

Original Article

Inhibition of adipocyte differentiation by phenformin and its underlying mechanism

Huizhi Sun¹, Nan Zhao², Jie Meng², Yanlei Li², Xian Lin², Yong Wang², Danfang Zhang², Jie Han¹, Chunchun Gan³, Xueyi Dong², Derun Tian¹

Departments of ¹Human Anatomy and Histology, ²Pathology, ³School of Pharmacy, Tianjin Medical University, Tianjin, China

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Abstract: Objective: To analyze the effect of phenformin (PHE) on differentiation of adipocyte and the underlying mechanism. Methods: Mouse 3T3-L1 cells were subjected to hormone-induced differentiation followed by PHE or metformin (MET) intervention. OB/OB mice were used as an obesity model. Adipose tissue specimens were collected and different groups of mice were compared to examine the adipocyte morphology and the degree of fatty liver. PCNA was used to detect the proliferation. qPCR was used to assay *PPAR γ* and its target genes mRNA in 3T3-L1 cells and mouse adipose tissue. Results: The PHE and MET groups showed decreased differentiation of preadipocytes to adipocytes. Furthermore, body weight gain, liver weight gain, PCNA staining index and the degree of fatty liver were lower in the PHE and MET treated mice. Results of real-time PCR revealed that PHE and MET had an inhibitory effect on the mRNA expression of *PPAR γ* and its target genes including *aP2*, *adipsin*, *LPL*, *adiponectin* and *CD36*. Conclusions: PHE and MET reduced weight gain of mice. The possible mechanism is that PHE and MET inhibit *PPAR γ* and its target genes, thereby suppressing adipocyte differentiation. The effect of PHE was stronger than that of MET.

Keywords: Phenformin, metformin, adipocyte differentiation, mechanism

Introduction

Obesity has an increasingly high incidence, and has emerged as an epidemic disease on a global scale, causing serious harm to human health. Approximately 2.3 billion adults in the world are currently overweight, and at least 700 million of them are obese. This problem is no longer exclusive to developed countries, as there has been a sharp rise in the incidence of obesity in many developing countries over recent years [1]. Obesity is the result of multiple factors such as physiology, biochemistry and lifestyle. The adipose tissue plays a critical role in the development and progression of obesity. In addition to providing energy storage sites, the adipose tissue can secrete various cytokines to intervene with the body's metabolism and vascular physiology, further participating in a range of diseases [2]. Therefore, the key to study obesity lies in the molecular regulation mechanism of adipocyte growth and differentiation.

Metformin (MET) is a biguanide medication with a history of over 50 years, which can lower blood glucose levels and improve insulin resistance. Studies have found that while MET lowers blood glucose levels, it simultaneously leads to weight loss in patients [3, 4]; however, the underlying molecular mechanism is still unclear. Phenformin (PHE) is another biguanide with a better hypoglycemic effect than MET. Nonetheless, few studies have assessed the therapeutic effect of PHE on obesity. Does PHE have an effect similar to MET? Can PHE regulate *peroxisome proliferator-activated receptor γ* (*PPAR γ*) gene expression to influence adipocyte differentiation and maturation, and thereby inhibit adipose tissue accumulation and achieve weight loss and a hypolipidemic effect?

To analyze the effect of PHE on differentiation of adipocyte and the underlying mechanism. In this study, an OB/OB mouse model was adopted to assess the effect of PHE and MET on adi-

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pogenesis. Furthermore, an *in vitro* culture was used to examine the effect of PHE and MET on the differentiation of 3T3-L1 preadipocytes and the underlying mechanism. The results help us to better understand the molecular mechanism of adipocyte differentiation. The potential effect of PHE providing another possible hypolipidemic drug for clinical treatment of obesity.

Materials and methods

Experimental materials

PHE, MET, human insulin, dexamethasone (DEX), 1-methyl-3-isobutyl xanthine (IBMX) and indomethacin were purchased from Sigma (St. Louis, MO, USA). DMEM medium and fetal bovine serum (FBS, SV30087.02) were purchased from Hyclone (Logan, UT, USA). Mouse anti-proliferating cell nuclear antigen (PCNA) and anti-endomucin monoclonal antibodies were purchased from Abcam (Cambridge, UK). Horseradish peroxidase conjugated anti-mouse IgG secondary antibody was purchased from Zhongshan (Beijing, China). Oil Red O was purchased from Solarbio (Beijing, China). Trizol reagents were purchased from Invitrogen (Carlsbad, CA, USA). Real time-PCR reverse transcription kit and SYBR real-time PCR kit were purchased from TaKaRa (Dalian, China).

Experimental animals

Sixty male OB/OB mice (7-9 weeks old) were purchased from Fukang Biotechnology Co., Ltd. (Beijing, China). Animals were assigned to six groups using a random number table: normal diet, high-fat diet, normal diet + high-dose PHE, high-fat diet + high-dose PHE, normal diet + high-dose MET and high-fat diet + high-dose MET. Mouse body weight was measured daily, and the treatment groups were given the biguanides by intragastric administration every other day. PHE (HY-16397A, Sigma) and MET (HY-17471, Sigma) were dissolved in autoclaved distilled water to a concentration of 25 mg/mL.

All of the experimental protocols were conducted in accordance with our Institutional Animal Care and Use Committee (IACUC) guidelines and were approved by the Tianjin Medical University IACUC committee.

Cell culture and drug-induced differentiation

The 3T3-L1 preadipocyte line was purchased from the Cell Bank of Chinese Academy of Sciences (Beijing, China). 3T3-L1 preadipocytes were cultured in high-sugar DMEM medium containing 10% FBS at 37°C and 5% CO₂ for 2 days. The medium was exchanged once every two days. When the cell monolayer reached above 80% confluence, the cells were subcultured and seeded into 6-well culture plates. Time zero was recorded when 3T3-L1 preadipocytes reached complete confluence. After 2 days of contact inhibition (i.e., day 0 of induced differentiation), hormones were added as differentiation-inducing agents. Differentiation medium I comprised: high-glucose DMEM medium containing 10% FBS, 1% penicillin-streptomycin mixture, 10 mg/L human insulin, 0.5 mmol/L IBMX, 1.0 μmol/L DEX and 0.1 mmol/L indomethacin. Differentiation medium I was removed 2 days later and replaced with differentiation medium II after 2 days of continuous culture. Differentiation medium II comprised: high-glucose DMEM medium containing 10% FBS, 1% penicillin-streptomycin mixture and 10 mg/L human insulin. Differentiation medium II was replaced 2 days later and the culture was incubated for 2 days. Thus, the induction of differentiation lasted 8 days to complete the process of differentiation. The experimental drugs were added at different concentrations of PHE (0.05 mM, 0.1 mM, 1 mM, 10 mM) or MET (0.05 mM, 0.1 mM, 1 mM, 10 mM) 1 day before the induction of differentiation, and the same drug concentrations were added during each medium exchange.

Oil red O and sudan IV staining

Oil red O (ORO) staining was used in combination with light microscopy to assess pre-adipocyte differentiation. Briefly, the treated differentiated 3T3-L1 cells were washed twice with PBS buffer and fixed in 10% neutral formalin overnight before staining with a 0.35% (w/v) ORO solution in isopropanol for 10 min. Excess stains were then removed by rinsing the cells under water and then dried before microscopic examination. For quantitative analysis, ORO was eluted with isopropanol and the absorbance was measured at 485 nm in a spectrophotometer.

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Table 1. Primers used in qRT-PCR

Gene	Primer	Product size (bp)	Annealing temperature (°C)
Mus GAPDH	Sense: 5'-CCTGGAGAAACCTGCCAAGT-3'	129	60
	Antisense: 5'-TGGGAGTTGCTGTGAAGTC-3'		
Mus PPAR γ	Sense: 5'-TGTCGGTTTCAGAAGTGCCTTG-3'	122	60
	Antisense: 5'-TTCAGCTGGTCGATATCACTGGAG-3'		
Mus aP2	Sense: 5'-AGCATCATAACCCTAGATGGCG-3'	114	58
	Antisense: 5'-CATAACACATTCCACCACCAGC-3'		
Mus adipsin	Sense: 5'-GCAACCGCAGGGACACTT-3'	106	57
	Antisense: 5'-TTGCCATTGCCACAGACG-3'		
Mus LPL	Sense: 5'-GTGGCCGAGAGCGAGAACAT-3'	196	58
	Antisense: 5'-GCTTTCCTCGGATCCTCTC-3'		
Mus adiponectin	Sense: 5'-TCCTGGAGAGAAGGGAGAGAAAAG-3'	83	58
	Antisense: 5'-TCAGCTCTGTCTTCCAACAT-3'		
Mus CD36	Sense: 5'-GGCCAAGCTATTGCGACATG-3'	124	58
	Antisense: 5'-CCGAACACAGCGTAGATAGAC-3'		

Sudan IV staining was another assessment of preadipocyte differentiation. The treated differentiated 3T3-L1 cells were stained with Sudan IV solution (composition (1 L): 5 g Sudan IV, 500 mL acetone, 500 mL 70% EtOH) and gently agitated for 15 min and then destained in 80% EtOH for a further 5 min.

Hematoxylin and eosin (HE) staining

Tissue section specimens were dewaxed and dehydrated with xylene-graded ethanol, followed by nuclear staining with hematoxylin, turning sections blue with 0.5% ammonia, and cytoplasm staining with eosin. The section specimens were dehydrated and clarified before mounting.

Morphological observations of 3T3-L1 preadipocytes

Inverted microscopy: After preadipocyte differentiation was induced, the round shape of cells and a gradually increased number of lipid droplets in the cells was directly observed under an inverted microscope.

Oil Red O staining: Oil Red O specifically stains lipids for identification of adipocytes and observation of the state of differentiation.

Section preparation

Tissue blocks from 60 OB/OB mice were fixed with formalin and embedded in paraffin. All

specimens were sliced into 4- μ m thick serial sections and heated in an oven at 70°C for 4 h.

Calculation of fatty liver index

After HE staining, fatty liver was assessed according to hepatocyte steatosis: 0 (<5%); 1 (5%~33%); 2 (34%~66%); 3 (>66%).

Immunohistochemical staining

Tissue section specimens were dewaxed and dehydrated with xylene-graded ethanol. Endogenous peroxidase was blocked using methanol-H₂O₂. The specimens were subjected to microwave retrieval (citrate pH 6.0) for 15 min, and then blocked with serum in a humid chamber for 30 min. One drop of working solution of the primary antibody was added and the specimens were incubated at 4°C overnight. Then, the secondary antibody was added and the specimens were incubated at 37°C for 1 h. PV6000 (Zhongshan) and the DAB colorization system were used for staining, followed by nuclear re-staining with hematoxylin. The specimens were dehydrated and clarified before mounting. The negative control was prepared using PBS instead of the primary antibody.

Real-time quantitative PCR

Total RNA was extracted from animal tissue using a total RNA extraction kit (DP431; Tiangen, Beijing, China) following the manufacturer's instructions. The extracted RNA was

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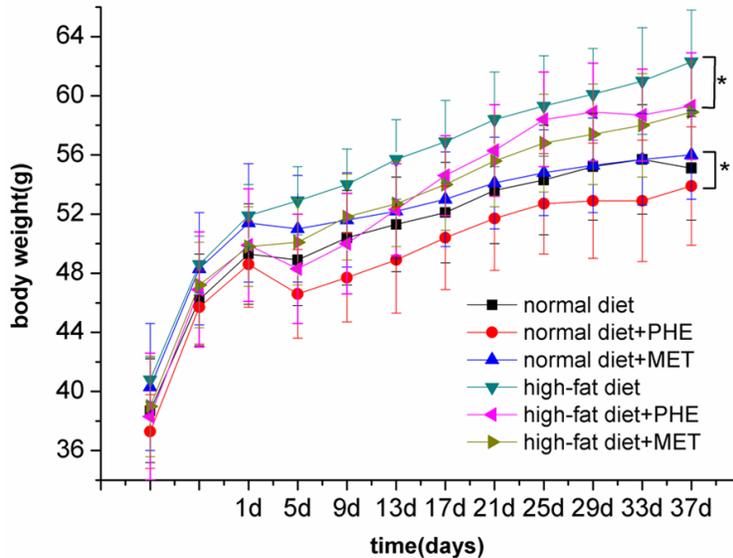


Figure 1. Body weight increased faster in high-fat diet group than normal diet group mice. In normal diet group mice, the body weight after administration of PHE was lower than that of the normal diet control group with time ($P<0.05$). In high-fat diet group mice, both administration of PHE and MET could decrease the mice body weight compare with the high-fat diet control group with time ($P<0.05$).

reverse transcribed to cDNA using a commercial kit (RR047A; TaKaRa). Primers for the target genes were designed using Beacon Designer 7.0 (Website OR Reference) and synthesized by Takara. Primer sequences are listed in **Table 1**. The PCR reaction (25 μ L) was performed with the following program: pre-denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing 58-60°C for 60 s (**Table 1**). The melting curve analysis was performed after completion of PCR amplification.

Statistical analysis

All data were evaluated using SPSS 22 (SPSS Inc., Chicago, USA). Differences were considered significant at $P<0.05$. The significant groups are marked with an asterisk in the figures.

Results

Effects of PHE and MET on body weight of OB/OB mice

Weight gain was more significant in mice in the high-fat diet group compared with the lower-fat diet group. During the induction of obesity with

high-fat diet, mice were given MET or PHE by intragastric administration and their body weight was recorded daily. The results showed that when mice were not obese in the early stage, the PHE and MET intervention groups had no difference in body weight compared with the high-fat diet control. However, when mice became overweight and even began to show obesity in the late stage (4 weeks later), PHE and MET played a role in reducing body weight. The weight gain trend slowed down in the PHE and MET intervention groups relative to the high-fat diet control ($P<0.05$; **Figure 1**).

Effects of PHE and MET on liver weight and fatty liver of OB/OB mice

Liver weight and the fatty liver index were significantly higher in the high-fat diet group compared with the normal diet group ($P<0.05$). In mice fed a high-fat diet, the PHE and MET intervention groups had markedly lower liver weight and fatty liver index compared with the high-fat diet control. The reduction in fatty liver weight was more significant in the PHE group compared with the MET group ($P<0.05$; **Figure 2**).

Effects of PHE and MET on adipocyte area and fat thickness in OB/OB mice

Experiments were performed using OB/OB mouse adipose tissue and 3T3-L1 preadipocytes. In the animal experiment, adipose tissue was taken from about 2 mm thick fat pad of the abdominal subcutaneous tissue of mice from the six groups. The results revealed that the fat thickness was significantly larger in the high-fat diet group compared with the normal diet group ($P<0.05$). In mice fed a high-fat diet, the PHE and MET groups had a markedly smaller fat thickness compared with the high-fat diet control ($P<0.05$). The reduction in fat thickness was more significant in the PHE group than in the MET group and the control group (**Figure 3A**).

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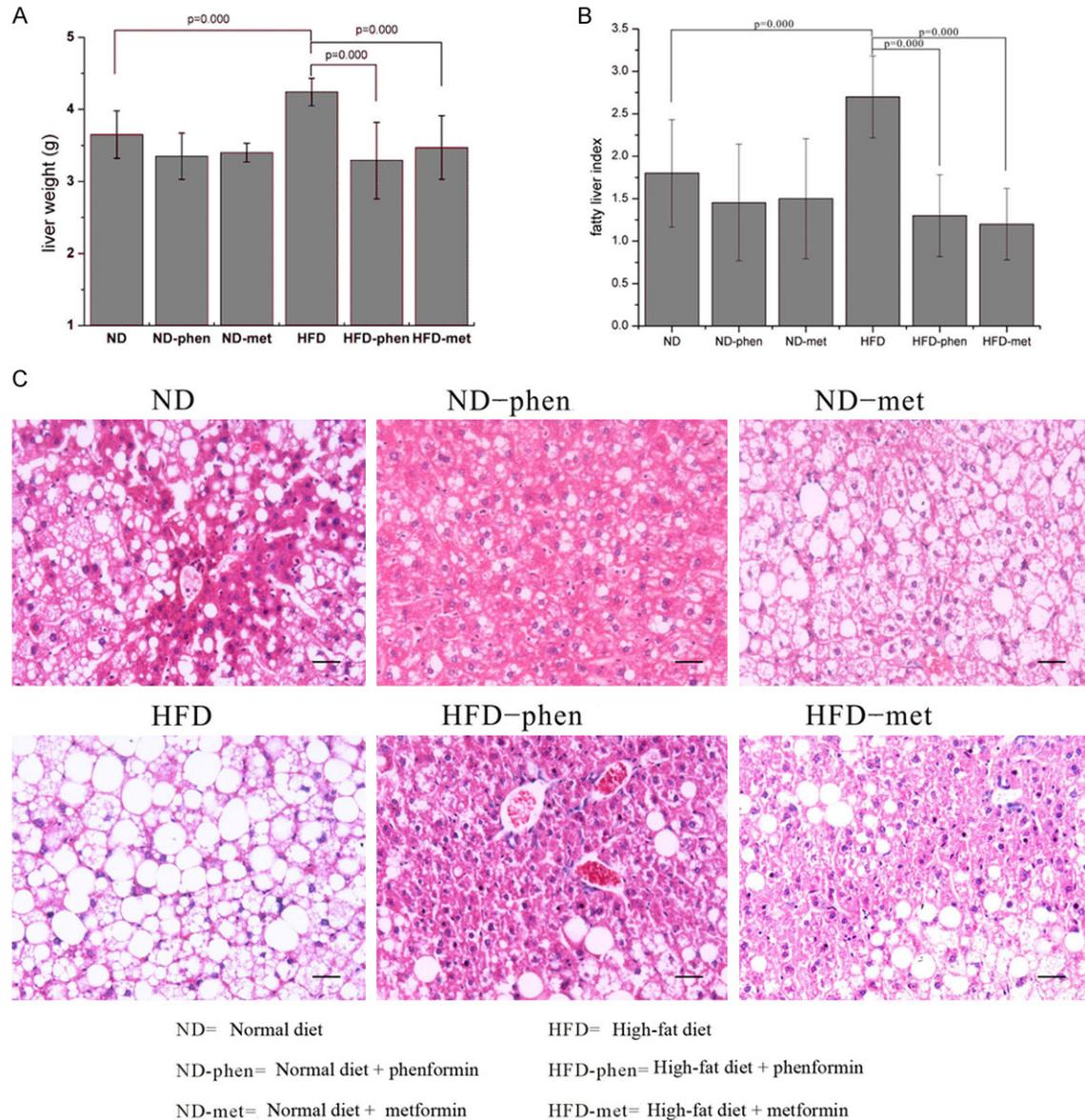


Figure 2. A. Liver weight was higher in the high-fat diet group compared with the normal diet group of mice. After administration of MET and PHE, the liver weight was significantly lower compared with the high-fat diet control ($P < 0.05$). B. Fatty liver index was higher in the high-fat diet group compared with the normal diet group of mice. After administration of MET and PHE, the fatty liver index was significantly lower compared with the high-fat diet control ($P < 0.05$). C. HE staining showing the condition of fatty liver in liver tissue of different diet and drug groups; scale bar = 200 μ m.

During the paraffin embedding and dehydration process, lipids stored in adipocytes were dissolved in organic solvents such as ethanol and xylene. Thus, fat vacuoles appeared in the section specimens, with the cell nucleus squeezed to one side. An image analysis system was adopted to select five fields of view, and the area of 100 adipocytes was measured in each field of view. The results showed that the adipo-

cyte area was significantly larger in the high-fat diet group compared with the normal diet group ($P < 0.05$). In mice fed a high-fat diet, the PHE and MET groups had a markedly smaller adipocyte area compared with the high-fat diet control ($P < 0.05$). Comparison of cell size in the different groups revealed that the high-fat diet group had adipocytes of uniform size, whereas varying adipocyte sizes (either large or small)

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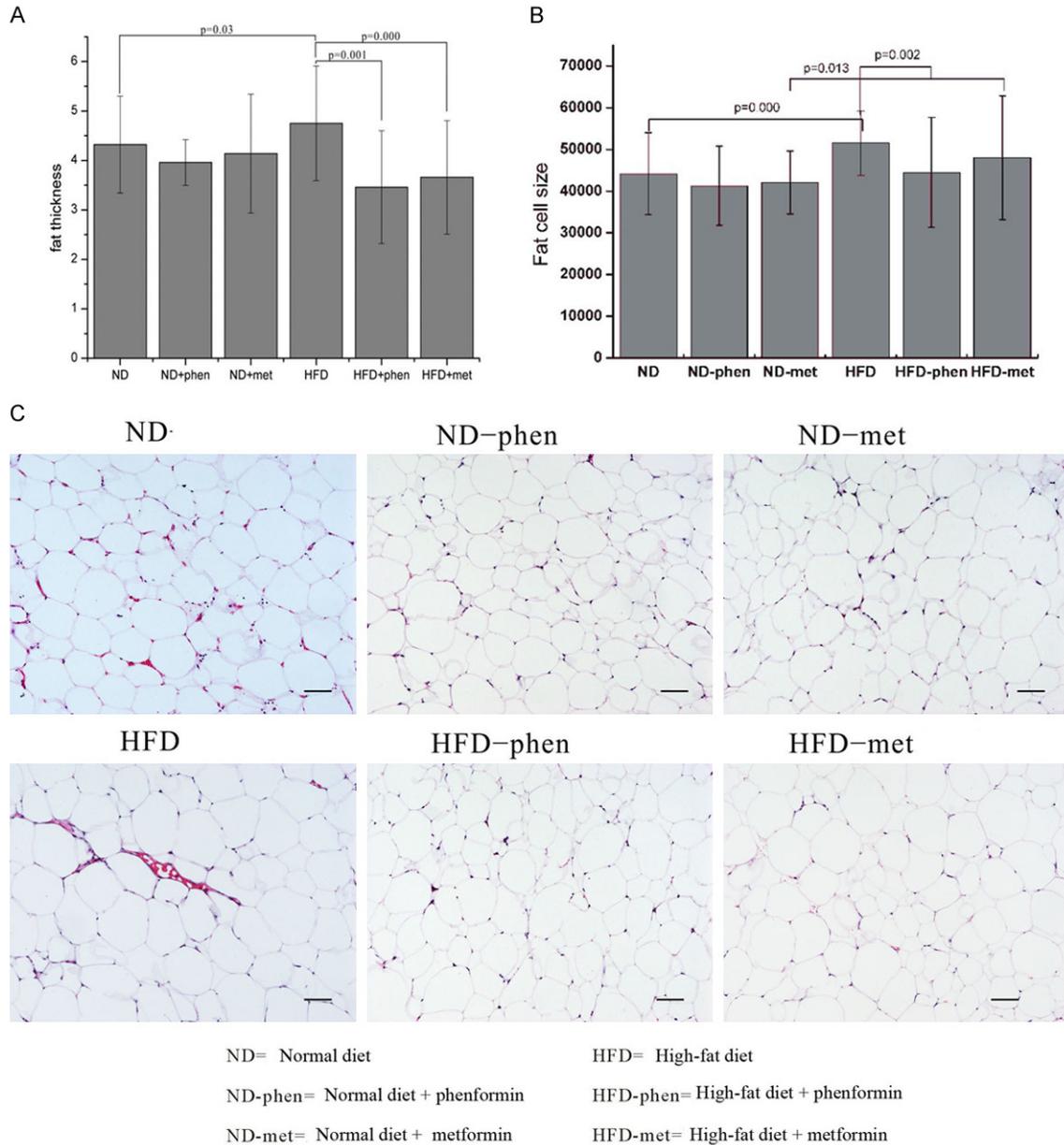


Figure 3. A. Fat thickness was larger in the high-fat diet group compared with the normal diet group of mice. Administration of PHE and MET significantly reduced the fat thickness compared with the high-fat diet control ($P < 0.05$). B. Adipocyte area was bigger in the high-fat diet group compared with the normal diet group of mice. Administration of PHE and MET significantly decreased the adipocyte area compared with the high-fat diet control ($P < 0.05$). C. HE staining showing adipocyte area in different diet and drug groups; scale bar = 200 μm .

were found in the PHE and MET groups (**Figure 3B** and **3C**).

Effects of PHE and MET on adipocyte proliferation

The PCNA staining index was slightly, but not significantly, lower in the high-fat diet group than in the normal diet group. In mice fed a high-fat diet, the PHE and MET groups had a

significantly lower PCNA staining index than the high-fat diet control ($P < 0.05$; **Figure 4**). The effect of PHE on the adipocyte proliferation is much stronger than that of MET.

Effects of PHE and MET on preadipocyte differentiation

After being seeded into 6-well culture plates, mouse 3T3-L1 preadipocytes were subjected

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A

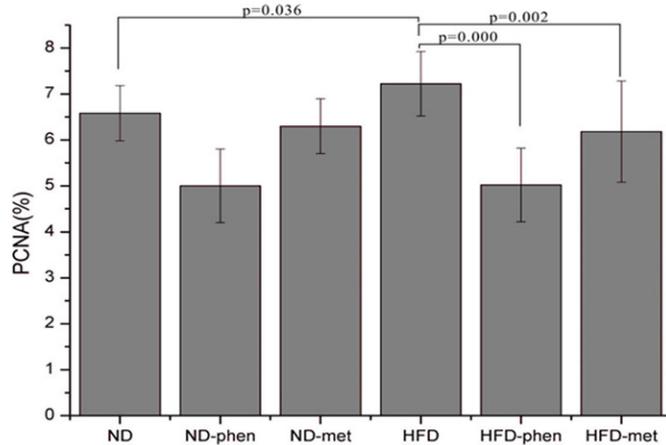
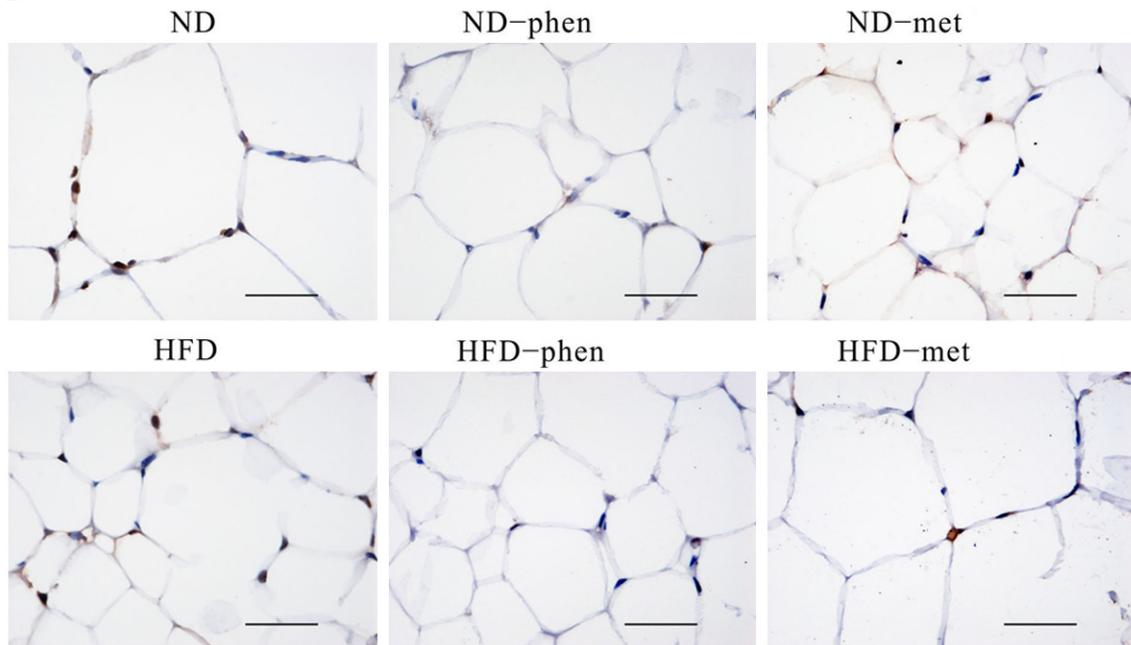


Figure 4. A. PCNA staining index was higher in the high-fat diet group compared with the normal diet group of mice. After administration of MET and PHE, the PCNA staining index was significantly lower compared with the high-fat diet control ($P < 0.05$). B. Immunohistochemical staining showing PCNA staining in different diet and drug groups; scale bar = 500 μ m.

B



ND= Normal diet

ND-phen= Normal diet + phenformin

ND-met= Normal diet + metformin

HFD= High-fat diet

HFD-phen= High-fat diet + phenformin

HFD-met= High-fat diet + metformin

to conventional induction of differentiation for 8 days. The experimental drugs were added at different concentrations of PHE (0 mM, 0.05 mM, 0.1 mM, 1 mM, 10 mM) or MET (0 mM, 0.05 mM, 0.1 mM, 1 mM, 10 mM) 1 day before the induction of differentiation, and the same drug concentrations were added during each medium exchange. Microscopy observation revealed that both PHE and MET had an inhibitory effect on adipocyte differentiation on different concentration, respectively. Results showed most of the cells (85%) were filled with fat drop-

lets in the control group (0 mM), indicating that the differentiation process was generally completed (**Figure 5A** and **5B**). Moreover, after Oil Red O and Sudan IV staining, the induced adipocytes appeared red. Furthermore, we found that that high concentration of PHE (1 mM, 10 mM) induced necrosis of adipocytes, while low concentration of PHE (0.05 mM, 0.1 mM) have significant inhibitory effect on adipocyte differentiation. In contrast, low concentration of MET (0.05 mM, 0.1 mM, 1 mM) didn't not have inhibitory effect, while high concentration of MET

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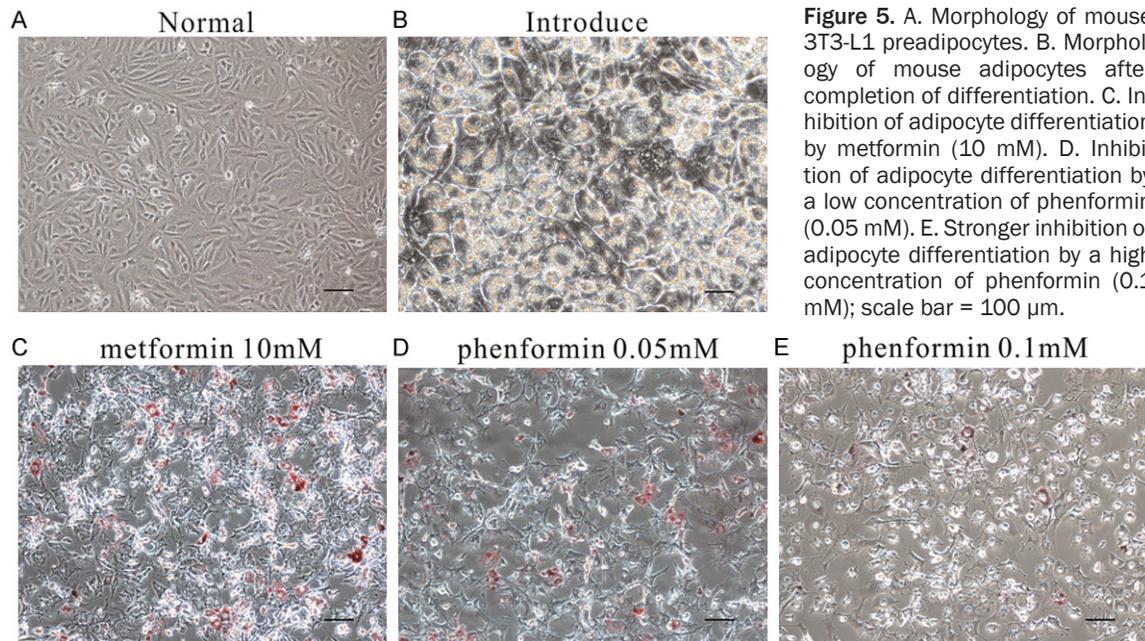


Figure 5. A. Morphology of mouse 3T3-L1 preadipocytes. B. Morphology of mouse adipocytes after completion of differentiation. C. Inhibition of adipocyte differentiation by metformin (10 mM). D. Inhibition of adipocyte differentiation by a low concentration of phenformin (0.05 mM). E. Stronger inhibition of adipocyte differentiation by a high concentration of phenformin (0.1 mM); scale bar = 100 μ m.

(10 mM) have significant inhibitory effect on adipocyte differentiation. Therefore, PHE (0.05 mM and 0.1 mM) or MET (10 mM) were chosen for further studies.

Taking MET as the reference drug (10 mM), PHE inhibited adipocyte differentiation in a dose-dependent manner. At a lower concentration (0.05 mM), adipocyte differentiation was suppressed by PHE compared with the control group of induction alone. At a higher concentration (0.1 mM), PHE exhibited a stronger trend of inhibiting adipocyte differentiation (**Figure 5C-E**). The result suggested that PHE may have a stronger effect than MET on preadipocyte differentiation.

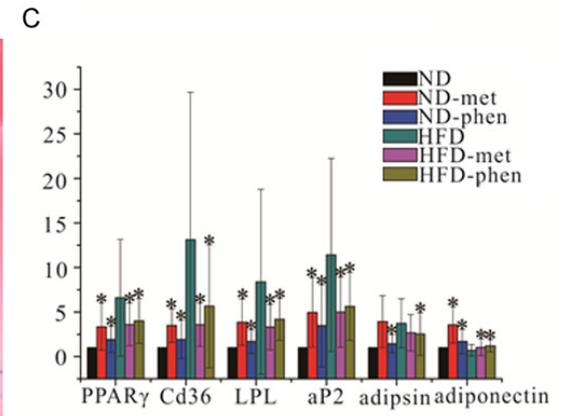
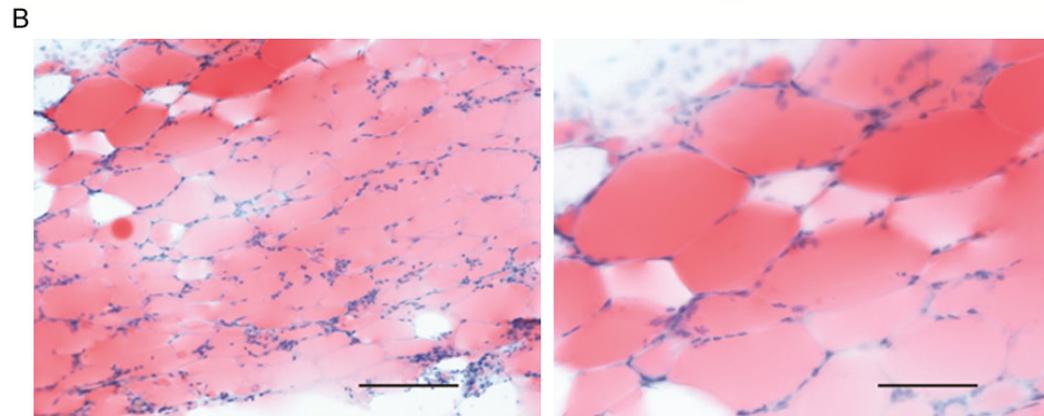
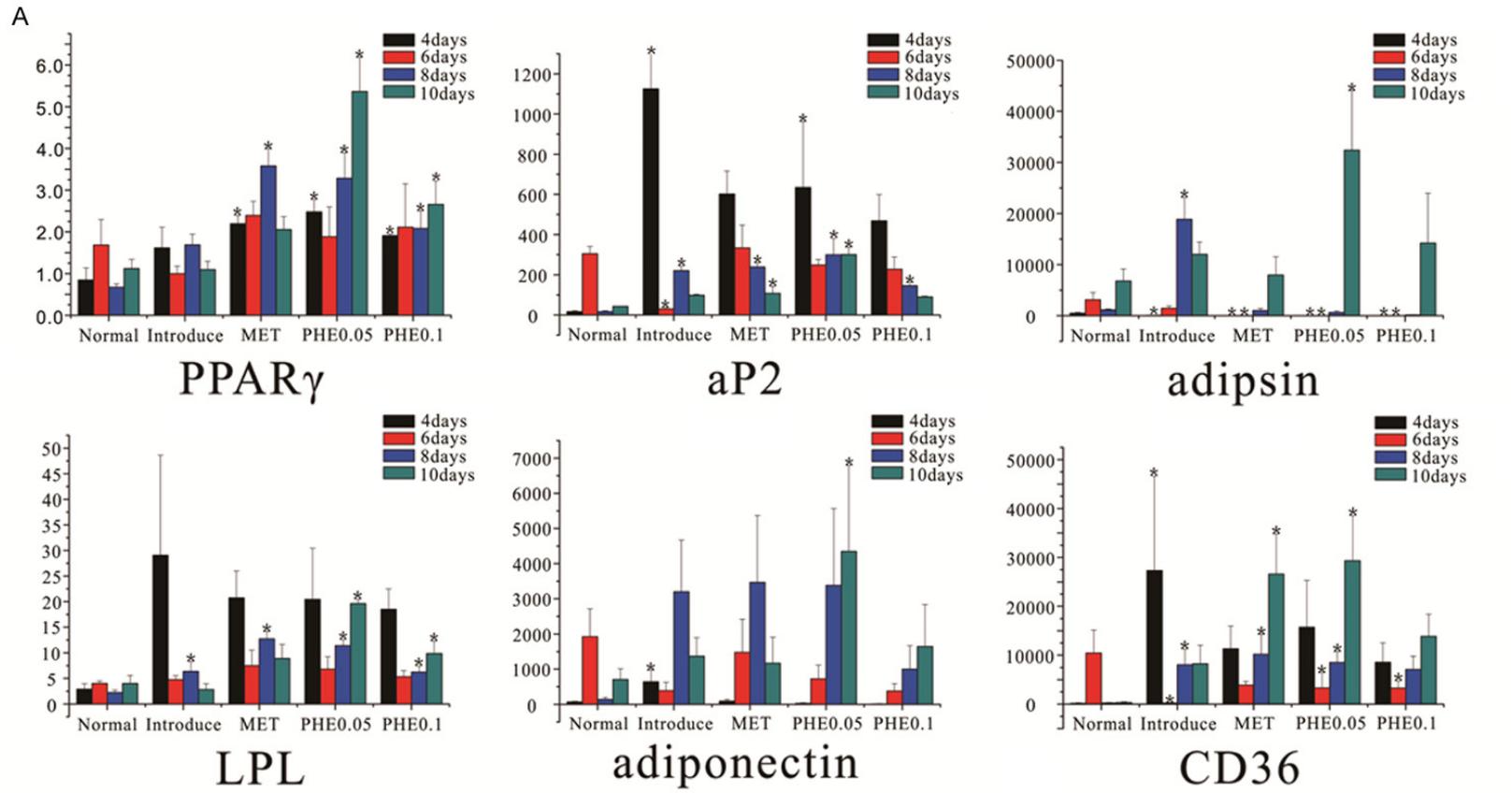
Role of PHE in preadipocyte differentiation

PPAR γ is an important transcription factor in the process of adipose differentiation, and it regulates the expression of adipocyte-specific molecules. To further investigate whether PHE plays an inhibitory role in relevant processes, we first used 3T3-L1 preadipocytes as a model and induced cell differentiation as performed in the above experiments. RNA samples were collected for mRNA analysis at 48 h intervals from day 4 of induction, i.e., at 4, 6 and 8 days of induction, and 2 days after induction. The mRNA expression changes in *PPAR γ* and its target genes including *aP2*, *adipsin*, *LPL*, *adipo-*

nectin and *CD36* were observed during the differentiation process using real-time PCR. 3T3-L1 cells without any inducer served as a negative control, MET as a reference drug, and *GAPDH* as an internal reference. Statistical analysis of the PCR data showed that the mRNA expression of *PPAR γ* and its target genes including *aP2*, *adipsin*, *LPL*, *adiponectin* and *CD36* were induced during the differentiation. We also found that PHE and MET had an inhibitory effect on the mRNA expression of *PPAR γ* and its target genes. The inhibition became more obvious with the increasing concentration of PHE (**Figure 6A**).

With regard to animal tissue, 2-mm thick fat pad was taken as adipose tissue from the abdominal subcutaneous tissue of mice from the six different groups. First, the animal tissue samples were proved to be adipose tissue by Sudan IV staining (**Figure 6B**). Next, the mRNA expression changes in *PPAR γ* and its target genes were examined between different groups by real-time PCR. The normal diet group served as a control and *GAPDH* as an internal reference. The real-time PCR data were statistically analyzed and the statistical results were consistent with the *in vitro* experimental data. Similarly, the results showed an inhibitory effect of PHE and MET on *PPAR γ* and its target genes' mRNA expression (**Figure 6C**).

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Figure 6. A. Metformin and phenformin inhibited the mRNA expression of *PPAR γ* and its target genes in 3T3-L1 pre-adipocytes; the inhibition by phenformin was dose-dependent. B. Adipose tissue stained red by Sudan IV; left: 20 \times ; right: 40 \times . C. Metformin and phenformin inhibited the mRNA expression of *PPAR γ* and its target genes in animal tissue.

Discussion

Conventional treatment of obesity is achieved through diet control. In recent years, research has shown that whilst biguanides lower blood sugar levels, these drugs can simultaneously lead to weight loss in patients; however, the underlying molecular mechanism is still unclear [5]. In this study, 8 weeks of high-fat diet induced a liver weight increase in mice. Compared with the high-fat diet control, the PHE and MET groups had a markedly lower liver weight, indicating that both drugs can reduce the amount of lipids and fatty acids entering the liver, and thereby improving the fatty liver condition.

The adipose tissue mainly comprises adipocytes. Thus, an increase in adipose tissue may be associated with an increased number and expanded area of adipocytes [6]. The current study revealed that whilst the proliferation index was significantly high in the high-fat diet group, it was lower in the PHE and MET groups. This result indicates that PHE and MET can inhibit adipocyte proliferation. Measurements of the individual adipocyte area in different experimental groups revealed that the adipocyte area was obviously smaller in the PHE and MET groups compared with the high-fat diet control. An interesting phenomenon is that the adipocytes appeared uniform in the high-fat diet control, while varying sizes of adipocytes were observed in the PHE and MET groups. This phenomenon shows that PHE and MET can inhibit the expansion of adipocyte area. Thus, PHE and MET can inhibit the proliferation of adipose tissue and suppress its area expansion.

Adipocytes are differentiated from preadipocytes, which are fibroblast-like cells in the vascular matrix of the adipose tissue [7, 8]. Human preadipocytes take around 20 days to initiate the expression of differentiation-specific genes, to produce adipocytes filled with lipid droplets. These differentiation-specific genes include two important adipogenesis transcription factor families: the enhancer binding protein family (CCAT α , β and δ) and the PPAR family (PPAR α and γ) [9, 10]. The PPAR family belongs to the

nuclear receptor transcription factors and many tissues in the human body secrete PPAR proteins. Among them, PPAR γ is the most specific to the adipose tissue and plays a key role in adipocyte differentiation. PPAR λ is activated by binding a ligand, and then it binds to its receptor to form a PPAR-RXR heterodimer. Furthermore, it binds a response element to activate its target genes, *aP2*, *adipsin*, *LPL*, *adiponectin* and *CD36*, ultimately promoting adipocyte differentiation and maturation [11]. Animal experiments have shown that once PPAR γ is activated in adipocytes, it can induce apoptosis in visceral and subcutaneous large adipocytes. Preadipocytes in subcutaneous fat differentiate into mature adipocytes, and thus increase the number of small adipocytes, which are more sensitive to insulin. This mechanism promotes an increase in insulin-dependent glucose uptake and a decrease in fat hydrolysis, resulting in increased insulin sensitivity. In the present study, the cell assays results revealed that PHE and MET can inhibit the differentiation of preadipocytes to adipocytes; such inhibition may be achieved by regulating *PPAR γ* gene expression to influence adipocyte differentiation and maturation. PPAR γ is a member of the nuclear receptor superfamily that belongs to transcription factors. Activation of PPAR γ affects the adipocyte differentiation pathway. PHE and MET can regulate the expression of *PPAR γ* and its downstream target genes, thereby affecting adipocyte differentiation. Similar results were obtained in the animal experiments.

In summary, PHE and MET can inhibit differentiation of preadipocytes to adipocytes and reduce accumulation of adipose tissue. The effect of PHE was stronger than that of MET and was possibly achieved through the following three mechanisms: 1) inhibition of adipocyte proliferation; 2) inhibition of adipocyte area expansion; and 3) inhibition of *PPAR γ* and its target genes *aP2*, *adipsin*, *LPL*, *adiponectin* and *CD36*.

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Disclosure of conflict of interest

None.

Address correspondence to: Derun Tian, Department of Human Anatomy and Histology, Tianjin Medical University, Tianjin 300070, China. Fax: 0086-22-83336813; E-mail: tianderun@aliyun.com

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