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**Original Article**

# Forskolin induced remodeling of lipid droplets in rat adipocytes

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**Abstract:** Adipose tissue is the main energy reserve of the body. When energy is required, adipocyte triglycerides stored in lipid droplets (LDs) are broken down by lipase, and free fatty acids are released to supply the physiological need. Intracellular LDs are active metabolic organelles in mammalian cells, particularly in adipocytes. The present study was aimed to investigate the morphological changes of LDs and the alternation of LD-associated perilipin family proteins during long-term lipolysis stimulated by forskolin. Primary differentiated adipocytes derived from epididymal fat pads of Sprague-Dawley (SD) rats were incubated in the presence or absence of 1 μmol/L forskolin for 24 h. Content of glycerol released to the culture medium was determined by a colorimetric assay and served as an index of lipolysis. Morphological changes of LDs were observed by Nile red staining. The mRNA level of perilipin family genes was detected by quantitative real-time PCR. The protein level and subcellular localization were examined by immunoblotting and immunofluorescence staining, respectively. The results showed that forskolin induced sustained lipolysis in differentiated adipocytes. The morphology of LDs changed in a time-dependent manner. Large clustered LDs became gradually smaller in size and eventually disappeared; in contrast, peripheral micro-LDs increased gradually in number until the cytoplasm was filled with numerous micro-LDs. The protein level of the perilipin family proteins showed obvious alternation. Mature adipocytes physiologically expressed a very low level of Plin2 protein, whereas in adipocytes stimulated with lipolytic forskolin, the protein and mRNA levels of Plin2 were significantly increased, and the increased Plin2 was specifically bound to the surface of LDs. During chronic stimulation of forskolin, the mRNA level of Plin3 was unchanged, but the mRNA levels of Plin1, Plin4 and Plin5 were significantly decreased. These results suggest that the morphology of LDs and perilipin family proteins on the surface of LDs are significantly altered during long-term lipolysis stimulated by forskolin, representing a dynamic process of the remodeling of LDs.

**Key words:** forskolin; adipocyte; lipid droplet; perilipin family proteins

# Forskolin引起大鼠脂肪细胞内脂滴重塑

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**摘 要**：脂肪组织是人体的主要能量储库，甘油三酯贮存在细胞内脂滴 (lipid droplets, LDs)中，越来越多的研究表明脂滴是细胞内代谢活跃的细胞器。本研究旨在探讨forskolin长时间刺激脂肪分解过程中脂滴形态和脂滴表面perilipin家族蛋白的改变。以Sprague-Dawley (SD)大鼠附睾脂肪垫来源的分化脂肪细胞为研究对象，给予1 μmol/L forskolin慢性刺激24 h，采用比色方法测定培养基中甘油的浓度；采用尼罗红染色观察细胞内脂滴形态的变化；采用荧光定量PCR检测perilipin家族蛋白mRNA水平的改变；采用免疫印迹和免疫荧光染色观察蛋白水平以及蛋白的亚细胞定位。结果表明，1 μmol/L forskolin孵育24 h可以持续刺激脂肪分解。伴随着脂肪分解的进行，细胞内脂滴形态逐渐发生改变，细胞内聚集存在的大脂滴逐渐减少，位于细胞周边的小脂滴逐渐增加，最终细胞内大脂滴全部消失，取而代之的是在细胞质中弥散存在的微小脂滴。在脂肪分解过程中，perilipin家族蛋白水平也发生明显变化。分化成熟的脂肪细胞几乎没有Plin2蛋白表达，而forskolin慢性刺激可以

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显著增加Plin2蛋白以及mRNA的水平，增加的Plin2蛋白特异性结合在脂滴表面。Forskolin慢性刺激对Plin3的mRNA水平无显著影响，但可以显著降低Plin1、Plin4和Plin5的mRNA水平。以上结果提示，在forskolin慢性持续刺激脂肪分解过程中，脂滴形态和perilipin家族蛋白均发生显著改变。

**关键词**：forskolin；脂肪细胞；脂滴；perilipin家族蛋白
**中图分类号**：R329.55

Adipose tissue is the major energy reserve for the body. When energy is required, the triglycerides stored in lipid droplets (LDs) of adipocytes undergo breakdown via the cooperation of hormone-sensitive lipase and adipose triglyceride lipase, and free fatty acids are released to meet physiological needs [1, 2]. Intracellular LDs are primary functional organelles that regulate cellular lipid metabolism and homeostasis [3, 4]. The surface of LDs is surrounded by a phospholipids monolayer, to which a number of proteins are bound and participate in the regulation of lipid metabolism [4, 5]. These LD-associated proteins are crucial to LDs structure and function. Perilipin family proteins (Plins), including Plin1–5, are the first family of proteins identified on the surface of LDs [6]. The LD coat is adapted to cellular and systemic energy needs, and the expression of Plins has tissue specificity [6]. Plin1 is expressed in white adipose tissue, brown adipose tissue, and steroidogenic tissue. Plin2 and Plin3 are ubiquitously expressed, Plin4 is highly expressed in white adipose tissue, and Plin5 is expressed in oxidative tissues including heart, liver, brown adipose tissue and skeletal muscle [7]. Plins can be segregated into two functional groups based on their subcellular localization. Exchangeable Plins, including Plin3–5, are stable both in the cytoplasm and at the surface of LDs. Constitutive Plins, including Plin1 and Plin2, are unstable in the cytoplasm and associated primarily with LDs [7, 8]. In adipocytes, the earliest deposits of neutral LDs are coated with Plin3 and Plin4, and as LDs gain more triglyceride and enlarge, these two Plins are replaced by Plin2, and ultimately by Plin1 [8]. Therefore, Plin1 is the main lipid associated protein in adipocytes, which constitutes about 0.25% of total protein in mature adipocytes [9] and plays fundamental roles in both lipid storage and triglyceride hydrolysis [10, 11].

Forskolin is an activator of adenylate cyclase and induces triglyceride hydrolysis through the cAMP-PKA signaling pathway without the activation of β-adrenergic receptors. It has been reported that being treated with $\beta_3$-adrenergic receptor agonist for several hours, LDs in 3T3-L1 adipocytes undergo morphological changes, and this phenomenon is known as the remodeling of LDs [12–14]. However, little is known about the remodeling of LDs and Plins during long-term triglyceride hydrolysis in adipocytes. The present study was aimed to investigate the dynamic changes of LD morphology and perilipin family proteins during the hydrolysis of triglyceride stimulated by forskolin for 24 h.

## 1 MATERIALS AND METHODS

### 1.1 *Cell culture and treatment*

Preadipocytes were isolated from epididymal fat pads of normal male Sprague-Dawley (SD) rats [15, 16]. Briefly, the fat pads were minced and digested in serum-free DMEM containing 0.8 mg/mL type I collagenase (C0130, Sigma, USA) and 1% defatted BSA, for 40 min at 37 °C in a water bath shaken at 120 cycles/min. The digestion mixture was filtered through 80 and 400 steel meshes to remove debris and floating primary mature adipocytes. Rat preadipocytes residing in the digestion mixture were collected by centrifugation at 800 *g* for 10 min and then plated and differentiated into adipocytes for 3 d in serum-free DMEM/F12 (1∶1) supplemented with 5 μg/mL insulin, 33 μmol/L biotin, and 200 pmol/L triiodothyronine, as we described previously [12, 13]. All reagents used for the induction of preadipocytes differentiation were purchased from Sigma Chemical, Co. (St. Louis. MO, USA). Thereafter, rat preadipocytes were differentiated initially (day 0) in differentiation cocktail. Seventy-two hours later (day 3) the medium was changed to DMEM/F12 with 5 μg/mL insulin, and replenished every 2 days. The cells were treated with forskolin on the day 6 of differentiation.

### 1.2 *Immunoblotting*

After treatment, cells were lysed in sample buffer containing 62 mmol/L Tris-HCl, pH 6.8, 2% SDS, 0.1 mmol/L sodium orthovanadate, and 50 mmol/L sodium fluoride. The protein content was determined by the BCA assay. Equal amounts of proteins were loaded and separated by 10% SDS-PAGE. The proteins were transferred to PVDF membranes (Millipore, Billerica, MA,

USA), and processed for analysis using enhanced chemiluminescence detection reagents (Applygen Technologies, Beijing). The primary antibodies for Plin1 or Plin2 were donated by professor C. Londos (U. S. National Institutes of Health), and horseradish peroxidase-conjugated secondary antibodies (A9044, A9169, and A8919) were purchased from Sigma Chemical Co. (St. Louis. MO, USA). Anti-β-actin (Bioss, Beijing) was served as a loading control. The optical density and area of the protein band were calculated using NIH ImageJ software from band densitometry. And the density values were expressed as tested proteins/β-actin ratio for each sample.

1.3 *Immunofluorescence staining*

Immunofluorescence staining was performed using cell cover slip. Briefly, the cover slip was fixed for 20 min with 4% paraformaldehyde, and permeabilized with 0.025% Triton X-100 and 0.5% BSA in PBS for 10 min, followed by 3 rinses with PBS each for 5 min. Nonspecific binding sites in cells were blocked with 1% BSA in PBS at room temperature for 1 h. For double immunofluorescence labeling, the cover slip was incubated in a mixture of Plin1 (rabbit anti-Plin1, 1:600) and Plin2 (goat anti-Plin2, 1:400) antibodies. Primary antibody was diluted in 1% BSA in PBS, and incubated at 4 °C for 48 h. After the 48 h-incubation, the cover slip was rinsed in PBS and then incubated with rhodamine-conjugated donkey anti-rabbit secondary antibody (805-026-180, Jackson, USA) and FITC-conjugated bovine anti-goat secondary antibody (711-001-003, Jackson, USA) at a dilution of 1:500 in 1% BSA in PBS for 2 h in the dark at room temperature with constant shaking, and cell nuclei were stained with Hoechst 33258, 3 rinses with PBS for 5 min each. Immunofluorescent signals were observed under a Nikon Eclipse 50i microscope.

1.4 *Evaluation of lipolysis by glycerol assay*

At 8:00 p.m. on day 5 of differentiation, rat differentiated adipocytes were washed twice with warm fDMEM (serum-free and phenol red-free DMEM) and then 2 mL fresh fDMEM were added overnight. At 8:00 a.m. on the next day (day 6 of differentiation), 100 μL culture media were removed as samples at 0 h, and then 1 μmol/L forskolin (F6886, Sigma, USA) was added to the media. 100 μL culture media were removed at each time points (1, 4, 8, 12 and 24 h). The culture media was collected and heated at 70 °C for 10 min to inactivate residue lipase activity [16, 17]. Glycerol was determined by the enzyme-coupled colorimetric assay (GPO Trinder reaction) from the absorption of 550 nm [16, 17], with use of a colorimetric assay kit (Applygen Technologies, Beijing). Data were expressed as micromoles of glycerol/well.

1.5 *Quantitative real-time PCR*

Total RNA was extracted from differentiated adipocytes using TRIzol reagent (Invitrogen, USA), the concentrations of RNA were measured, and 2 μg RNA was reverse-transcribed using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo Fisher Scientific, USA). Then, cDNA was amplified by real-time PCR. The PCR solution contained 1 μL specific primers (0.5 μmol/L each), 10 μL 2×BRYT Green Taq qPCR master mix (A6002, Promega, USA) and 100 ng cDNA with a final volume of 20 μL. The reaction conditions for amplifying cDNA were 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The target mRNA level was analyzed and normalized to the internal control 18S rRNA and was calculated using the following equation: fold change = $2^{-\Delta\Delta Ct}$. Real-time PCR was performed in duplicate in an Mx3000 Quantitative PCR System (Stratagene). The primer sets were listed in Table 1.

1.6 *LDs staining with Nile red*

Cover slips of differentiated adipocytes were fixed for 20 min in 4% paraformaldehyde, and then the cells were stained with Nile red (Applygen Technologies, Beijing). Saturated solution of Nile red was prepared in

Table 1. Real-time PCR primers

| Genes | Forward sequence (5'–3') | Reverse sequence (3'–5') | Product length (bp) |
|---|---|---|---|
| 18S | CGCTAGAGGTGAAATTCTTG | GGAACTACGACGGTATCTGA | 125 |
| Plin1 | TGCGCAAGAAGAGCTGAGTA | AGAGGCCAACCTGAAGGAGT | 128 |
| Plin2 | AGCTCCACTCCACTGTCCAT | TCGTAGCCGACGATTCTCTT | 121 |
| Plin3 | CAACCAGGCCTATGAGCACT | CTCTGGTCCACACCCTGTTT | 126 |
| Plin4 | ACACTGGAAGGCATGTACCC | TGGTCTGGAGTGTTGCAGTC | 122 |
| Plin5 | AAATCAGAGGAGCTGGTGGA | ACGCACAAAGTAGCCCTGTT | 132 |

acetone and kept at −20 °C until use. The cells were stained with Nile red solution in PBS diluted to 1:1 000 from the saturated solution for 10 min [18]. After the cells were rinsed 2 times with PBS, fluorescent signals were observed under a Nikon Eclipse 50i microscope. The LDs stained with Nile red originally showed yellow/gold fluorescence with a 450 nm to 490 nm excitation filter and 520 nm barrier filter [19].

1.7 *Statistics analysis*

Results shown in the figures were representative of at least three independent experiments. Data were expressed as mean ± SEM and statistically analyzed using Prism 5.0 program. The results were analyzed by Student's *t* test or one-way analysis of variance followed by a Bonferroni *post hoc* test for multiple comparisons. $P < 0.05$ was considered statistically significant.

## 2 RESULTS

### 2.1 Forskolin induced sustained lipolysis in differentiated adipocytes

On day 6 of differentiation, rat differentiated adipocytes derived from epididymal fat pads of SD rats were incubated in the presence or absence of forskolin (1 μmol/L) for 1, 4, 8, 12 and 24 h, separately. Glycerol concentration in the culture media was determined as an index of triglyceride hydrolysis. The results showed that the glycerol concentration in the culture media from forskolin-treated group increased in a time-dependent manner, which indicated that forskolin induced sustained lipolysis during the 24 h-incubation (Fig. 1).

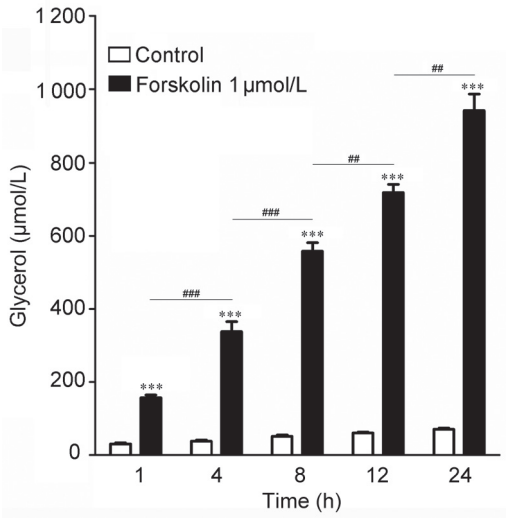


Fig. 1 Forskolin induced sustained lipolysis in differentiated adipocytes. On day 6 of differentiation, rat differentiated adipocytes were treated with or without 1 μmol/L forskolin for different times. Glycerol concentration in the culture medium was assayed. The data are expressed as mean ± SEM. $n = 4$. $^{***}P < 0.001$ *vs* control group at the same time point; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

### 2.2 Morphological changes of LDs during sustained stimulation of forskolin

The morphological changes of LDs were monitored during the 24 h-forskolin treatment. It is shown that untreated differentiated adipocytes had clustered large LDs, and the morphology of LDs in the adipocytes treated with forskolin changed in a time-dependent manner. The obvious remodeling of LDs was detectable at 6 h after treatment, at which moment micro-LDs appeared at the periphery of the large perinuclear LDs. By 12 h, the number of large LDs in the adipocytes were reduced, and the large LDs were surrounded by numerous medium-sized or small-sized LDs. Remodeling of LDs was enhanced further during the following 12 h, and small-sized LDs were increased gradually in number. By 24 h, large LDs were almost absent, and a huge number of micro-LDs diffused throughout the cytoplasm (Fig. 2*A*). The process of LDs remodeling is slow and needs a relatively long time. In order to better show the remodeling process of LDs, a typical cell was chosen as a representative of each time point (Fig. 2*B*). It is evident that the remodeling of LDs during lipolysis is a chronic and progressive process.

### 2.3 The mRNA levels of Plin family proteins were differentially regulated during sustained stimulation of forskolin

Given the fact that Plin family proteins play a significant role in stabilizing LDs and controlling lipolysis [6], we then sought to determine whether sustained forskolin stimulation could alter the mRNA level of Plin family proteins. Quantitative real-time PCR data showed that the mRNA levels of Plin1, Plin4 and Plin5 were decreased by 9 folds, separately ($P < 0.001$). The mRNA level of Plin2 was increased by 8 folds ($P < 0.001$), and the mRNA level of Plin3 slightly increased without statistical significance. Interestingly and perhaps surprisingly, Plin2 was the only Plin family protein which was significantly elevated at mRNA level (Fig. 3). The results indicated that Plin2 might play an important role in adipocytes under sustained lipolytic stimulation.

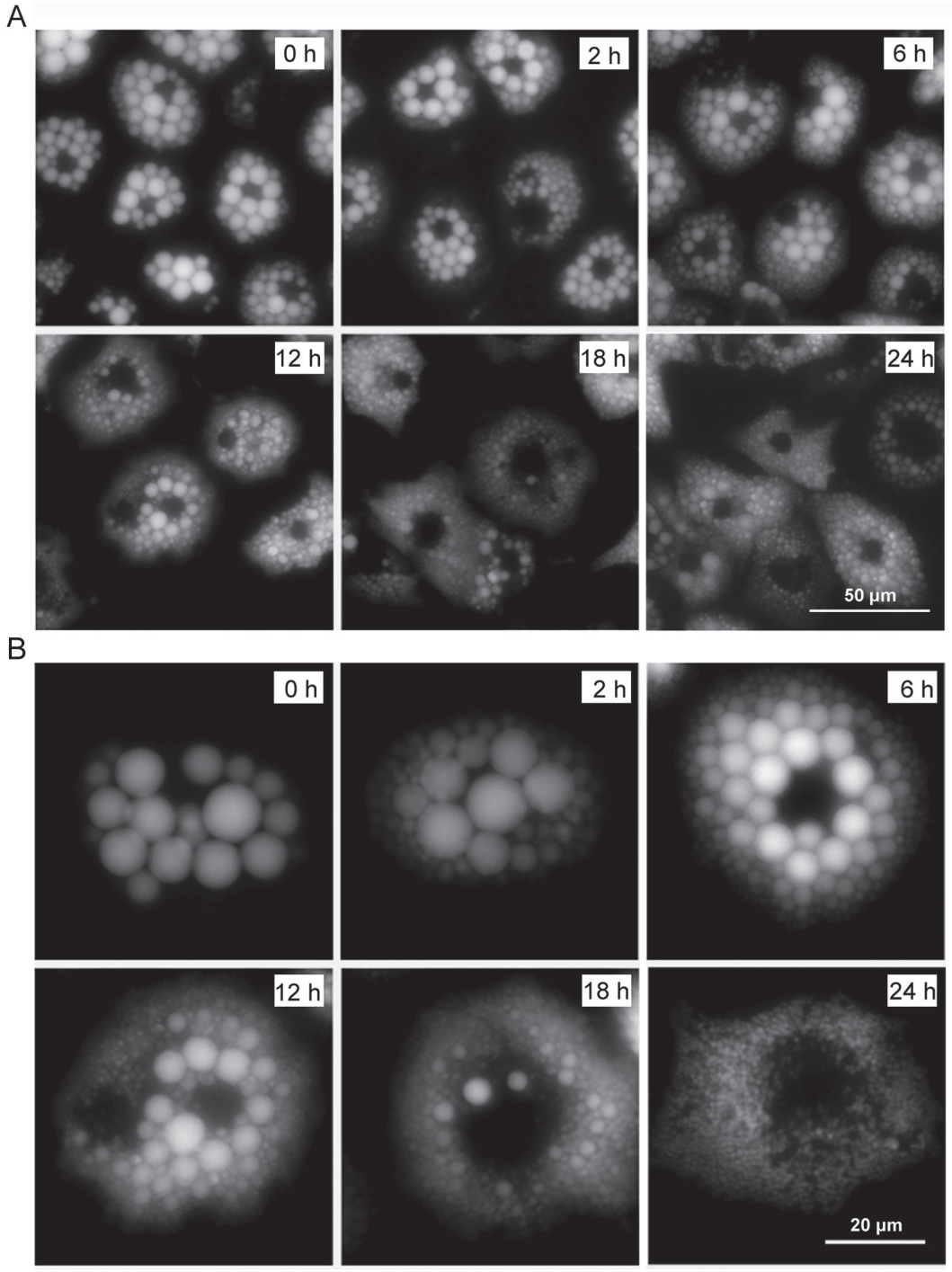


Fig. 2. Effects of forskolin on the morphology of lipid droplets (LDs) in differentiated adipocytes. On day 6 of differentiation, rat differentiated adipocytes were treated with 1 μmol/L forskolin for 2, 6, 12, 18 and 24 h, and then fixed and stained with Nile red. The morphology of LDs was visualized with fluorescence microscopy. *A*: Morphological changes of a group of adipocytes at the indicated time point. Scale bar, 50 μm. *B*: Morphological changes of LDs in a representative cell. Images were representative of three independent experiments. Scale bar, 20 μm.

### 2.4 Forskolin increased the protein level of Plin2 in a time-dependent manner

We further investigated the protein changes of Plin1 and Plin2 during the sustained stimulation of forskolin. On lipolysis stimulated with forskolin or isoproterenol, Plin1 was hyperphosphorylated as shown by its electro-

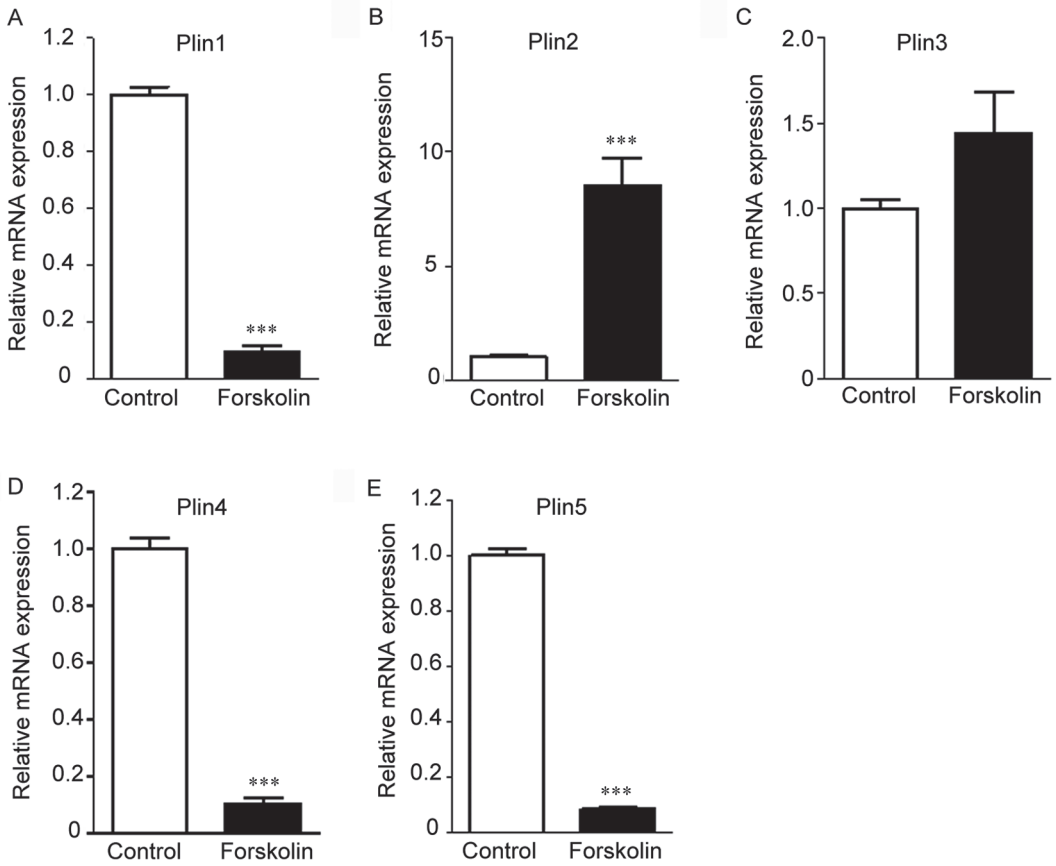


Fig. 3. Effects of forskolin on mRNA level of perilipin family proteins in rat differentiated adipocytes. On day 6 of differentiation, rat differentiated adipocytes were treated with or without 1 μmol/L forskolin for 24 h, and the mRNA levels of the perilipin family proteins were examined by quantitative real-time PCR. *A*: Plin1; *B*: Plin2; *C*: Plin3; *D*: Plin4; *E*: Plin5. The data were expressed as mean ± SEM. $n = 3$. $^{***}P < 0.001$ *vs* control.

phoretic shift from 65 to 67 kDa [20]. Such a change in migration is characteristic of increased phosphate incorporation in Plin1. The phosphorylation of Plin1 was maximized at 1 h and maintained for 24 h (Fig. 4). While, the protein level of Plin2 increased in a time-dependent manner, it was obviously increased at 8 h by 1.5 folds, and reached the highest level at 24 h by ~2.7 folds (Fig. 4). The results from immunoblot were in accordance to that from quantitive real-time PCR.

### 2.5 Plin2 co-localized with Plin1 on LDs in forskolin-treated adipocytes

The sub-cellular localization of Plin2 was further investigated by double immunofluorescence staining. The results showed that in the adipocytes of control group Plin1 localized on the surface of large LDs, while Plin2 was almost undetectable. However, in the adipocytes stimulated with forskolin, Plin2 was significantly elevated. Plin2 together with Plin1 decorated all the visible LDs in the cytoplasm (Fig. 5).

## 3 DISCUSSION

In the present study, we presented novel data showing that the morphology of LDs changed in a time-dependent manner. Large clustered LDs became gradually smaller in size and eventually disappeared, in contrast, peripheral micro-LDs increased gradually in number until the cytoplasm diffused with numerous micro-LDs. Our experiment showed that the remodeling of LDs was a chronic and progressive process, which was consistent with most of the published papers [12, 14, 21–23], but inconsistent with reports of Ariotti *et al*. [24]. Ariotti *et al*. reported that the remodeling of LDs reaches maximal level at 30 min after isoproterenol (10 μmol/L) treatment and no significant change in micro-LD numbers was observed over the subsequent 6 h. The discrepancies in remodeling process may be due to different model systems, and the types and dose of stimulators used in the experiments.

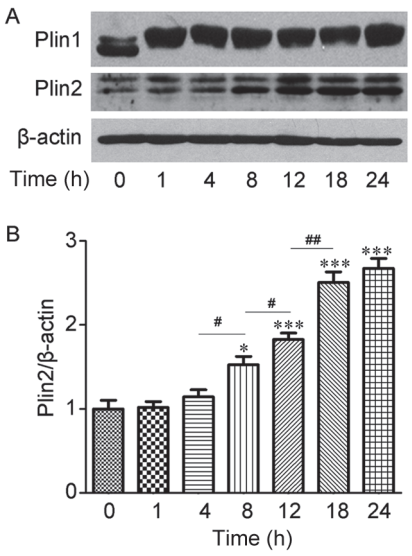


Fig. 4. Forskolin increased the protein level of Plin2 in rat adipocytes. On day 6 of differentiation, rat differentiated adipocytes were treated with 1 μmol/L forskolin for 1, 4, 8, 12, 18 and 24 h, and the protein levels of Plin1 and Plin2 at the indicated time were examined by immunoblot. *A*: Images were representative of four independent experiments. *B*: Average protein level of Plin2 was quantified as ratios to β-actin. Data were presented as mean ± SEM of four independent experiments. $^{*}P < 0.05$, $^{***}P < 0.001$ *vs* control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

The present study also showed that perilipin family proteins were altered obviously with the chronic stimulation of forskolin. Plin1 is the main LD associated protein in mature white adipocytes, and the protein level of Plin2 is very low [9–12]. However, the protein and mRNA levels of Plin2 were significantly increased via the chronic stimulation of forskolin, and the increased Plin2 together with Plin1 decorated all the visible LDs in the cytoplasm. In addition, the mRNA level of Plin3 was unchanged, but the mRNA levels of Plin1, Plin4 and Plin5 were significantly reduced. Therefore, Plin2 was the only up-regulated perilipin family protein during the forskolin-stimulated lipolysis.

Plin2 is unstable without the combination with LDs and would be degraded via ubiquitin-proteasome pathway [10]. It had been speculated that the relative surface coverage of perilipin thins during LDs remodeling, which results in Plin2 binding to the LDs and thus stabilization and up-regulation [6, 12, 13]. However, little evidence supported this assumption. Extreme temporal accordance between Plin2 elevation and LDs remodeling in our findings suggested that they are closely related phenomena during lipolysis. Micro-LDs were significantly increased during sustained lipolysis, which not

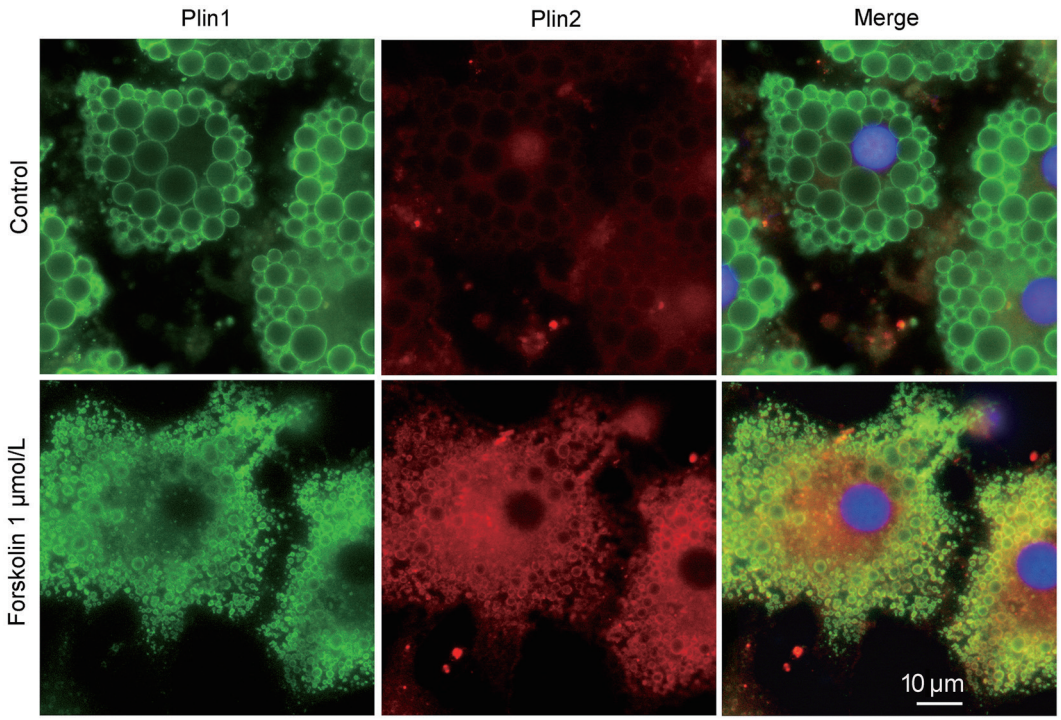


Fig. 5. Plin2 co-localized with Plin1 on the lipid droplets (LDs) in forskolin treated adipocytes. On day 6 of differentiation, rat adipocytes were treated with or without 1 μmol/L forskolin for 24 h. The cells were stained with Plin1 (green), Plin2 (red) antibodies and Hoechst 33258 (blue). Images were representative of three independent experiments. Scale bar, 10 μm.

only provides a larger surface to facilitate hydrolysis, but also provides space for Plin2 binding. In addition, the present study showed that the phosphorylation of Plin1 maintained for 24 h during the sustained stimulation of forskolin. The phosphorylation of Plin1 changed its conformation and reduced its ability to cover the surface of LDs [9, 11], which may spare relatively more LD surface for Plin2 binding.

Plin1 is the central regulator of adipose LD hydrolysis [7, 9, 11]. Under basal conditions, unphosphorylated Plin1 serves a barrier to lipases. Upon β-adrenergic stimulation, Plin1 and hormone sensitive lipase (HSL) are phosphorylated by protein kinase A (PKA), and the scaffold structure of LDs is re-organized. Phosphorylated HSL binds to phosphorylated Plin1 at the surface of LDs. In addition, CGI-58 (comparative gene identification-58), the co-activator of ATGL (adipose tissue triglyceride lipase), dissociates from phosphorylated Plin1 and recruits ATGL to the surface of LDs, and then lipolysis is activated [7]. Thus, unphosphorylated Plin1 protects triglycerides in LDs from hydrolysis by lipase. While phosphorylated Plin1 participates in the recruitment of lipases and is required for maximal lipolytic activity [25]. It is reported that lipolytic rates in cells expressing either Plin1 or Plin2 have a distinct hierarchy [7]. Basal cells expressing Plin1 are less active than Plin2-expressing cells, which are less active than stimulated Plin1 cells, and lipolysis in Plin2-cells is unchanged by PKA-activation [7, 26]. Furthermore, Plin2 has been proven to reduce the association of ATGL with LDs [27]. These findings suggested that LDs coated with Plin2 are more protective to lipases than those with phosphorylated Plin1. Thus, the increased Plin2 on the surface of LDs during lipolysis induced by forskolin may slow down the turnover of triglyceride and help to stabilize the structure of LDs.

In addition, it has been reported that micro-LDs were converted into larger LDs, after the removal of the lipolytic stimulus [24]. The recovery process is a microtubule-dependent LDs clustering and fusion sequence, and is proposed to be mediated by an interaction between Plin2 on the LD surface and the microtubule motor protein dynein [28]. Therefore, the elevation of Plin2 during lipolysis may also play an important role in the reformation of large LDs in the post-lipolytic process.

In conclusion, during the long-term lipolysis, the morphology of LDs in adipocytes changed significantly, and the macro-LDs disappeared, being replaced by numerous micro-LDs throughout the cytoplasm. The perilipin family proteins are also significantly altered. Plin2 is the only one which increased significantly among the five members in the perilipin protein family, and the elevation of Plin2 protein might be related to the enlarged surface area of LDs and involved in the remodeling of LDs. The function of Plin2 during lipolysis in adipocytes remains to be further studied.

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