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Acetyl-keto-β-boswellic acid induces lipolysis in mature adipocytes

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ABSTRACT

Recently, it was reported that naturally occurring pentacyclic triterpenoids such as ursolic acid have anti-adiposity property. We studied if acetyl-keto-β-boswellic acid (AKBA), an established anti-inflammation and anti-cancer pentacyclic triterpenoid which has similar chemical structure to ursolic acid, may modulate adipocyte phenotype. 3T3-L1 murine adipocytes and human subcutaneous adipocytes were treated with AKBA in different concentrations in vitro. AKBA triggered significant lipolysis in 3T3-L1 adipocytes as shown by reduced neutral lipids in cytosol and increased free fatty acids in culture medium. Increased lipolysis by AKBA was accompanied by up-regulation of lipolytic enzymes, adipocyte triglyceride lipase (ATGL) and hormone sensitive lipase (HSL), and a decreased expression of lipid droplet stability regulator perilipin. In addition, AKBA treatment reduced phenotypic markers of mature adipocyte aP2, adiponectin and glut-4 in mature adipocytes. Further studies revealed that AKBA down-regulated PPAR-γ and C/EBP-α expression in a dose and temporal dependent manner in mature adipocytes. In human adipocytes, AKBA likewise mobilized lipolysis accompanied by down-regulation of PPAR-γ2 expression and loss of phenotypic markers of mature adipocytes.

1. Introduction

Boswellic acids, a group of pentacyclic triterpene molecules, has been used as anti-tumor and anti-inflammation supplements for the treatment of inflammatory bowel disease, arthritis, psoriasis, asthma and many types of cancers for years [1–3]. Previous clinical trials indicated that boswellic acids had lower toxicity and were well tolerated in humans [4].

The anti-obesity property of pentacyclic triterpenoid draws increasing attention in recent years. For example, betulinic acid, a natural pentacyclic triterpenoid, showed anti-obesity effect in animal models [5]. Recently, both Rao et al. and Kunkel et al. reported that ursolic acid, another pentacyclic triterpenoid rich in the wax of apple peel, also reduced fat weight and prevented high fat diet- induced obesity in mice [6,7]. These studies prompted us to hypothesize that pentacyclic triterpenoid extracted from plants may modulate adipocyte phenotype directly or indirectly. In this work, we found that acetyl-keto-β-boswellic acid (AKBA), a naturally occurring, orally active pentacyclic triterpenoid compound, mobilized lipolysis in both mouse and human adipocytes.

2. Materials and methods

2.1. Materials and cell culture

Acetyl-keto-β-boswellic acid was from ChromaDax Incorporation (reagent grade, purity >90%, Santa Ana, CA). Oil Red O, Dulbecco's Modified Eagle Medium (DMEM), isobutylmethylxanthine (IBMX), insulin and dexamethasone were purchased from Sigma Aldrich (St Louis, MO). Glut-4, aP2, adiponectin, adipocyte triglyceride lipase (ATGL), hormone sensitive lipase (HSL), perilipin, PPARγ and actin antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). C/EBP-α antibody was purchased from Cell Signaling (Danvers, MA). High capacity reverse transcriptase and SYBR green-based quantitative RT-PCR master mix were from Applied Biosystems (Grand Island, NY). 3T3-L1 preadipocytes were obtained from ATCC (Rockville, MD) and maintained in DMEM medium supplemented with 10% bovine serum, 1 mM sodium pyruvate, 100 u/ml penicillin and streptomycin.

Abbreviations: AKBA, acetyl-keto-β-boswellic acid; C/EBP, CCAAT/enhancer-binding proteins; PPAR-γ, peroxisome proliferator-activated receptor γ; IBMX, isobutylmethylxanthine; ATGL, adipocyte triglyceride lipase; HSL, hormone sensitive lipase.

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2.2. Isolation of human preadipocyte from subcutaneous adipose tissue

To isolate preadipocytes from vascular- stromal compartment of human adipose tissue, about 1 g of human subcutaneous fat tissue was obtained from patients undergoing bariatric surgery. Adipose tissue was minced into pieces about 2 mm in diameter and digested with type I collagenase for 1 h. After filtering through 250 μm nylon mesh, preadipocytes were separated from mature adipocytes by centrifuge. Preadipocytes in the pellet fraction would be washed three times by Krebs-Ringer bicarbonate buffer and propagated in gelatin-coated cell culture dish in PromoCell medium (Heidelberg, Germany) supplemented with 20% fetal bovine serum (FBS). The protocol was approved by our Institutional Domain Review Committee and written consent has been obtained from each patient.

2.3. Induction of differentiation in 3T3-L1 and human preadipocytes

To induce differentiation, 3T3-L1 preadipocytes were plated in DMEM medium supplemented with 10% FBS. Two days after confluency (day 0), cells were stimulated with differentiation induction medium which contained 0.5 mM isobutylmethylxanthine (IBMX), 167 nM insulin and 1 μM dexamethasone for two days (day 2), followed by incubation with insulin medium (DMEM supplemented with 167 nM insulin) for another 2 days (day 4). Cells were then maintained in DMEM medium supplemented with 10% FBS for another two days (day 6). By day 6, 90% of the preadipocytes would be differentiated into mature adipocytes characterized by accumulation of lipid droplets in cytosol.

To induce differentiation in human preadipocytes, cells were plated at the density of $3 \times 10^4/cm^2$ on gelatin-coated dish and cultured in complete PromoCell medium for 2 to 3 days. Differentiation was then induced by adipogenic DMEM medium which contained 10% FBS, 1 μM dexamethasone, 0.5 mM IBMX and 200 μM indomethacin for 8–12 days [8]. At day 10 of differentiation induction, lipids droplets were obviously accumulated in cell cytosol.

2.4. Oil red O staining

Adipocytes were fixed by 10% formalin for 1 h and permeabilized by 60% isopropanol for 5 min. Fixed cells were stained with Oil red O solution in 20% isopropanol for 1 h, followed by washing with H_2O for 4 times. To quantify the abundance of neutral lipids after image capturing, Oil red O was eluted from the cells by 100% isopropanol and absorbance of optical density was read at 500 nm wave length.

2.5. Assay of free fatty acids in culture medium

Free fatty acids released into culture medium by adipocytes were assayed by an enzyme based method. Fatty acids were converted to their CoA derivatives and were subsequently oxidized with concomitant generation of color. The assay was performed as instructed by the manufacturer (BioVision, Mountain View, CA). Palmitic acid was used as a standard.

2.6. Quantitative RT-PCR

Total RNA was extracted by RNeasy kit from Qiagen (Valencia, CA). First strand cDNA was transcribed by high capacity reverse transcriptase and cDNA of target genes was amplified by SYBR green-based PCR master mix from Applied Biosystems (Grand Island, NY). Primers used for amplification of target genes will be provided upon request. Expression of β-actin mRNA was used as a housekeeping control. The relative abundances of mRNA expression were calculated by $2^{-\Delta\Delta Ct}$ method.

2.7. Immunoblot

Cells were lysed by RIPA buffer (Tris 50 mM, pH 7.0, NaCl 150 mM, SDS 0.1%, sodium deoxycholate 0.5% and NP-40 1%) supplemented with proteinase inhibitor cocktail (Roche). Electrophoresis was performed using 12% or 15% SDS/polyacrylamide gel and 40 μg proteins were loaded in each lane. Proteins were transferred to nitrocellulose membrane by electro-blotting. After blocking with 5% non-fat milk in PBS-T buffer for 1 h, membranes were incubated with primary antibodies of different concentrations overnight at 4 °C, followed by incubation with secondary antibodies conjugated to horseradish peroxidase for 1 h at room temperature. Chemiluminescence was captured by ChemiDoc XRS system (Bio-Rad, Hercules, CA) after adding chemiluminescent substrate. To control for loading variations, β-actin was used as a housekeeping control.

2.8. Statistical analysis

Data were expressed as mean ± SD. Student *t* test was used to compare means of two independent groups. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. AKBA mobilized lipolysis in 3T3-L1 adipocytes

At the end of differentiation induction, mature 3T3-L1 adipocytes were treated with 30 μM AKBA for 48 h and triglycerides in lipid droplets were assayed by Oil red O staining. As shown in Fig. 1A and B, content of triglycerides in adipocytes was decreased more than 50% after treatment with AKBA compared with vehicle-treated controls. Consistently, free fatty acids in cell culture medium were increased 9-fold, indicating that AKBA treatment significantly increased lipolysis in 3T3-L1 adipocytes (Fig. 1C). AKBA at the dosage used in the current study did not induce obvious cytotoxicity in non-proliferative quiescent mature adipocytes. Assay of caspase-3 cleavage by immunoblots suggested that AKBA at the dose of 40 μM did not induce obvious apoptosis in 3T3-L1 adipocytes (Supplementary Fig. 1).

3.2. Lipolysis triggered by AKBA was accompanied by increased lipolytic enzymes and a decreased perilipin expression

Hydrolysis of triglycerides in lipid droplets in adipocytes was determined by both lipid droplets stability regulators and lipolytic enzymes [9,10]. ATGL and HSL account for 90% of neutral lipids lipolysis in adipocytes [11,12]. Expression of HSL increased mildly in a dose-dependent manner in AKBA-treated adipocytes and changes of ATGL showed similar trend (Fig. 2A). The significantly increased lipolysis induced by AKBA in adipocytes looked seemingly inconsistent with the mild increase of ATGL and HSL enzyme expression. We studied if AKBA reduced the integrity of lipid droplets and increased the access and actions of lipolytic enzymes. As expected, expression of perilipin, one of the most important regulators of lipid droplet stability, was down-regulated by about 50% in 3T3-L1 adipocytes treated by 20 μM and 40 μM AKBA for 24 h (Fig. 2B).

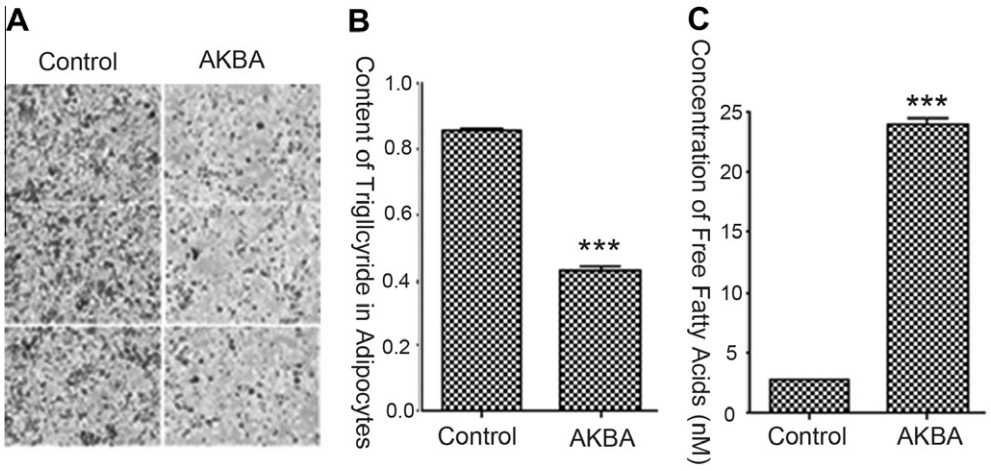

Fig. 1. AKBA induced lipolysis in 3T3-L1 adipocytes. 3T3-L1 preadipocytes were induced to mature adipocytes as described. At the end of differentiation induction, cells were incubated with fresh culture medium containing 10% FBS and 30 μM AKBA for 48 h. (A) Content of triglycerides as stained by Oil Red O; (B) staining of Oil Red O was also eluted by isopropanol and quantified by spectrophotometer; (C) changes of concentrations of free fatty acids in culture medium after treatment by AKBA; $^{***}p < 0.001$ compared to untreated control.

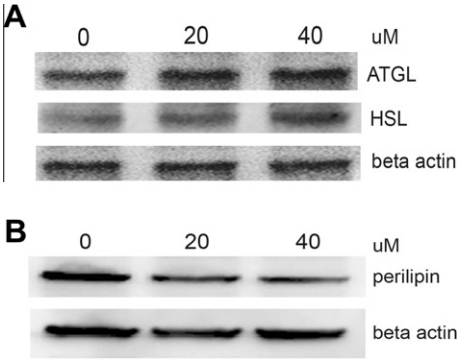

Fig. 2. Changes of ATGL, HSL and perilipin in adipocytes treated by AKBA. Mature 3T3-L1 adipocytes were treated by 0, 20 and 40 μM AKBA for 24 h. Expression of ATGL, HSL (A) and perilipin (B) was assayed by immunoblot (representative of 3 independent experiments). Beta actin was used as a housekeeping control.

3.3. AKBA- induced lipolysis was accompanied by loss of phenotypic markers of mature adipocytes and deceased PPAR-γ and C/EBP-α expression

Next, we asked if AKBA modulated phenotype of mature adipocytes. We analyzed expressions of three phenotypic markers of mature adipocyte, aP2, adiponectin and glut-4 in 3T3-L1 adipocytes treated by AKBA. As shown in Fig. 3A, expressions of adiponectin, aP2 and glut-4 were down-regulated in a dose-dependent manner in AKBA-treated adipocytes as assayed by immunoblots. These data suggested a loss of terminal differentiation in 3T3-L1 adipocytes. Next, we analyzed the expression of PPAR-γ and C/EBP-α, two master transcription factors which determine terminal differentiation in adipocytes. 3T3-L1 adipocytes were incubated with 0, 20 and 40 μM AKBA for 24 h and changes of PPAR-γ and C/EBP-α proteins were analyzed by immunoblots. As shown in Fig. 3B, PPAR-γ and C/EBP-α were down-regulated in a dose-dependent manner in AKBA-treated adipocytes. Further, mature 3T3-L1 adipocytes were treated with 30 μM AKBA for 0, 24 and 48 h. Immunoblot results showed that AKBA suppressed expression of both PPAR-γ and C/EBP-α in a temporal- dependent manner (Fig. 3C).

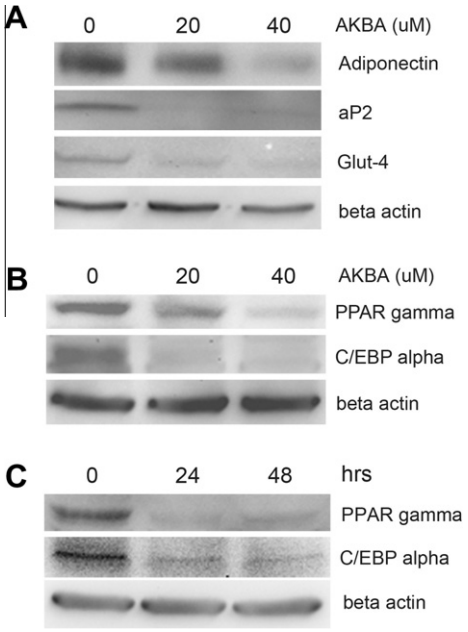

Fig. 3. (A) Changes of phenotypic markers of mature adipocytes treated by AKBA. Mature 3T3-L1 adipocytes were treated by 0, 20 and 40 μM AKBA for 24 h. Expressions of adiponectin, aP2 and glut-4 proteins were analyzed by immunoblots and β- actin was used as a housekeeping control; (B and C) Changes of PPAR-γ and C/EBP-α in AKBA-treated 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated by 0 μM, 20 μM and 40 μM AKBA for 24 h (B) or by 30 μM AKBA for 0 hrs, 24 h and 48 h, respectively. Changes of PPAR-γ and C/EBP-α were assayed by immunoblot and β-actin was used as housekeeping control (representative of 3 independent experiments).

3.4. AKBA induced lipolysis in human adipocytes

Next, we asked if AKBA could induce lipolysis in human adipocytes. Subcutaneous preadipocytes isolated from 5 subjects undergoing bariatric surgery were induced to differentiate. At the end of differentiation induction, cells were incubated with fresh culture medium which contains 30 μM AKBA for 24 h. As shown in Fig. 4A, triglycerides in lipid droplets in human adipocytes were markedly decreased after AKBA treatment. Concomitantly, free fatty acids released by adipocytes in culture medium were increased 4-fold, indicating an increased lipolysis in the presence of AKBA (Fig. 4B). Consistent with the observations obtained in murine 3T3-L1 adipocytes, PPAR-γ was significantly decreased in AKBA-treated adipocytes accompanied by a significant reduction

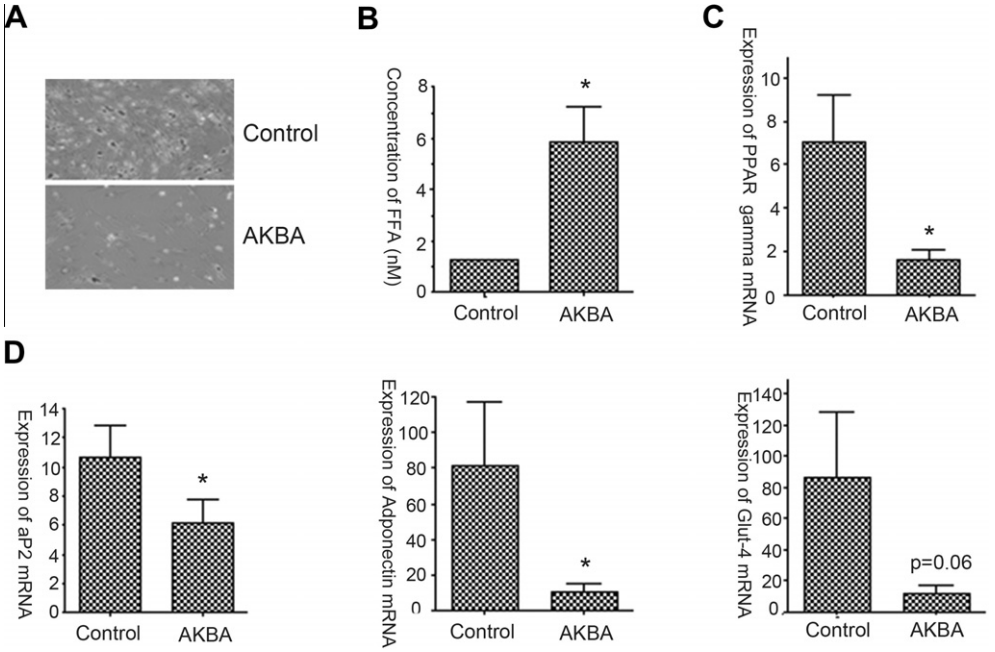

Fig. 4. (A and B) Increased lipolysis in human adipocytes treated by AKBA. Preadipocytes isolated from human subcutaneous adipose tissue were differentiated into mature adipocytes as described in Materials and Methods ($n = 5$). Mature human adipocytes were treated with 30 μM AKBA for 24 h. (A) Oil red O staining of triglycerides in adipocytes treated by AKBA ($n = 5$); (B) changes of free fatty acids in culture medium after treatment by 30 μM AKBA in human adipocytes for 24 h; (C) changes of PPAR-γ mRNA expression were assayed by quantitative RT-PCR in human adipocytes after treatment by 30 μM AKBA for 24 h ($n = 5$); (D) Changes of aP2, adiponectin and glut-4 mRNA expression assayed by quantitative RT-PCR in human adipocytes treated by 30 μM AKBA for 24 h ($n = 5$). $^*p < 0.05$ compared to vehicle-treated controls.

in phenotypic markers of adipocytes including aP2, adiponectin and glut-4 (Fig. 4C and D).

4. Discussion

Two independent groups recently reported that ursolic acid may have anti-obesity property although the underlying mechanisms were unknown [6,7].These studies prompted us to speculate that pentacyclic triterpenoids like ursolic acids and boswellic acids may modulate phenotype of adipocytes, directly or indirectly.

In this study, we found that AKBA, a naturally occurring, orally active pentacyclic triterpenoid extracted from boswellia serrata, induced lipolysis in both mouse and human adipocytes. Mature adipocytes treated by AKBA showed reduced triglyceride in lipid droplets and increased release of free fatty acids. We also found that AKBA-induced lipolysis was accompanied by a mildly increased ATGL and HSL expression, two key enzymes involved in 90% of neutral lipolysis [11]. However, it seems that the mildly increased expression of ATGL and HSL might not be able to fully explain the multitude change of lipolysis observed in our study. The rate of lipolysis in adipocytes is determined by both the expression of lipolytic enzymes and the integrity of lipid droplets [13]. Perilipin, a target gene of PPAR-γ, is one of the most important regulators of lipid-droplet stability. Overexpression and knockdown studies showed that perilipin stabilizes lipid droplets and shields lipid droplets from active lipases such as ATGL and HSL [9]. Although expression of lipolytic enzymes was only mildly increased by AKBA, we found that perilipin expression was significantly down-regulated in AKBA-treated adipocytes. Therefore, it is reasonable to propose that the stability of lipid droplets in AKBA-treated adipocytes was compromised due to the down-regulation of perilipin and thus facilitated the access and actions of lipolytic lipases ATGL and HSL. Further studies showed that AKBA also modulated the phenotype of adipocytes, as showed by reduced expression of phenotypic markers of mature adipocytes aP2, adiponectin and glut-4.

PPAR-γ and C/EBP-α are master regulators of terminal differentiation in adipocytes and they are also the key transcription factors for the maintenance of adipocyte phenotype [10,14,15]. Expression of PPAR-γ is correlated with the degree of lipid accumulation in adipocytes. Cells with a 50% reduction in PPAR-γ expression showed a 50% loss in adipogenic capacity [16]. Based on our observation in the present study, it is reasonable to speculate that down-regulation of PPAR-γ and C/EBP-α at least in part attributed to loss of mature adipocyte phenotype induced by AKBA. Also, it is possible that reduced expression of PPAR-γ promoted lipolysis through down-regulation of its target gene perilipin in AKBA-treated adipocytes [13]. Taken together, we found that AKBA, a naturally occurring pentacyclic triterpenoid, induced lipolysis in adipocytes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.136.

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