., 1998). Studies have revealed that KPF has the ability to inhibit lipogenesis and promote lipolysis (Torres-Villarreal et al., 2019). Furthermore, KPF exerts anti-obesity effects by inhibiting intestinal inflammation and harmonising the balance of the intestinal microbiota (Bian et al., 2022). In addition, KPF attenuates the activation of hypothalamic microglia associated with obesity, thereby reducing body weight in obese mice (Romero-Juárez et al., 2023). However, little is known about its effect on the thermogenic adipose tissue. Given the pivotal role of white adipose tissue browning in lipid metabolism, we investigated whether KPF could indeed combat obesity by modulating the AMPK/SIRT1/PGC-1α pathway.

In the present study, we tested this hypothesis using a high-fat-diet induced obese mouse model and an in vitro differentiation assay of primary adipocytes. We showed that KPF increased the formation of beige cells by activating AMPK. Furthermore, KPF promoted the differentiation of brown-like adipocytes in vitro, whereas AMPK inhibition reversed this dominant phenotype.

2. Materials and methods

2.1. Materials

Kaempferol (purity >98%), purchased from Shanghai yuanye Bio-Technology Co., Ltd (Shanghai, China), was dispersed in 1/1000 dimethyl sulfoxide (DMSO) for in vitro experiments and dissolved in sodium carboxymethylcellulose (CMC-Na) (0.2%) for in vivo experiments. Dexamethasone (50-02-2), 3-isopropyl-1-methylxanthine (28822-58-4), Indometh8acin (53-86-1), Insulin (11070-73-8), and Oil Red O staining solution (1320-06-5) were sourced from Sigma-Aldrich (St. Louis, MO). Collagenase (LS004196) was procured from Worthington (New Jersey, USA). MEM-α medium (12571063), DMEM-F12 medium (10100147C), Penicillin-streptomycin (15070063), and fetal bovine serum (FBS) (10100147C) were obtained from Gibco (California, USA). Compound C (CC) (BML-275), AICAR (13417) was purchased from Med Chem Express (California, USA). Protease Inhibitor Cocktail (CW2200) and Phosphatase Inhibitor Cocktail (CW2383) were acquired from Jiangsu Cowin Biotech Co., Ltd. (Jiangsu, China). The Enhanced BCA Protein Assay Kit (P0009), Cell Counting Kit-8 (CCK-8) (C0037), RIPA Lysis Buffer (P0013B), and SDS-PAGE Sample Loading Buffer (P0015F) were sourced from Beyotime (Shanghai, China). Rabbit antibodies for β-actin (93473), AMPK (2532), p-AMPK (50081), PGC-1α (2178), PPARγ (2435), and Mouse antibodies for SIRT1 (8469) were obtained from Cell Signaling Technologies (Danvers, MA), while Rabbit antibodies for UCP1 (ab234430), PRDM16 (ab191838) was procured from Abcam (Cambridge, United Kingdom). Secondary antibodies of horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (L3012) and HRP-conjugated anti-mouse IgG (L3032) were obtained from Signalway Antibody (Maryland, USA).

2.2. Animal models and treatments

Male C57BL/6 J mice (3 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China). All mice had access to food and distilled water provided ad libitum. According to the previous study of our group, the dose of KPF 50 mg/kg can achieve the best effect of inhibiting the weight gain of mice (Zhang et al., 2023). Therefore, KPF at a dose of 50 mg/kg was chosen for subsequent studies. After one week of acclimatization, the mice were randomly divided into four groups: normal control group (NCD), normal diet KPF group (NCD+KPF), high fat diet (HFD) and high fat diet KPF group (HFD+KPF). Normal diet and high-fat diet were fed libitum for 14 weeks. KPF (50 mg/kg) was prepared using 0.2% CMC-Na solution.

Treatment started concomitantly with the administration of NCD and HFD and consisted of daily oral introduction with KPF (50 mg/kg/d body weight) in mice with a safe dose. Mice in the control group received an equal volume of 0.2% CMC-Na. Bodyweight was measured weekly. The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Animal Care and Use Committee of Zhejiang Chinese Medical University and conducted according to institutional guidelines and protocols (Hangzhou, China. Permission number: 20220221–09). Glucose tolerance test (GTT) and insulin tolerance test (ITT) measurements were performed as previously described (Hou et al., 2018).

2.3. Cell culture and treatment

Primary adipocytes were isolated from inguinal fat of male C57BL/6 J mice and cultured in MEM-α medium (20%FBS) in a humidified atmosphere at 37 °C and 5% CO_2 as described previously (Hou et al., 2018) and used in experiments upon 80% confluence was reached. Primary adipocytes were cultured and differentiated according to the protocols described in the article (Cheng et al., 2023). The primary preadipocytes were induced to differentiate into mature adipocytes. After the induction of differentiation was completed, the cells were treated with 10, 20, and 40 μM KPF for 48 h to observe the effect of KPF on lipid deposition. And attached to the cell culture diagram for detailed explanation (Fig. S1K). To investigate the activation and inhibition of AMPK, the AMPK inhibitor CC and AICAR (AMPK agonist, 5-aminoimidazole-4-carboxamide ribonucleotide) was infused into adipocytes at a concentration of 20 μM for 4 h, respectively.

2.4. Cell viability assay

In the cytotoxicity experiment, primary adipocytes were incubated with serum-free medium containing 5, 10, 20, 40, 60, 80, 100 μM KPF. Complete cell fusion required 48 h before induction. After the induction of differentiation was completed, the cells were treated with KPF for 48 h, and then CCK-8 solution was added to incubate for another hour. Finally, the absorbance of 96-well plates was obtained at 450 nm using a microplate reader.

2.5. Histology and biochemica analysis

According to standard protocols, adipose tissue sections were stained with hematoxylin-eosin (H&E) staining to assess fat morphology and oil red O for lipid assessments. Serum concentrations of triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) were measured using kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.6. Western blotting

Proteins were extracted from mouse adipose tissue and primary adipocytes using RIPA buffer without EDTA-containing protease inhibitors and phosphatase inhibitors. Equal amounts of protein were loaded on 10% SDS-PAGE gel electrophoresis and transferred to PVDF membranes. Membranes were soaked in 5% skim milk for 1.5 h, washed 3 times with TBST, and then incubated overnight in a refrigerator at 4 °C with the following primary antibodies: UCP1 (1:1000), p-AMPK (1:1000), AMPK (1:1000), SIRT1 (1:1000), and PGC1α (1:1000). The membranes were finally incubated with secondary antibodies for 1 h at room temperature. All blots were imaged on an ECL chemiluminescence system.

2.7. Measurement of oxygen consumption in isolated white adipose tissue

About 10 mg of mouse iWAT was weighed, cut into pieces, and transferred to the volume cell of Strathkelvin instrument equipped with ex vivo tissue oxygen consumption detection buffer (100 mM sodium pyruvate added to bovine serum albumin and glucose powder, constant volume to 50 mL with PBS). The slope was recorded under the action of electromagnetic stirrers, and the oxygen consumption was calculated.

2.8. Statistical analysis

Data represent mean ± SD. T-test was used for comparisons between two groups, and one-way ANOVA of variance was used for comparison between multiple groups. Statistical analysis was performed with GraphPad Prism 8.0 software (GraphPad Software, Inc., La Jolla, CA, USA). $p < 0.05$ indicates a significant difference and $p < 0.01$ indicates an extremely significant difference.

3. Results

3.1. KPF prevents HFD-induced obesity

To investigate the effects of KPF on systemic metabolic physiology, we first treated male mice under NCD or HFD conditions with KPF (50 mg/kg) for 14 weeks (starting at 4 weeks of age). Compared to mice fed a normal diet, HFD-induced mice grew larger and showed a marked accumulation of abdominal fat. Remarkably, the body size of KPF fed mice was significantly reduced under the same diet condition (Fig. 1A). Consistent with the somatic observations, the high-fat diet significantly increased body weight, whereas KPF attenuated weight gain in mice on both diets (Fig. 1B–E). In addition, our previous study showed that KPF reduced body weight in obese mice by increasing basal metabolic level and energy expenditure independent of food intake (Zhang et al., 2023).

In order to determine whether weight loss in mice was associated with changes in adipose tissue, we isolated inguinal white adipose tissue (iWAT) and epididymal white adipose tissue (eWAT) from mice. The results confirmed that the weight of the adipose tissue of the mice was reduced to varying degrees after the drug intervention. Specifically, both iWAT and eWAT showed a significant decrease in weight after KPF treatment, with iWAT being the most significant (Fig. 1F–I). In addition, the pathological morphology of adipose tissue in which significant differences existed was investigated by H&E staining. Under both dietary conditions, the cell volume of mouse iWAT was significantly reduced by KPF treatment (Figs. S1A and B), and a large number of smaller aggregated vesicles appeared, showing browning characteristics (Fig. 1J). On the other hand, we observed that the reduction of adipocyte volume in eWAT was not significant (Figs. S1C and D), and the browning characteristic did not appear (Fig. 1J).

3.2. KPF improves glucose tolerance and insulin tolerance and reduces serum lipid levels

Glucose tolerance and insulin sensitivity serve as pivotal indicators for evaluating glucose and lipid metabolism, with obesity intricately linked to aberrant glucose and lipid handling. The intraperitoneal glucose- and insulin-tolerance tests (IP-GTT and IP-ITT, respectively) were carried out. After intraperitoneal injection of glucose, the KPF-treated mice in the NCD group exhibited significantly lower blood glucose levels than the control group only at 15 min (Fig. 2A), in contrast to the KPF-treated HFD-fed mice whose blood glucose levels were significantly lower than those of the control group at multiple time intervals (at 15, 30, and 60 min, respectively) (Fig. 2C), but whose area under the curve (AUC) of the GTT was significantly lower for both dietary scenarios (Fig. 2B–D). After insulin injection, blood glucose levels in the KPF-treated group were significantly lower than in the control group at 15 min, while the AUC was significantly lower than in the control group (Fig. 2E–H). Furthermore, a slight but not significant decrease in insulin content was observed after KPF treatment. It was shown that changes in glucose tolerance were not associated with increased insulin secretion (Figs. S1E and F). Collectively, these findings underscore KPF's ability to rapidly reduce and maintain glucose levels

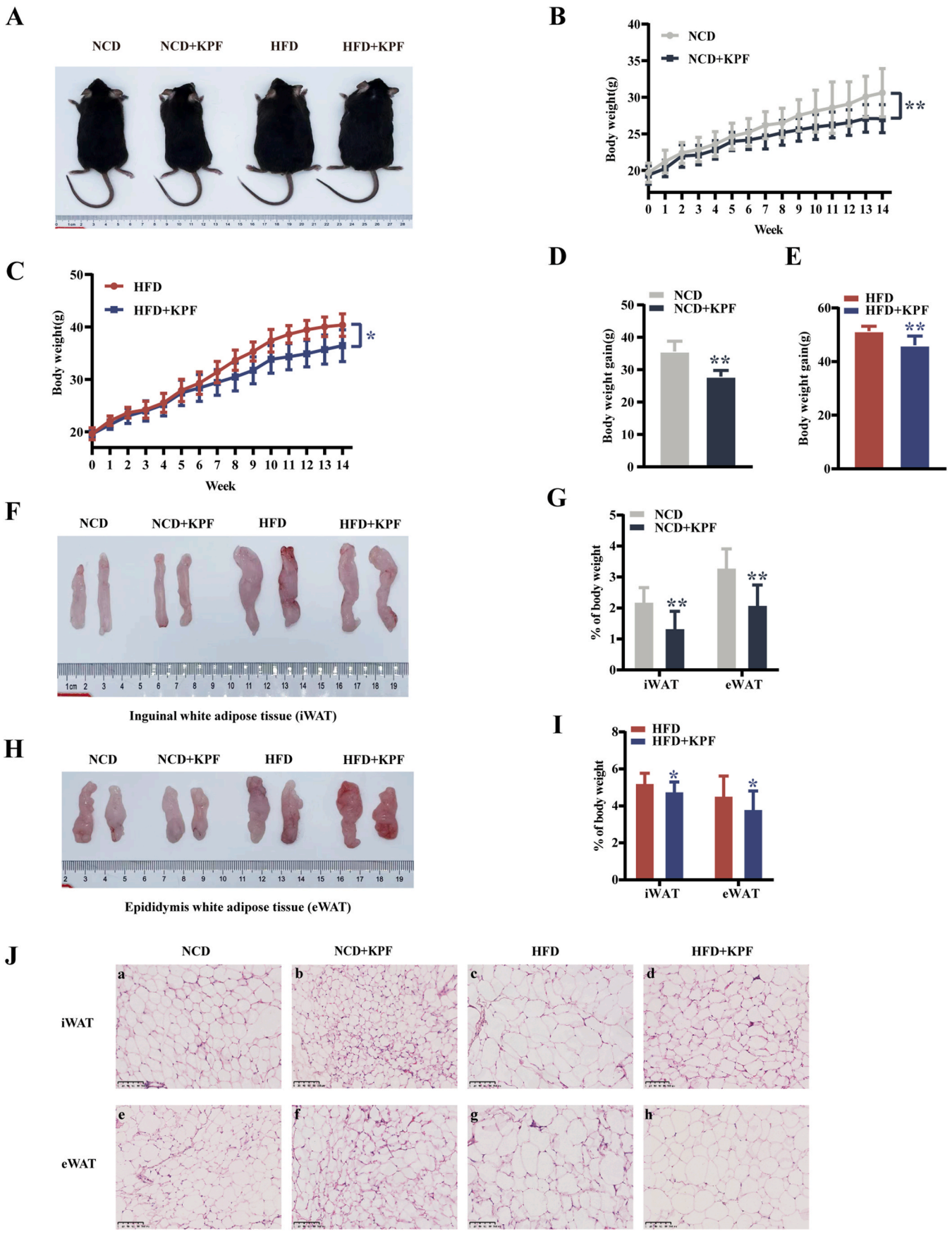

Fig. 1. KPF ameliorates body weight in NCD-fed and HFD-fed mice. (A) Representative image of the whole body. The effects of KPF on body weight (B, C), body weight gain (D, E), iWAT (F, G, I), eWAT (H, G, I). (J) Representative light microscopy images of H&E staining sections of iWAT and eWAT from KPF treated and untreated NCD and HFD male mice. Data shown are mean ± SD (n = 8). *$p < 0.05$, **$p < 0.01$, vs. Control.

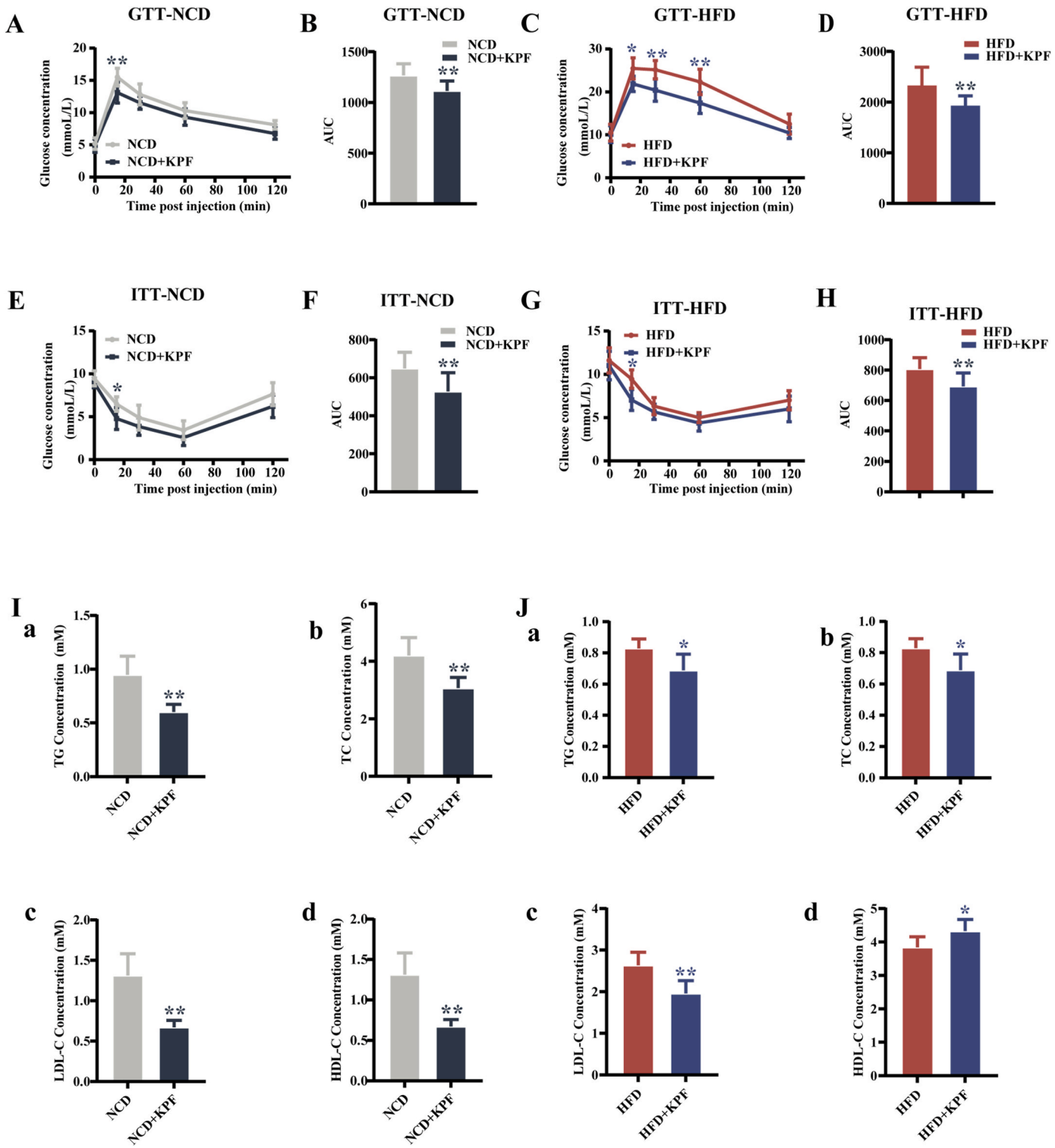

Fig. 2. KPF improved GTT, ITT, and lipid levels in mice. (A, B, C and D) GTT after 13 weeks on NCD (A and B) or HFD (C and D). (E, F, G and H) ITT after 13 weeks on NCD (E and F) or HFD (G and H). (I, J) Serum levels of TG, TC, LDL and HDL in each group of mice. Data shown are mean ± SD (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$, vs. Control.

within a stable range, and improve insulin sensitivity, thereby exerting a significant corrective effect on impaired glucose tolerance and insulin sensitivity associated with obesity.

Obesity is not only characterised by an excessive accumulation of fat, but is often accompanied by a disorder of lipid metabolism. This condition is mainly characterised by elevated levels of total cholesterol (TC), triglycerides (TG) and low-density lipoprotein cholesterol (LDL-C) and reduced levels of high-density lipoprotein cholesterol (HDL-C). Compared with vehicle treatment, KPF significantly reduced the levels of TC, TG and LDL-C in both dietary conditions, but increased the levels of HDL-C in HFD (Fig. 2I and J).

3.3. KPF induces white fat browning by AMPK/SIRT1/PGC-1α signaling pathway

Histopathological examination suggested that KPF may play a role in promoting iWAT browning, which is often associated with an increase in energy metabolism with a corresponding increase in oxygen consumption. To further confirm this effect, we examined the oxygen consumption of each adipose tissue in vitro. The results of our oxygen consumption analyses showed that oxygen consumption within iWAT was significantly increased in NCD- and HFD-fed mice treated with KPF (Fig. 3A–C). Although oxygen consumption in eWAT increased slightly, this change did not reach statistical significance (Fig. 3B–D).

Next, we examined the protein expression of the brown fat marker gene UCP1, and the results showed that KPF significantly increased the protein expression of UCP1 in iWAT, demonstrating that KPF promoted the occurrence of browning in iWAT. However, no such change was observed in BAT (Figs. S1G–J). In order to explore the specific mechanism by which KPF regulates the occurrence of iWAT browning, we examined the proteins on the AMPK-related pathway, and the results showed that KPF significantly increased the expression of p-AMPK and promoted the expression of SIRT1 and PGC-1α in iWAT of NCD mice (Fig. 3E and F), consistent with NCD, KPF increased the ratio of p-AMPK to AMPK and promoted the increase of downstream SIRT1 expression in HFD mice (Fig. 3G and H). In addition, activation of PGC-1α induced the expression of PRDM16 (PR domain containing 16), a regulator of energy expenditure. Therefore, we examined the protein expression of PRDM16 separately for NCD and HFD mice, which appeared significant after KPF treatment (Fig. 3E–H).

3.4. KPF effect on SVF-derived adipocyte differentiation

During the induction process of the stromal vascular fraction, preadipocytes gradually differentiate into mature adipocytes, where fatty acids are converted into triglycerides and accumulate as lipid droplets. We evaluated whether the adipocyte model was successfully established using Oil Red O staining. The results revealed the emergence of a multitude of transparent, spherical, or elliptical lipid droplets within the cells post-induction (Fig. 4A and B). Additionally, there was a marked elevation in TG content, clearly confirming the successful establishment of the primary adipocyte model (Fig. 4C). To assess the potential cytotoxic effects of KPF, we performed an CCK-8 assay in adipocytes. The results showed that the concentration of KPF less than or equal to 40 μM had no significant effect on cell viability (Fig. 4D). Based on the results of CCK-8, doses of 10 μM, 20 μM, and 40 μM KPF concentrations were selected for subsequent experiments. Compared with the control group, the KPF treatment group showed a significant reduction in transparent lipid droplets and the appearance of distinct multilocular structures. In addition, the number and density of lipid droplets decreased as the dose of KPF was gradually increased from 10 μM to 40 μM (Fig. 4E). Oil Red O staining results also showed a gradual decrease in the number of red lipid droplets with increasing dose of KPF (Fig. 4F). Consistent with the Oil Red O staining results, measurement of TG accumulation using a colourimetric assay showed a significant decrease in TG accumulation in primary adipocytes following KPF treatment (Fig. 4G). These results suggest that KPF can reduce lipid accumulation in a concentration-dependent manner.

To unravel the underlying mechanism of kaempferol's involvement in lipolysis, we carried out a thorough examination of UCP1, AMPK, SIRT1, PGC-1α, PPARγ and PRDM16 expression through western blotting in vitro. The results showed that there was no significant difference in AMPK protein expression level among the experimental groups. The levels of PGC-1α, SIRT1, p-AMPK/AMPK, PRDM16, PPARγ and UCP1 were all significantly increased after KPF treatment (Fig. 4H and I).

To investigate the mechanism, we treated iWAT with AICAR and CC. We found that KPF and AICAR increased the expression of UCP1, p-AMPK, and SIRT1, while this effect was inhibited after CC treatment (Fig. 5A–D). This collective evidence strongly suggests that KPF may orchestrate the browning of adipocytes by triggering AMPK phosphorylation, enhancing the expression of SIRT1, and upregulating the levels of PGC-1α, a pivotal mitochondrial regulatory factor.

4. Discussion

Kaempferol is widely distributed in many edible vegetables, fruits, and plant species commonly used in traditional medicine, such as: strawberries, onions, carrots, horseradish, broccoli, cucumber, and galangal (Romero-Juárez et al., 2023; Alam et al., 2020). Numerous studies have confirmed that kaempferol is extremely high in some commonly consumed foods, for example, onion leaves contain up to 832 mg of kaempferol per kg, papaya buds contain up to 453 mg/kg of kaempferol, pumpkin contains 371 mg/kg of kaempferol, cauliflower and black tea contain 270 mg/kg and 118 mg/kg of kaempferol, respectively (Miean and Mohamed, 2001). In this study, we selected a dose of 50 mg/kg of KPF for animal experiments, which was chosen based on our preliminary experiments (Zhang et al., 2023). According to the body surface area method, the dose administered to mice was approximately 12.3 times the human dose. At an intake of 50 mg/kg, a 70 kg adult would receive 284.6 mg, which can be achieved through consumption of certain foods.

Projections show that more than half of the world's adults could be affected by obesity by 2030, highlighting the urgent need for safe and effective weight loss interventions (Juszczak et al., 2020). Previous studies have shown that KPF reduces lipid accumulation in 3T3-L1 and human mesenchymal stem cells (hMSC) derived adipocytes in a dose-dependent manner by inhibiting lipid synthesis and increasing lipolysis (Torres-Villarreal et al., 2019; Gómez-Zorita et al., 2017). Similar to the report, in the present study we found that KPF reduced the number of lipid droplets in primary adipocytes, decreased lipid accumulation and induced a shift from large lipid droplets to small polycyclic lipid droplets in a dose-dependent manner (40 μM had the best effect). Other studies have shown that KPF exerts anti-obesity effects in vivo by regulating intestinal flora homeostasis, modulating obesity-associated activation of hypothalamic microglia and regulating peroxisome proliferator-activated receptor γ (PPARγ) (Bian et al., 2022; Romero-Juárez et al., 2023; Tang et al., 2021). In the present study, we found that KPF reduced the body weight of NCD and HFD-fed mice and decreased the percentage of iWAT and eWAT, which exerts anti-obesity effects.

At its core, obesity stems from the excessive accumulation of adipose tissue, pivotal in maintaining energy balance, and thus holds a substantial role in the quest to combat this issue. The role of adipose tissue is profound in impacting overall health. For example, visceral fat contributes to an increased influx of free fatty acids into the liver, which increases the risk of metabolic disorders, including disorders of glucose and lipid metabolism, diabetes, and hypertension (Choi et al., 2021). Conversely, brown adipose tissue, which is rich in mitochondria, counteracts obesity and its related complications by dissipating excess body fat as heat (Wu et al., 2017; Rajbhandari et al., 2018). In light of these considerations, the browning of white adipose tissue emerges as a crucial avenue of research pertaining to adipose tissue (Moreno-Aliaga and Villarroya, 2020; Pan and Chen, 2022). We found that the number of lipid droplets of primary adipocytes isolated from iWAT was significantly reduced by KPF treatment, and the cell size of iWAT adipose tissue became smaller, with these changes accompanied by the appearance of multicompartmental structures, suggesting that browning of white adipose tissue may have occurred, as shown by oil red O staining and H&E staining.

Located on the inner mitochondrial membrane, UCP1 is a transmembrane carrier protein that plays a key role in mitochondrial respiration by uncoupling the proton gradient, thereby converting energy expenditure into heat production, accompanied by increased oxygen consumption (Ikeda and Yamada, 2020; Ježek et al., 2019). Increased

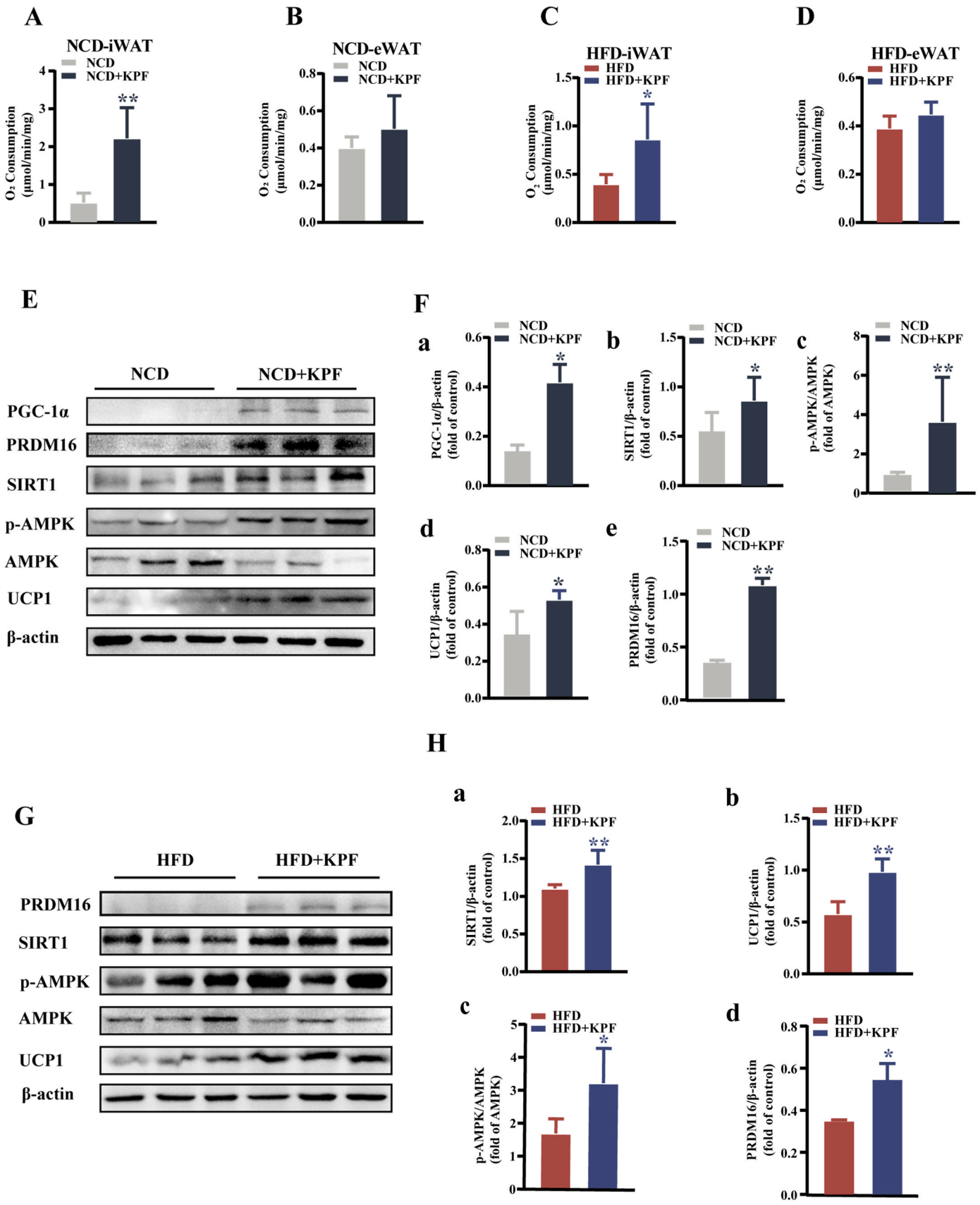

Fig. 3. KPF increased energy expenditure and induced white adipose tissue Browning via AMPK/SIRT1/PGC-1α pathway. (A, B, C and D) Ex vivo oxygen consumption of iWAT and eWAT homogenates from NCD (A, B) or HFD (C, D) mice. (E) Immunoblot of the cell related protein in iWAT from mice on NCD. (G) Immunoblot of the cell related protein in iWAT from mice on HFD. Specific bands were quantified and are presented as graphs (F, H). Data shown are mean ± SD (n = 3–6). *$p < 0.05$, **$p < 0.01$, vs. Control.

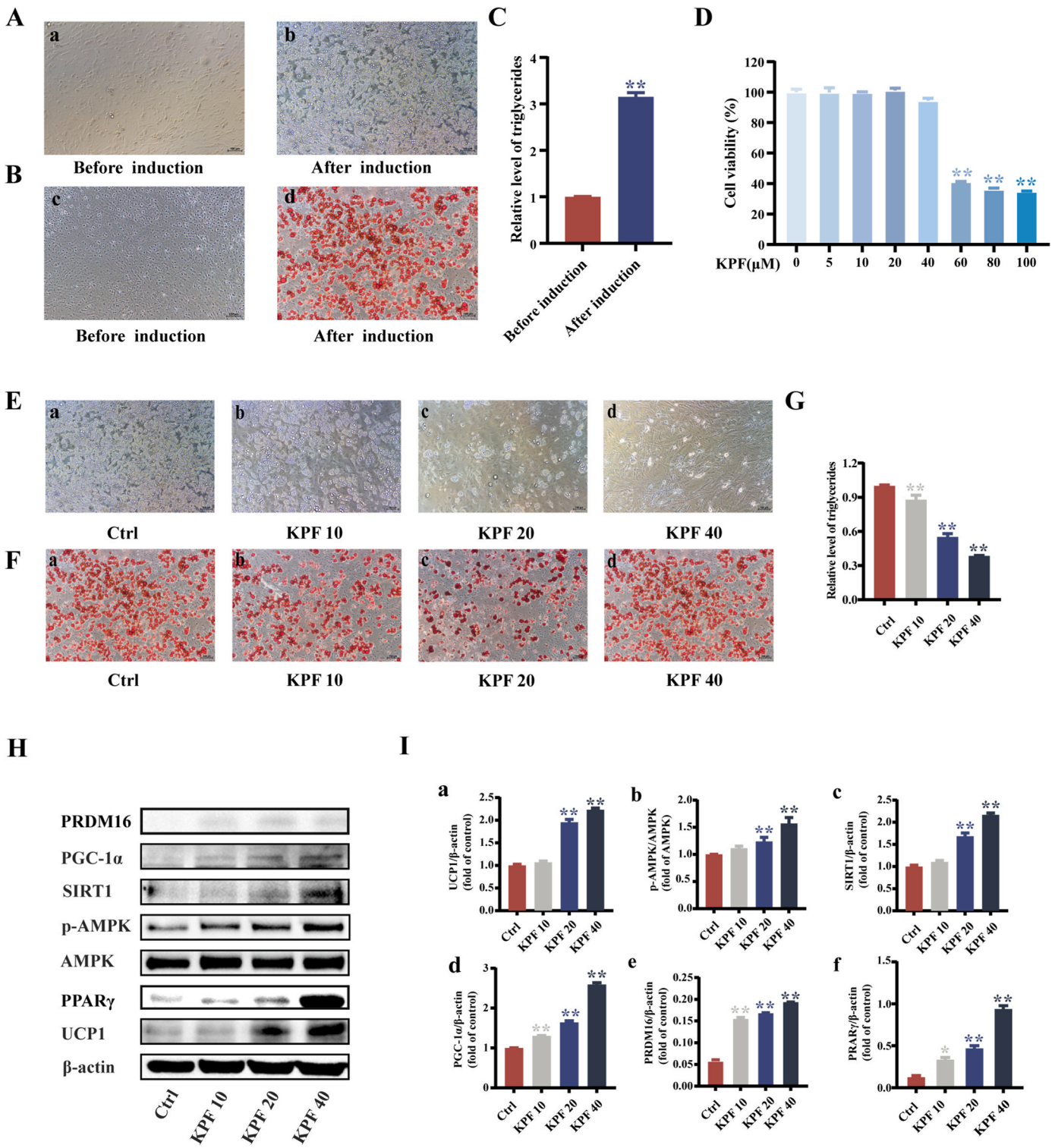

Fig. 4. KPF increased lipolysis in mature adipocytes and promoted the differentiation of iWAT pre-adipocytes into beige cells. (A, B and C) Identification of the primary adipocyte model. (D) KPF on cell viability in iWAT, determined using a 48 h CCK-8 assay. (E, F and G) Effects of lipid droplet morphology and triglycerides in primary adipocytes of KPF. (H) Immunoblots of the indicated protein levels in differentiated adipocytes. Specific bands were quantified and are presented as graphs (I). Data shown are mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, vs. Control.

UCP1 expression was observed in the iWAT of both NCD-fed and HFD-fed mice treated with KPF, as well as in primary adipocytes isolated from iWAT after 48 h of KPF treatment. In addition, the oxygen consumption of iWAT was significantly enhanced as measured by ex vivo tissue oxygen consumption. These are evidence that browning occurs. Obesity is closely related to abnormal glucose and lipid metabolism, and the browning of white adipose tissue promotes the improvement of glucose and lipid metabolism (Cheng et al., 2021). In the present study, we found that glucose tolerance and insulin sensitivity were improved in KPF treated mice under two diet conditions. It also significantly improved the levels of TG, TC, HDL-C and LDL-C in mice. Similar to the results of the present study, it has been found that kaempferol can

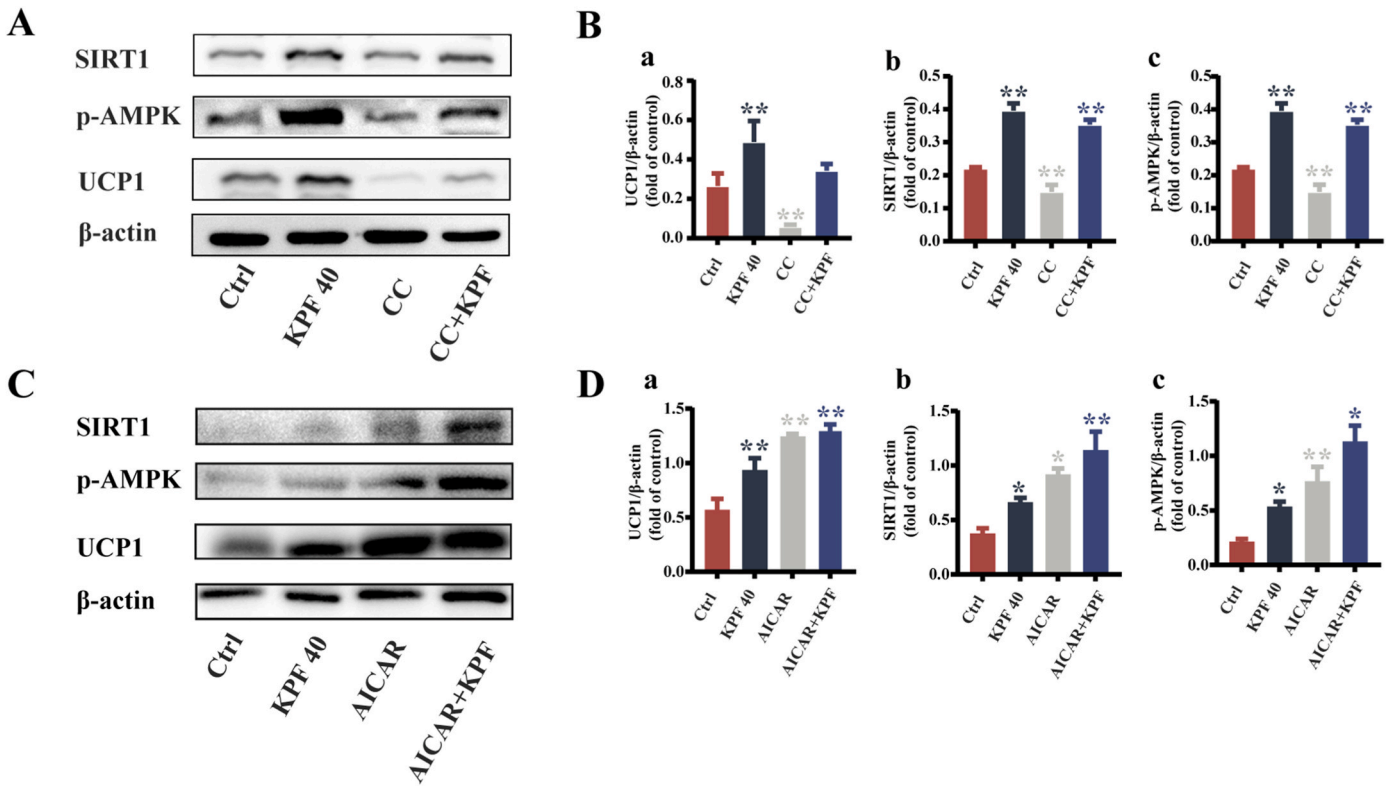

Fig. 5. KPF promotes white adipose tissue browning in an AMPK-dependent manner. (A) Immunoblotting of indicated protein levels in differentiated adipocytes after AMPK inhibition. (C) Immunoblotting of indicated protein levels in differentiated adipocytes after AMPK activation. Specific bands were quantified and are presented as graphs (B, D). Data shown are mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, vs. Control.

inhibit hepatic gluconeogenesis and increase insulin sensitivity to improve hyperglycemia in mice fed a high-fat diet (Alkhalidy et al., 2018; Zang et al., 2015). It can significantly improve the dyslipidemia induced by high-fat diet and prevent the formation of fatty liver (Li et al., 2019).

Studies have illuminated the intricate regulation of UCP1 expression, which involves a diverse array of transcription factors, including PPARγ, activated transcription factor 2 (ATF2), thyroid hormone receptors (THR), and notably, PGC-1α (Chang et al., 2019). PGC-1α is a mitochondrial regulatory protein that directly binds to the promoter region of UCP1 gene to activate UCP1 transcription. Subsequently, the increased UCP1 facilitates proton leakage across the mitochondrial inner membrane, thereby increasing energy metabolism (Bertholet et al., 2022; Pettersson-Klein et al., 2018). PRDM16, the key activator of PPARγ (peroxisome proliferator-activated receptor γ) in the process of WAT, forms a complex with PPARγ to regulate the expression of UCP1 and other browning genes, thereby inhibiting the genes of WAT and promoting the browning of WAT (Qiang et al., 2012). PGC-1α is a co-activator of PPARγ, and its expression marks the appearance of beige cells in white adipose tissue, which are involved in energy expenditure and lipid metabolism (Wu et al., 1999). In addition, brown adipocyte specific markers PRDM16, PGC1α and UCP1 induce thermogenesis (Harms and Seale, 2013). AMPK is an important regulator of energy balance and can regulate PGC-1α expression and activity through a variety of pathways, thereby promoting mitochondrial biosynthesis and oxidative phosphorylation. AMPK accomplishes this by phosphorylating PGC-1α, amplifying its transcriptional prowess, and directly influencing its expression (Herzig and Shaw, 2018). Meanwhile, SIRT1 is an NAD (+) - dependent deacetylase, augments PGC-1α's transcriptional capabilities through deacetylation. AMPK augments SIRT1's activity by elevating the cellular NAD+/NADH ratio, thereby culminating in the deacetylation of downstream targets and their resultant augmented activity (Xu et al., 2021). This tandem collaboration with AMPK further bolsters PGC-1α expression, and the interactions between AMPK and SIRT1 form a vital nexus in the orchestration of cellular metabolism and energy balance, thus constituting a pivotal therapeutic target in the fight against obesity (D. Kim et al., 2022). Our study showed unequivocally establishes that KPF significantly amplifies the protein expression of p-AMPK, PGC-1α, and SIRT1, concomitant with an elevated p-AMPK/AMPK ratio. We evaluated the main effect of AMPK on the regulation of UCP1 expression by KPF. Studies have shown that AICAR induces AMPK activation, increases UCP1 expression, and induces a transition to beige adipocytes in white adipose tissue (H.J. Kim et al., 2022; Lee et al., 2023). However, the AMPK inhibitor CC reversed this phenomenon (Lee et al., 2023). Therefore, we used AICAR and CC to activate and inhibit AMPK, respectively, and KPF affected the protein expression of UCP1 and significantly increased and restored it. This cascade of events signifies that KPF augments mitochondrial function while fostering adipocyte browning, courtesy of the heightened activation of AMPK phosphorylation sites alongside augmented SIRT1 activity.

5. Conclusions

Collectively, our results support that KPF protects against obesity by inducing the formation of beige cells and increasing adipose tissue thermogenesis through the AMPK/SIRT1/PGC-1α pathway, which broadens the understanding of the biological mechanisms of KPF. Therefore, KPF would be a promising pharmacological agent against obesity and its associated metabolic diseases. Consumption of KPF rich foods in daily life is also a potential option for the treatment of obesity.

CRediT authorship contribution statement

Changyu Xu: Conceptualization, Data curation, Formal analysis, Writing – original draft. **Xiaoxi Zhang:** Investigation, Funding acquisition, Project administration, Resources, Writing – review & editing.

Yihuan Wang: and. **Yan Wang:** Investigation, Methodology. **Yixuan Zhou:** Software. **Fenfen Li:** and. **Xiaoli Hou:** Supervision, Validation, Visualization. **Daozong Xia:** Funding acquisition, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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