Published in final edited form as:

J Nutr Biochem. 2019 May ; 67: 201–211. doi:10.1016/j.jnutbio.2019.02.013.

Phytanic acid activates PPAR α to promote beige adipogenic differentiation of preadipocytes

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Abstract

A better understanding of the mechanisms of beige and brown adipogenesis is needed for developing strategies to prevent and treat obesity and associated metabolic disorders. Phytanic acid (PA) exists in a wide range of foods, especially in milk fat and marine foods, but its effects on obesity and beige adipogenesis remain poorly defined. The objective is to investigate the effects and regulatory mechanisms of PA in the beige adipogenesis. In 3T3-L1 preadipocytes, PA elevated the expression of brown adipogenic markers, suggesting that PA promotes beige adipogenic differentiation in committed adipogenic cells. In uncommitted C3H10T1/2 cells, while PA increased PGC1 α expression, it did not increase brown adipogenic regulators PRDM16 or UCP1 expression, suggesting that PA had no significant effects on brown adipocyte commitment. PA also enhanced mitochondrial biogenesis and oxygen consumption. Promotion of both mitochondriogenesis and beige adipogenic differentiation were blocked by using PPAR α antagonist or with *Ppara* knockdown, showing that PA-mediated beige/brown adipogenic differentiation is dependent on PPAR α . Additionally, the PA-regulated effect is independent on β 3-adrenergic receptor. Taken together, PA promotes beige adipogenic differentiation, but not the commitment of progenitor cells to the brown adipocyte lineage. PPAR α is a key mediator during PA-induced beige/brown adipogenic differentiation.

Keywords

Beige adipogenesis; Brown adipogenic differentiation; Phytanic acid; PPAR α

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Author contributions

H.W. and M.D. designed the study and wrote the manuscript. H.W. performed the experiments and analyzed the data. X.M. and M.D. reviewed and edited the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

1 Introduction

Based on morphological and functional differences, adipose tissue can be classified as white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is mainly responsible for storage of energy in the form of triglycerides droplets [1]. Excessive and abnormal WAT deposition may lead to metabolic diseases, such as obesity, type 2 diabetes and cardiovascular disease [1, 2]. In contrast, BAT dissipates energy in the form of heat due to the presence of numerous mitochondria and abundant uncoupling protein 1 (UCP1) [3]. Recently, brown-like adipocytes (termed beige adipocytes) have been discovered in WAT [3, 4]. Similar to classic brown adipocytes, beige adipocytes dissipate calories as heat, and importantly, they are highly inducible [4]. Therefore, promoting beige adipogenesis and the browning of WAT through nutritional and pharmacological interventions are attractive strategies for preventing and treating obesity-linked dysfunctions [5].

Beige adipocytes are derived from PDGFR α ⁺ cells within WAT [4, 6]. Both brown and beige adipogenesis is regulated by transcriptional regulators including PR domain zinc-finger protein 16 (PRDM16), peroxisome proliferator-activated receptor (PPAR) γ and PPAR γ coactivator 1 α (PGC-1 α) [7-9]. In addition, fibroblast growth factor 21 (FGF21) and bone morphogenetic protein 7 (BMP7) play important roles in brown adipogenesis [10, 11]. Micro RNA (i.e. miR-196a) could also have positive effects on the browning of WAT [12]. In addition, pharmacological and nutritional agents, such as resveratrol [13], retinoic acid [14], curcumin [15], berberine [16], cryptotanshinone [17], sulforaphane [18] and phytol [19], enhance WAT browning by activating brown adipocytes-related signaling pathways.

Phytanic acid (PA) is a branched-chain fatty acid that is produced by the degradation of phytol, a constituent of chlorophyll [20]. As a result, PA is abundant in a variety of foods especially in marine foods, beef and dairy products [20]. The daily consumption per person is estimated at 50 to 100 mg [21]. PA acts via (PPARs) to regulate glucose metabolism in rat primary hepatocytes [22]. PA also induced white adipocyte differentiation [23]. Besides, PA has been found to activate *Ucp1* gene transcription and brown adipocyte differentiation in HIB-1B cells and mouse primary brown preadipocytes [24-26]. A phytol-enriched diet may boost PA levels in the liver of mouse, thereby leading to activation of PPAR α [26]. To date, effects of PA on the formation of brown and beige adipocytes have only been sparsely explored and the underlying mechanism is unknown.

PPAR α regulates fatty acid oxidation in many organs [27]. It has been showed that PPAR α agonist fenofibrate promoted the expression of brown adipocyte marker genes in subcutaneous WAT and ultimately resulted in beige adipocyte formation [28, 29]. PPAR α could also cooperate with SIRT1 to increase metabolic activity and promote browning of WAT [30]. PA is considered as a ligand of mouse PPAR α [31], but whether PPAR α is involved in PA-mediated brown or beige adipogenesis remains undefined. The objective of this work is to explore the effects of PA on beige adipogenesis. Excitingly, we found that PA promotes beige adipogenic differentiation of preadipocytes but not uncommitted progenitor cells, and PPAR α is a key mediator of PA-induced beige adipogenic differentiation.

2 Materials and Methods

2.1 Antibodies and chemicals

Antibodies against β -actin (#4967), AMPK α (#5832), and phospho-AMPK α at Thr172 (#2535) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against FABP4 (ab92501), PPAR γ (ab41928), PGC1 α (ab54481), PRDM16 (ab106410), UCP1 (ab10983), and PPAR α (ab3484) were purchased from Abcam (Cambridge, UK). Goat anti-rabbit IgG HRP (A0208) and goat anti-mouse IgG HRP (A0216) secondary antibodies were bought from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China).

Insulin (91077C), indomethacin (I7378), dexamethasone (D4902), PA (P4060), 3-isobutyl-1-methylxanthine (I5878), triiodothyronine (T3) (I2877), DMSO (D2650), Polybrene (H9268) and Oil-Red O (O0625) were purchased from Sigma (St Louis, MO, USA). DMEM (11960-044) and PierceTM ECL Western Blotting Substrate (#32109) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). GW6471 (4618) and SR59230A (1511) were purchased from Tocris Bioscience (Avonmouth, Bristol, UK). Mito Stress Test Kit (103015-100) was purchased from Agilent Technologies (Wilmington, USA).

2.2 Cell culture and induction of adipogenesis

3T3-L1 and C3H10T1/2 cell lines were purchased from China Infrastructure of Cell Line Resource (Beijing, China). The cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (called basic medium) in a humidified atmosphere containing 5% CO₂ at 37 °C. For inducing beige/brown adipogenesis, confluent cells were cultured in the basic medium containing 1 μ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 5 μ g/mL insulin, 50 nM T3 and 125 μ M indomethacin (called differentiation medium) with or without PA for 2 days and then switched to the basic medium containing 5 μ g/mL insulin and 50 nM T3 with or without PA for 6 days. The medium was changed every other day. To induce adipogenic commitment, C3H10T1/2 cells were pretreated with 50 ng/mL BMP7 [10, 11] or PA until the cells became confluent. The confluent cells were cultured in the differentiation medium for 2 days and then switched to the basic medium including 5 μ g/mL insulin and 50 nM T3 for 6 days. The medium was changed every other day. Unless otherwise specified, PA was used at 50 μ M to treat cells. To inhibit PPAR α , 10 μ M GW6471, a PPAR α inhibitor, was used. To inhibit β 3-AR, 10 μ M β 3-AR antagonist SR59230A was used.

2.3 Isolation and differentiation of primary brown adipocytes

Isolation of primary brown preadipocytes were performed as described previously [32]. Briefly, the interscapular brown adipose tissue was dissected from mice, minced, and digested for 20 min at 37°C in the digestion medium (1.5 U/mL Collagenase D, 2.4 U/mL Dispase II and 10 mM CaCl₂). Digested tissue was filtered through a 70 μ m cell strainer and the cell suspension was then centrifuged for 10 min at 700 g to pellet cells. Primary brown preadipocytes were resuspended in complete culture medium containing DMEM/F12, Pen/Strep and 10% FBS, and then plated onto culture dishes. The differentiation of primary brown adipocytes were performed as mentioned above.

2.4 Oil-Red O staining

Oil-Red O staining was conducted as previously described [33, 34]. Briefly, differentiated cells were fixed with 4% paraformaldehyde (PFA) and stained with 0.15% Oil-Red O dye. The stained Oil-Red O was then extracted with isopropanol and the absorbance was spectrophotometrically measured at 530 nm.

2.5 Quantitative real-time PCR

Total RNA was extracted from cells using RNeasy pure Cell Kit (Qiagen biotech, Beijing, China) according to the manufacturer's protocol and cDNA was synthesized from 1–2 µg of total RNA using M-MLV Reverse Transcriptase (Promega, Fitchburg, WI, USA). The cDNAs were used for quantitative real-time PCR analysis with SYBR™ Select Master Mix (Applied Biosystems™, Waltham, MA, USA). The relative expression level was calculated after normalization to *β-actin* reference using 2^{-CT} method. Primer sequences are shown in Table 1.

2.6 Western blotting

Cells were lysed in the cell lysis buffer (Beyotime, Haimen, Jiangsu, China) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) on ice for 20 min and the supernatant was collected after centrifugation at 10000 g for 15 min. The protein concentration was measured using the BCA Protein Assay Kit (Beyotime, Haimen, Jiangsu, China). The samples were boiled at 100 °C for 10 min to denature the protein. The proteins were subjected to SDS-polyacrylamide gel and transferred to PVDF membranes (Millipore, Billerica, MA, USA). These membranes were in 5% bovine serum albumin for blocking and incubated overnight at 4 °C with primary antibodies. Secondary antibodies were then incubated for 1 h at room temperature. Protein bands were detected by SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Waltham, MA, USA) and images were captured by Amersham Imager 600 imaging system (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Gray analysis was performed using image J software and the protein band density was normalized to the *β-actin* expression.

2.7 Mitochondrial DNA (mtDNA) Measurement

Mt DNA measurement was conducted as previously described [35, 36]. Genomic DNA was isolated with TIANamp Genomic DNA Kit (Tiangen biotech, Beijing, China) according to the manufacturer's protocol and then used for quantitative real-time PCR analysis with SYBR™ Select Master Mix (Applied Biosystems™, Waltham, MA, USA). The mtDNA was amplified using primers specific for the mitochondrial *COX2* gene and normalized to genomic DNA by amplification of the *β-globin* nuclear gene. Primer sequences are shown in Table 1.

2.8 Oxygen consumption rate measurements

3T3-L1 cells were plated in an XF24-well microplate (Seahorse Bioscience) and differentiated into beige adipocytes. Oxygen consumption rate (OCR) was measured using an XF24 analyser (Seahorse Bioscience) according to the manufacturer's instructions. 1 µM

oligomycin, 1 μ M FCCP and 0.5 μ M rotenone/antimycin were injected to detect ATP production, maximal respiration and non-mitochondrial respiration, respectively.

2.9 PPAR α gene silencing in 3T3-L1 cells and C3H10T1/2 cells

To knockdown PPAR α , shRNA and control scramble lentivirus were designed and synthesized by Genomeditech Company. For lentivirus production, 293T cells were transfected with the vector using Lipofectamine 3000 (Invitrogen, Waltham, MA, USA) and the conditioned medium was centrifuged at 4,500g for 20 min 2 days later. The supernatant was used to infect 3T3-L1 and C3H10T1/2 cells in the presence of 8 μ g/mL polybrene. Infected cells were selected 2 days later using 2 μ g/mL puromycin (Sigma, St Louis, MO, USA). The sequences used for shRNA-mediated experiments were: sh Ppara No.1, 5'-GGAAGCCGTTCTGTGACATCA-3'; sh Ppara No.2, 5'-GCTAAAGTACGGTGTGTATGA-3'.

2.10 Statistical Analyses

The data were generated from three independent experiments and three parallels were used in each experiment. All data were reported as means $\pm$ standard deviations (SD). Differences between groups were calculated using the Student's t test or one-way ANOVA (for multiple comparison). $P < 0.05$ was considered to be statistically significant.

3 Results

3.1 PA promotes beige adipogenesis of 3T3-L1 preadipocytes, but not in C3H10T1/2 progenitor cells.

To determine the optimal concentration of PA in inducing beige adipogenesis, 3T3-L1 cells were treated with PA at various concentrations including 10 μ M, 50 μ M and 100 μ M. 100 μ M PA caused toxicity to cells and 50 μ M PA was used for subsequent experiments for its strong effect on beige adipogenesis based on Oil-Red O staining. PA treatment (Fig. 1A) increased the density of multilocular lipid droplets during 3T3-L1 beige adipogenesis (Fig. 1B and 1C). Consistently, PA promoted protein contents of adipogenic markers PPAR γ and FABP4 in 3T3-L1 cells (Fig. 1D and 1E). To explore the effect of PA on the commitment of C3H10T1/2, PA was used to treat cells for 2 days before the differentiation of progenitor cells into brown adipocytes (Fig. S1A). BMP7 profoundly stimulates brown adipogenesis [10]. Here, we compared the effects of PA with BMP7, and we found that BMP7 promoted brown adipogenic differentiation as shown by increased lipid droplets, while PA did not affect the lipid accumulation (Fig.S1B and S1C). We also tested the effect of PA on the differentiation of C3H10T1/2 into brown adipocytes (Fig. S1D). We found that PA did not enhance the lipid accumulation in this process either (Fig. S1E and S1F). These findings demonstrated that PA had no obvious effects on lipid accumulation in C3H10T1/2 mesenchymal progenitor cells under brown adipogenic induction. Taken together, these data suggest that PA stimulates the beige adipogenesis in committed preadipocytes but not in uncommitted progenitor cells.

3.2 PA promotes formation of beige adipocytes.

Furthermore, in 3T3-L1 cells, PA elevated the mRNA expression of brown adipocyte marker genes including *PGC1 α* , *Prdm16*, *Ucp1* and *Ppara* (Fig. 2A), and also markedly increased beige adipocyte markers such as *Fgf21*, *Cd137* and *Tbx1* (Fig. 2B). Consistently, PGC1 α , UCP1 and PPAR α protein contents in PA-treated cells were 2-fold higher than those of control cells (Fig. 2c and 2d). However, the protein content of PRDM16, a key brown adipogenic regulator [7], remained unchanged (Fig. 2C and 2D). These data demonstrate that PA has positive effects on promotion of beige adipogenic differentiation of committed preadipocytes. To test whether PA treatment could promote white differentiated adipocytes beiging, PA was used to culture differentiated adipocytes (Fig. 2E). Followed by PA treatment for 2 and 5 days respectively, PGC1 α protein expression was examined. The PGC1 α content was not significantly changed in the PA presence (Fig. 2F and 2G), indicating that PA hardly plays a role in the differentiated white adipocytes beiging, and it mainly functions in beige adipogenesis using committed but undifferentiated 3T3-L1 as the starting point.

Next, we determined whether PA could stimulate the browning of C3H10T1/2 progenitor cells. PA notably increased *PGC1 α* expression, but not other brown fat markers such as *Ucp1* and *Prdm16* or beige adipocyte markers *Fgf21*, *Cd137* and *Tbx1* (Fig. S2A and S2B). Similarly, PGC1 α protein content was elevated in PA-treated group while PRDM16 and UCP1 contents were not significantly changed (Fig. S2C and S2D). These data indicate that PA did not trigger the differentiation of progenitor cells to brown adipocyte lineage while it promoted the expression of PGC1 α that is a master regulator of mitochondrial biogenesis [37].

Because C3H10T1/2 cells are multipotent, to further examine the effect of PA on lineage differentiation of C3H10T1/2 cells, we tested marker gene expression of other lineages. Both 10 μ M and 50 μ M PA treatment did not alter white adipocyte markers including *Resitin*, *Psat1*, *Zfp423*, *Retnla* (Fig. S2E), nor chondrogenesis markers *Sox9*, *Col1a1*, *Col2a1* and myogenesis marker *MyoD* (Fig. S2F). Taken together, these results demonstrate that PA had no noticeable effects on other lineage differentiation of C3H10T1/2 cells to white adipocytes, myocytes and chondrocytes.

3.3 PA increases mitochondrial biogenesis and oxygen consumption.

Mitochondrial biogenesis is key for brown fat thermogenesis and browning of WAT [1]. Treatment with PA increased mtDNA content in both 3T3-L1 (Fig. 3A) and C3H10T1/2 (Fig. S3A) during brown adipogenic induction, suggesting that PA increased mitochondrial biogenesis. As a master regulator of mitochondrial biogenesis, PGC1 α was substantially elevated by PA treatment at both mRNA and protein levels (Fig. 2A, 2C, S2A and S2C). In agreement, mitochondrial transcription factors (i.e. *Tfam* and *Tfb2m*), nuclear respiratory factors (i.e. *Nrf1* and *Nrf2*) and components of the mitochondrial electron transport chain (i.e. *Uqcrc1*, *Sdhb*, *Cox7a* and *ATP5a1*) were also elevated in the PA treatment group (Fig. 3B and S3B). Additionally, using a Seahorse XF-24 extracellular flux analyzer, we observed that basal mitochondrial respiration and proton leak significantly increased in PA-treated

beige adipocytes (Fig. 3C and 3D), indicating that PA could promote the oxygen consumption and uncoupled respiration during beige adipogenesis.

Taken together, these data demonstrated that PA promoted mitochondrial biogenesis and oxygen consumption during beige adipogenesis.

3.4 PA stimulates the phosphorylation of AMPK.

AMPK, an energy sensor of cells, is crucially involved in energy metabolism and is highly expressed during browning [13, 35, 38]. To understand whether AMPK was involved in the PA-mediated browning process, we tested the effect of PA on the phosphorylation of AMPK α (p-AMPK). PA increased the AMPK α phosphorylation in differentiated 3T3-L1 and C3H10T1/2 cells without obvious effects on total AMPK α content (Fig. 4 and S4). Moreover, the ratio of p-AMPK to AMPK was upregulated in the PA-treated group (Fig. 4 and S4). These data suggested that the AMPK α signaling pathway was activated in response to PA.

3.5 PPAR α antagonist eliminates browning effects of PA.

Given the previous findings that PPAR α has a positive role in the browning process [28, 29] and PA was a natural PPAR α ligand [31, 39], we hypothesized that PPAR α was required for PA-mediated beige adipocyte formation. PPAR α antagonist GW6471 was used to inhibit PPAR α . Remarkably, GW6471 abolished the lipid accumulation in both 3T3-L1 (Fig. 5A and 5B) and C3H10T1/2 cells (Fig. S5A and S5B), suggesting that GW6471 inhibited beige and brown adipocyte differentiation. Besides, compared with PA-treated group, brown adipocytes and mitochondrial biogenesis marker proteins (UCP1 and PGC1 α) were down-regulated in the presence of GW6471 (Fig. 5C, 5D, S5C and S5D). The master brown adipogenic regulator PRDM16 remained unchanged (Fig. 5C, 5D, S5C and S5D). Taken together, these results indicate that PPAR α inhibition eliminates PA-mediated browning effects in both committed preadipocytes and uncommitted progenitor cells.

The elevated phosphorylation of AMPK α was also reduced while total AMPK was not changed by GW6471 (Fig. 5E, 5F, S5E and S5F), suggesting that PA enhances the phosphorylation of AMPK α during the browning process, at least in part, via PPAR α .

3.6 Knockdown of PPAR α abolishes PA- mediated brown and beige adipogenesis.

To exclude the possible nonspecific effects of inhibitor, we further knocked down *Ppara* gene to confirm the roles of PPAR α in PA-mediated beige adipogenesis. We introduced a short hairpin (sh) RNA for PPAR α into 3T3-L1 cells, which effectively knocked down PPAR α protein levels (Fig. 6A and 6B). Following *Ppara* knockdown in 3T3-L1 cells, the enhanced lipid accumulation by PA treatment was largely abolished (Fig. 6C and 6D). The upregulation of UCP1 and PGC1 α protein contents in PA-treated group were profoundly decreased (Fig. 6E, 6F, S6A and S6B), while the effect of *Ppara* knockdown on the PRDM16 protein expression was not significant (Fig. 6E and 6F). PPAR γ content was also decreased in the PA presence (Fig. 6G, 6H, S6C and S6D). These data indicate that PPAR α is required for brown-adipocyte specific marker expression in the PA-mediated browning process. Additionally, the elevated phosphorylation of AMPK α was eliminated while total

AMPK was not significantly changed (Fig. 6I and 6J), indicating that PA enhances the phosphorylation of AMPK α through activation of PPAR α during beige adipogenesis.

To further test PPAR α effects on PA-mediated brown adipocyte differentiation, we also introduced shRNA for PPAR α into C3H10T1/2 cell line (Fig. S6E). In knockdown cells, lipid accumulation was not significantly changed (Fig. S6F and S6G), while elevation of PGC1 α by PA treatment was decreased (Fig. S6H and S6I). The data suggest that PPAR α is necessary for PGC1 α expression in the PA-mediated mitochondriogenesis from C3H10T1/2 progenitor cells. Taken together, PPAR α is a key factor during PA-mediated beige adipogenesis, primarily through enhancing PGC1 α expression and mitochondriogenesis.

3.7 PA-mediated beige adipogenesis is independent on β 3-adrenergic receptor.

To explore whether PA-mediated beiging is through β 3-adrenergic receptor (β 3-AR) independent mechanism, the β 3-AR antagonist SR59230A was used for PA-stimulated beige adipocyte differentiation. Through Oil-Red O staining, we found that with the addition of SR59230A, lipid droplets were slightly reduced compared with that in PA-treated cells. However, the lipid droplets were still significantly higher compared with the control group (Fig. 7A and 7B). Additionally, in the PA treatment group, adipocyte markers PPAR γ and FABP4 protein contents were both higher regardless of the presence of SR59230A or not (Fig. 7C and 7D). The upregulation of UCP1, PGC1 α and PPAR α protein contents in PA-treated group were not downregulated with the addition of SR59230A (Fig. 7E and 7F). Similarly, PA-mediated PGC1 α upregulation in the differentiation of primary brown adipocytes did not significantly change in the presence of SR59230A (Fig. 7G and 7H).

Taken together, β 3-AR antagonist SR59230A did not abolish the PA-promoted browning of 3T3-L1 cells and primary brown adipocytes. PA-stimulated beige adipogenesis is independent on β 3-AR.

4 Discussion

Due to their ability to effectively burn lipids for thermogenesis, enhancing brown and beige adipogenesis is a promising method to treat obesity and associated metabolic diseases [40, 41]. PA is a bioactive component of milk and dairy products, marine foods and meat from ruminant animals [20]. It has been reported to increase brown adipogenesis in HIB-1B cells and primary brown preadipocytes, but mechanisms remain undefined [24, 25]. Brown/beige adipogenesis can be separated into the commitment of progenitor cells into preadipocytes, and the further differentiation of preadipocytes into mature brown/beige adipocytes. The major difference between white and beige adipocytes is the expression of UCP1. New beige adipocytes are induced from PDGFR α ⁺ progenitor cells through a two-step process, adipogenic commitment and differentiation [42]. Beige adipogenesis is initiated by PRDM16, which induces the expression of brown/beige-specific genes, including UCP1, while white adipocytes is initiated by the expression of Zinc Finger protein (ZFP) 423 [4, 6]. To our knowledge, the effect of PA on beige adipogenic commitment and differentiation has not been systemically explored. In this study, we found that PA promoted multilocular lipid accumulation and gene expression of beige (i.e. *Fgf21*, *Tbx1* and *Cd137*) and brown (i.e. *Ucp1*, *PGC1 α* and *Ppara*) fat cell markers. In addition, protein expression levels of PGC1 α ,

UCP1 and PPAR α in beige adipocytes, which are known as the brown adipocytes-selective markers, were elevated by PA treatment. These findings strongly support that PA promotes the formation of beige adipocytes.

Interestingly, the master brown adipocyte regulator PRDM16 were not increased in the protein level following PA stimulation, suggesting that PA might not have significant effects on the early stage of brown adipogenesis. Consistently, we found that, in uncommitted C3H10T1/2 progenitor cells, PA did not increase lipid accumulation and UCP1 and PRDM16 protein expression levels, nor the expression of other brown adipocyte-selective markers (i.e. *Ucp1*, *PGC1 α* and *Prdm16*), white adipocyte markers (i.e. *Resitin*, *Psat1*, *Zfp423* and *Retnla*) and markers of chondrogenesis and myogenesis (i.e. *Sox9*, *Col1a1*, *Col2a1* and *MyoD*). In combination, these data show that PA positively affects the beige adipogenic differentiation but not the commitment of progenitor cells.

Given our observation that PA increased the mRNA and protein levels of PGC1 α , which is a key regulator of mitochondrial biogenesis regulator, we further analyzed mitochondriogenesis which was promoted by PA treatment. Consistently, AMPK was also activated, which is in agreement with the effect of phytol in the browning process [19]. This phenomenon suggests that phytol and its metabolite PA may share signal pathways to promote beige adipocyte differentiation.

It has been reported that PA was a natural ligand of PPAR α in the human diet [31, 39]. Thus, the mediatory role of PPAR α was further tested by using antagonist and knockdown. Inhibition of PPAR α decreased the promotion effects of PA in the browning process, demonstrating that PA requires PPAR α to promote beige adipocyte differentiation. Consistently, in the mouse model, a phytol-enriched diet elevated PA levels and activated PPAR α in the liver and BAT, which increase thermogenesis [26]. Our results were consistent with these findings, and we further discovered that PA promotes beige adipocyte differentiation but not commitment. In addition, we established the mediatory role of PPAR α through both using antagonist and also gene knockdown.

Our study also indicated that as a PPAR α ligand, PA promoted the phosphorylation of AMPK α possibly via PPAR α , which is similar to the previous reports that PPAR α activators such as fenofibrate and WY14643 could activate AMPK signaling pathway [43, 44]. AMPK plays an important role in regulating energy homeostasis in cells [27]. AMPK activation impedes hepatic lipogenesis and adipocyte differentiation and promotes glucose uptake in muscle [45, 46]. Therefore, PPAR α agonists including PA may activate AMPK to exert beneficial effects on the prevention of metabolic syndrome.

Activation of β 3-AR has been shown to have profound effects on browning [47, 48]. However, we found that PA maintained the ability to stimulate the browning of 3T3-L1 cells and primary brown adipocytes in the presence of β 3-AR antagonist, SR59230A, demonstrating that PA-mediated browning is independent on β 3-AR.

In conclusion, our data revealed that PA mainly boosts beige adipogenesis via stimulation of PPAR α pathway, and it functions primarily to enhance beige/brown adipogenic differentiation not the commitment. Moreover, the effects of PA on beige adipogenesis is

independent on $\beta 3$ -AR. Compared to these chemosynthesis drugs [49], PA, a natural agent, has low toxicity and is safer for the promotion of beige adipogenesis. In our study, we used 50 μ M PA for testing, which represents the high end of physiological PA level and is consistent the previous report [5]. The concentration of PA in the plasma of healthy human varies from 0.04–9.88 μ M [3], whereas, in Refsum disease, patient levels have been reported to be 240–1400 μ M [4]. PA is readily available through food (i.e. milk and dairy products, beef and fatty fish), which is in contrast to synthetic agonists of PPAR α such as fibrates (i.e. clofibrate, fenofibrate and bezafibrate), available only as dietary supplements [20, 39]. Because of the wide availability of PA in diets, increasing PA intake provides an effective method for enhancing beige adipocyte differentiation, thus preventing or reducing obesity and its associated metabolic dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 31371753), and the Beijing Dairy Industry Innovation Team (BAIC06–2018) to XM, and NIH R01HD067449 to MD.

Abbreviations:

AMPK	AMP-activated protein kinase
BAT	Brown adipose tissue
PA	Phytanic acid
PPARα	Peroxisome proliferator-activated receptor α
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator 1 α
UCP1	Uncoupling protein 1
WAT	White adipose tissue

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Figure 1. Effects of PA on the lipid accumulation and the expression of adipogenic markers in differentiated 3T3-L1 cells.

3T3-L1 preadipocytes were induced into beige adipocytes through the treatment of PA for 8 days. (A) Confluent 3T3-L1 cells were cultured in the differentiation medium with or without PA for 2 days and then switched to the basic medium including insulin and T3 with or without PA for 6 days. (B) Oil-Red O staining of differentiated 3T3-L1 white adipocytes. Bar, 100 μ m. (C) The absorbance of the stained Oil-Red O at 530 nm. (D) Western blotting analysis of adipogenic markers FABP4 and PPAR γ in the differentiated 3T3-L1 white adipocytes. β -actin was used as the control. (E) Relative quantitative analysis of protein bands. Data are shown as means $\pm$ SD of three independent experiments. **p 0.01 versus control; ***p 0.001 versus control.

Figure 2. PA promotes beige adipocyte biogenesis.

3T3-L1 preadipocytes were induced into beige adipocytes through the treatment of PA for 8 days. (A and B) Quantitative real-time PCR analysis of brown (A) and beige (B) adipocyte marker genes in beige adipocytes from 3T3-L1 white adipocytes. Data are shown as means $\pm$ SD of three independent experiments. (C) Western blotting analysis of brown adipogenic markers in beige adipocytes. β -actin was used as the control. (D) Relative quantitative analysis of protein bands. (E) Confluent 3T3-L1 cells were cultured in the differentiation medium for 2 days, switched to the basic medium including insulin and T3 for 4 days and then changed to the medium (DMEM and 10% FBS) with or without PA for 2 days and 5 days, respectively. (F and G) Western blotting analysis of PGC1 α content and relative quantitative analysis of protein bands. Data are shown as means $\pm$ SD of three independent experiments. *p 0.05 versus control; **p 0.01 versus control; ***p 0.001 versus control; ns indicates no significant differences between PA treatment and the control group.

Figure 3. PA stimulates mitochondrial biogenesis.

3T3-L1 preadipocytes were induced into beige adipocytes through the treatment of PA for 8 days. (A) Mitochondrial DNA content analyzed by quantitative real-time PCR in differentiated 3T3-L1 white adipocytes treated with PA. Relative expression values were normalized to control cells. (B) Quantitative real-time PCR analysis of mitochondrial marker genes in beige adipocytes. Data are shown as means $\pm$ SD of three independent experiments. (C and D) The oxygen consumption rate (OCR) in differentiated beige adipocytes from 3T3-L1 cells ($n=3$). The OCR was measured before and after injection of 1 μ M oligomycin (the ATP synthase inhibitor), 1 μ M FCCP (the uncoupling agent) and 0.5 μ M Antimycin A/rotenone (the inhibitor of complex III and complex I). Basal respiration was measured before addition of oligomycin. Proton leak (uncoupled respiration) and ATP production were measured after addition of oligomycin. Maximal respiration was measured after

addition of FCCP. *p 0.05 versus control; ***p 0.001 versus control; ns indicates no significant differences.

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Figure 4. PA has a positive effect on the phosphorylation of AMPK.

3T3-L1 preadipocytes were induced into beige adipocytes through the treatment of PA for 8 days. (A) Western blotting analysis of p-AMPK and AMPK in beige adipocytes. β -actin was used as the control. (B) Relative quantitative analysis of protein bands. Data are shown as means $\pm$ SD of three independent experiments. *p < 0.05 versus control.

Figure 5. PPARα antagonism inhibits PA-stimulated browning of white adipocytes.

3T3-L1 preadipocytes were induced into beige adipocytes through the treatment of PA or GW6471 for 8 days. (A) The PPARα antagonist GW6471 inhibits PA-mediated browning effects. Oil-Red O staining of differentiated 3T3-L1 white adipocytes. Bar, 100 μm. (B) The absorbance of the stained Oil-Red O at 530 nm. (C and E) Western blotting analysis of PGC1α, UCP1, PPARα, PRDM16 (C) and p-AMPK/AMPK (E) in beige adipocytes. β-actin was used as the control. (D and F) Relative quantitative analysis of protein bands. Data are shown as means ± SD of three independent experiments. *p 0.05 versus control; **p 0.01 versus control; ## p 0.01 versus PA treatment; ### p 0.001 versus PA treatment.

Figure 6. PPARα is necessary for the effect of PA on beige adipocyte biogenesis.

3T3-L1 preadipocytes with knockdown of PPARα were induced into beige adipocytes through the treatment of PA for 8 days. (A) The target sequences used for shRNA-mediated experiments. (B) Quantitative real-time PCR analysis of *Ppara* (left) and western blot analysis of PPARα (right) in shRNA control and shRNA PPARα 3T3-L1 cells. (C) Oil-Red O staining of differentiated 3T3-L1 cells. Bar, 100 μm. (D) The absorbance of the stained Oil-Red O at 530 nm. (E, G and I) Western blotting analysis of brown adipogenic markers (E), PPARγ (G), AMPK and p-AMPK (I) contents in the indicated cells. β-actin was used as the control. (F, H and J) Relative quantitative analysis of protein bands. *p 0.05 versus control; **p 0.01 versus control; ns indicates no significant differences between PA treatment and the control group.

Figure 7. PA-stimulated browning of white adipocytes is independent on $\beta 3$ -AR.

3T3-L1 preadipocytes were induced into beige adipocytes through the treatment of PA and SR59230A for 8 days. (A) Oil-Red O staining of differentiated 3T3-L1 white adipocytes. Bar, 100 μm. (B) The absorbance of the stained Oil-Red O at 530 nm. (C and E) Western blotting analysis of adipocyte markers (C) and brown adipocyte markers (E) in beige adipocytes. β -actin was used as the control. (D and F) Relative quantitative analysis of protein bands. Data are shown as means $\pm$ SD of three independent experiments. (G and H) Western blotting analysis of PGC1 α during differentiation of primary brown adipocytes (G) and relative quantitative analysis of protein bands (H). Data are shown as means $\pm$ SD of three independent experiments. *p 0.05 versus control; **p 0.01 versus control; ***p 0.001 versus control; # p 0.05 versus PA treatment; ## p 0.01 versus PA treatment; ns indicates no significant differences.

Table 1

The primer sequences

Gene	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')
β -actin	GATCTGGCACCACACCTTCT	GGGGTGTGAAGGTCTCAA
PGC1 α	ATGTGTCGCCTTCTTGCTCTTC	GGACCTTGATCTTGACCTGGAATATGG
Prdm16	CAGCAACCTCCAGCGTCACATC	GCGAAGGTCTTGCCACAGTCAG
Ucp1	ACTCAGGATTGGCCTCTACGACTC	GCATTCTGACCTTCACGACCTCTG
Ppara	CTTCACGATGCTGCTCCTTGATG	GATGTCACAGAACGGCTTCCTCAG
Cidea	GCCGTGTTAAGGAATCTGCTGAGG	GGATGGCTGCTCTTCTGTATCGC
Tbx1	GGCAGACGAATGTTCCCCAC	CATCCACTGTGCGCCCTTAG
Cd137	TGGTCATTGTGCTGCTGCTAGTG	CAGTTCGGCTGTCCACCTATGC
Ffg21	CTGCTGCTGGCTGTCTCTCTG	GTGTCTTGGTCGTCATCTGTGTAGAG
Resistin	TCCTGTGGCTCTGCCTGTGG	GCTGCTGTCCAGTCTATCCTTGC
Retnla	ACTTCTTGCCAATCCAGCTAACTATCC	GTCCAGTCAACGAGTAAGCACAGG
Psat1	CTTCCCCTGCTGTCGCCTTA	AGCACACTGATGCCGAGTCC
Zfp423	GAGAGTGCTGAGGACCTGGAGAG	GGTTGGCGACGTGGATCTGAATC
Tfam	CCATGCAAGGCTTTTCTCTCAG	TGGGATTGTCAGCTCAAAGT
Cox7a	CGTGTGGCAGAGAAGCAGAA	GCCCAGCCCAAGCAGTATAA
Sox9	GAGTGTCCTTAGCCTTCCT	CCAAGAGCCTGGGGAATTAATCA
MyoD [40]	CGCCACTCCGGGACATAG	GAAGTCGTCTGCTGTCTCAAAGG
Col1a1 [17]	GCATGGCCAAGAAGACATCC	CCTCGGGTTCCACGTCTC
Col2a1 [17]	CCACACCAAATTCCTGTTCA	ACTGGTAAGTGGGGCAAGAC
Nrf1 [16]	AGCACGGAGTGACCCAAAC	TGTACGTGGCTACATGGACCT
Nrf2 [35]	TGAAGTTCGCATTTTGATGGC	CTTTGGTCCTGGCATCTCTAC
Tfb1m [35]	ATAGAGCCCAAGATCAAGCAG	TGTAACAGCCTTCCAGTGC
Uqerc1 [35]	ATCAAGGCACTGTCCAAGG	TCATTTTCCTGCATCTCCCG
Sdhb [35]	ACCCCTTCTCTGTCTACCG	AATGCTCGCTTCTCCTTGATG
ATP5a1 [35]	CATTGGTGATGGTATTGCGC	TCCCAAACACGACAACCTCC
COX2 [35]	ATAACCGAGTCGTTCTGCCAAT	TTTCAGAGCATTGGCCATAGAA
β -globin [36]	GAAGCGATTCTAGGGAGCAG	GGAGCAGCGATTCTGAGTAGA