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ORIGINAL PAPER

Interleukin-6 stimulates lipolysis in porcine adipocytes

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Abstract Interleukin (IL)-6 stimulates lipolysis in human and rodents adipocytes. However, the mechanism regulating this process is little known. In this study, we demonstrated that IL-6 increased lipolysis in differentiated porcine adipocytes by activation of extracellular signal-related kinase (ERK), which was inhibited by specific ERK inhibitor PD98059. IL-6 treatment did not elevate intracellular cAMP and specific PKA inhibitor H89 did not affect IL-6-induced lipolysis, which suggested that protein kinase A (PKA) pathway was not involved in IL-6-induced lipolysis. Also, the expressions of perilipin A and PPARγ2 were significantly reduced in response to IL-6 treatment, but the expressions of peroxisome proliferators-activated receptor gamma coactivator-1 alpha (PGC-1α), carnitine-palmitoyl-transferase-1 (CPT-1), and uncoupling protein 2 (UCP2) were significantly elevated. In conclusion, these results suggested that chronic high dose of IL-6 directly stimulated lipolysis in porcine adipocytes through activation of ERK, subsequently repressing perilipin A and promoting PGC-1α expression.

Keywords Interleukin-6 · Lipolysis · Porcine adipocytes · ERK · Perilipin A · PGC-1α

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Abbreviations

IL-6	Interleukin-6
PPARγ2	Peroxisome proliferators-activated receptor-gamma 2
HSL	Hormone-sensitive lipase
ATGL	Adipose triglyceride lipase
PGC-1α	Peroxisome proliferators-activated receptor gamma coactivator-1 alpha
CPT-1	Carnitinepalmitoyl-transferase-1
UCP2	Uncoupling protein 2
FFA	Free fatty acids
Semi-q RT-PCR	Semi-quantitative reverse transcription polymerase chain reaction
ERK	Extracellular signal-related kinase
PKA	Protein kinase A
cAMP	Cyclic adenosine monophosphate
MAPK	Mitogen-activated protein kinase

Introduction

The enzymatic hydrolysis of stored neutral lipid in adipocytes is an exquisitely regulated process for body fat deposition and metabolic health. Generally, the released free fatty acids (FFA) from adipocyte lipolysis act as fuel to provide energy for peripheral tissues during times of energy need, such as fasting and exercise [1]. Furthermore, the released FFA also functions as modulators of glucose and insulin action and production [2], which may involve insulin resistance [3]. Thus, in addition to its roles in body energy homeostasis and fat deposition, the regulation of adipocyte lipolysis is vital to metabolic health.

Interleukin (IL)-6 is a multifunctional cytokine, which is secreted by many different cell types and tissue [4]. Human

adipose tissue is a major site of IL-6 secretion, accounting for 15–35% of the circulating levels [5, 6]. Furthermore, plasma concentrations of IL-6 increase with obesity [6]. Recent studies have shown that plasma IL-6 levels are closely related to adipocyte lipolysis. For example, in patients with type 2 diabetes, circulating IL-6 and FFA levels are significantly elevated [7], suggesting that IL-6 may act as an important inducer of lipolysis. Moreover, infusion of recombinant human IL-6 into healthy humans and the patients with type 2 diabetes for 3 h increases plasma FFA concentrations and FFA turnover [8, 9]. In addition, after human breast adipocytes and 3T3-L1 adipocytes were cultured with IL-6 for 24 h, lipolysis showed a significant increase [9, 10]. Recently, Wallenius and his coworkers demonstrated that IL-6-deficient mouse developed mature-onset obesity, which was due to an impaired ability to oxidize fatty acids. However, after the null mice were treated with IL-6 for 18 days, there was a significant decrease in body weight [11, 12]. Taken together, these studies demonstrated that high dose of IL-6 could stimulate lipolysis and fat oxidation in human or mouse adipocytes in vivo and in vitro. However, the involved molecular mechanisms remain unclear up to now.

Adipocyte lipolysis is performed through certain signaling pathways. The conventional pathway leading to lipolysis is the cAMP-dependent protein kinase A (PKA) pathway. The increased intracellular cAMP levels lead to the activation of PKA, which then phosphorylates hormone-sensitive lipase (HSL) and perilipin, and subsequently the lipolysis is enhanced [13, 14]. However, whether PKA pathway mediates IL-6-induced lipolysis remains unclear up to now. In addition, extracellular signal-related kinase (ERK) 1/2 pathway has been shown to mediate IL-6-induced serine phosphorylation of insulin receptor substrate-1, another possible mechanism by which IL-6 induces insulin resistance [15]. Meanwhile, the ERK1/2 pathway has been shown to regulate lipolysis in 3T3-L1 adipocytes by phosphorylating HSL [16], a main enzyme in hormone-regulated lipolysis [17]. So we hypothesize that ERK1/2 pathway may regulate IL-6-induced lipolysis in porcine adipocytes.

Perilipin A is an important structural and functional protein on the surface of lipid droplets in adipocytes and plays a key regulatory role in adipocyte lipolysis [14, 18, 19]. In addition, FFA oxidation can decrease the end products of lipolysis, and helps to keep and promote lipolysis. However, it is unclear how IL-6 regulates the expressions of perilipin A and the genes relevant to FFA oxidation to affect lipolysis in porcine adipocytes.

Pigs have abundant body fat and have many similar physiological characters to human. Thus, pigs have been considered as an ideal medical model for researching human obesity and metabolic diseases [20]. In this study, we investigated the mechanisms by which chronic high dose of IL-6 stimulated lipolysis in porcine adipocytes. First, we evaluated the effect of IL-6 on lipolysis in porcine adipocytes. Then, we examined the signaling pathways leading to lipolysis. Finally, we studied the expression of genes relevant to lipolysis and FFA oxidation.

Results

Effect of IL-6 treatment on lipolysis in differentiated porcine adipocytes

To investigate the mechanisms by which IL-6 stimulates lipolysis in porcine adipocytes, we firstly examined the effect of IL-6 on lipolysis. As shown in Fig. 1a, treatment with 50 ng/ml IL-6 for 48 h resulted in a significant increase in glycerol release compared with the control group (31.6% increase, $P < 0.05$). The maximal glycerol release was seen in 100 ng/ml IL-6 treated group, and was about 64.5% higher than that in the control. Furthermore, as shown in Fig. 1b, the adipocytes released more glycerol in 48 h than 24 h in response to the same dose of IL-6 (50 or 100 ng/ml) treatment. These data indicated that IL-6 stimulated lipolysis in porcine adipocytes in a dose- and time-dependent manner.

Identification of IL-6 receptor and gp130 in porcine adipocytes

To determine whether IL-6 stimulates lipolysis directly, mRNA expression of IL-6 receptor (IL-6R) and gp130, the IL-6 receptor system that mediates IL-6 signal, were checked in cultured porcine adipocytes. As shown in Fig. 2, porcine adipocytes expressed IL-6R and gp130, indicating that IL-6 could directly stimulate lipolysis in porcine adipocytes.

Effect of PKA pathway on IL-6-stimulated lipolysis

To verify whether the activation of cAMP-dependent PKA mediated IL-6-stimulated lipolysis in porcine adipocytes, specific PKA inhibitor H89 was used to block activation of the PKA pathway. As shown in Fig. 3a, treatment of adipocytes with isoprenaline (ISO) (10 μM) caused a significant increase in glycerol release, and this effect was completely blocked by the PKA inhibitor H89, showing that PKA mediated the ISO-induced lipolysis. In contrast, H89 pretreatment did not prevent IL-6-stimulated lipolysis. To further clarify the role of cAMP, we directly measured intracellular cAMP levels after IL-6 (100 ng/ml) or ISO (10 μM) treatment. As shown in Fig. 3b, ISO, but not IL-6, caused a significant increase in cAMP levels. These data showed that IL-6-induced lipolysis does not involve the conventional cAMP-dependent pathway.

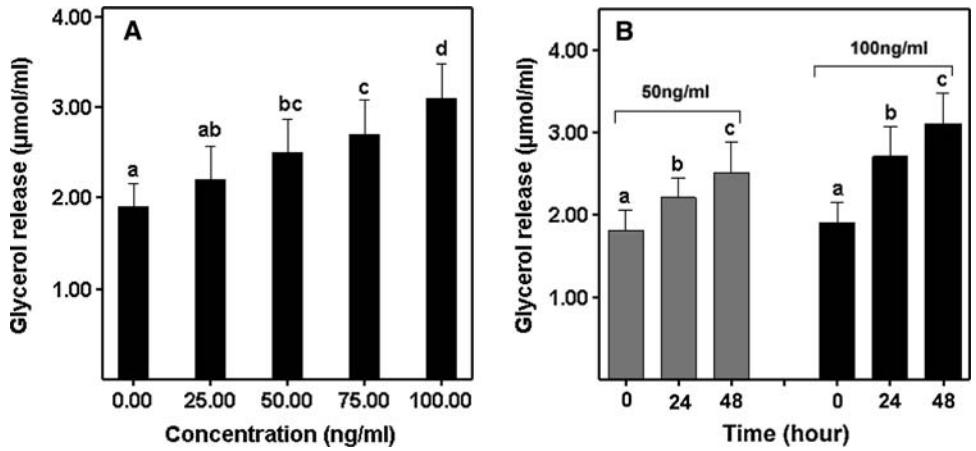


Fig. 1 Dose- and time-dependent effect of IL-6 on lipolysis in porcine adipocytes. After differentiated porcine adipocytes were incubated with various concentrations of IL-6 (25–100 ng/ml) for 24–48 h, and then glycerol release was determined as a measure of lipolysis. **a** Dose-dependent effect of IL-6 on lipolysis in porcine adipocytes. The results are the means ± S.E. of triplicate measurements. Values with different superscripts are significantly different ($P < 0.05$). **b** Time-dependent effect of IL-6 on lipolysis in porcine adipocytes. The results are the means ± S.E. of triplicate measurements. Values with different superscripts for the same dose of IL-6 are significantly different ($P < 0.05$)

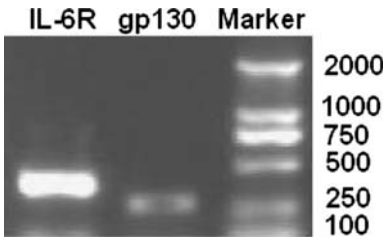


Fig. 2 Identification of IL-6R and gp130 in porcine adipocytes. Total RNA was extracted from differentiated porcine adipocytes on day 8, and then mRNA expression of IL-6R and gp130 was detected using RT-PCR

Effect of ERK pathway on IL-6-induced lipolysis

To determine the relationship of the ERK pathway and IL-6-mediated lipolysis, the dose-dependent effects of IL-6 on ERK1/2 activation was examined. As shown in Fig. 4a, IL-6 treatment increased ERK1/2 phosphorylation, whereas it did not affect the expression of total ERK. The increase was detectable at 50 ng/ml treatment, and was maximal at 100 ng/ml treatment. Furthermore, pretreatment of porcine adipocytes with the ERK inhibitor PD98059 (50 μM) significantly blocked IL-6 (100 ng/ml)-stimulated ERK1/2 phosphorylation.

To study the possible role of the ERK1/2 pathway in IL-6-induced lipolysis, specific ERK inhibitor PD98059 was used to block activation of the ERK pathway. As shown in Fig. 4b, pretreatment with low-dose PD98059 (20 μM) could only partially inhibit IL-6-stimulated lipolysis. However, high-dose PD98059 (50 μM) could fully inhibit IL-6-stimulated lipolysis. Combined with the inhibitory effect of PD98059 on ERK activation (Fig. 4a), these findings provided the evidence that ERK activation mediated IL-6-stimulated lipolysis.

Effect of IL-6 on expression of the related genes to lipolysis

Hydrolysis of triacylglycerol in adipocytes is performed by catalysis of lipases and regulation of several important protein factors. In order to explore the effect of IL-6 on expression of related genes to lipolysis, we analyzed the protein and mRNA expression of perilipin A, PPARγ2, and HSL. As shown in Fig. 5a, IL-6 treatment significantly decreased protein expression of perilipin A and PPARγ2 in comparison to the control, whereas it did not affect the expression of HSL protein. Meanwhile, IL-6 treatment significantly decreased the mRNA expression of perilipin A and PPARγ2 (Fig 5b, c), but the mRNA expression of HSL was significantly increased ($P < 0.05$). The results indicated that IL-6 stimulated lipolysis in porcine adipocytes by transcriptional repression of perilipin A, and the expression of HSL might involve the post-transcriptional events.

Effect of IL-6 on expression of related genes to FFA oxidation

FFA oxidation helps to decrease the end products of lipolysis, and subsequently maintain and promote lipolysis. In order to explore the effect of IL-6 on expression of related genes to FFA oxidation, we analyzed the mRNA expression of PGC-1α (peroxisome proliferators-activated receptor gamma coactivator-1 alpha), CPT-1 (carnitine-palmitoyl-transferase-1), and UCP2 (uncoupling protein 2). As shown in Fig. 6a and b, IL-6 treatment significantly

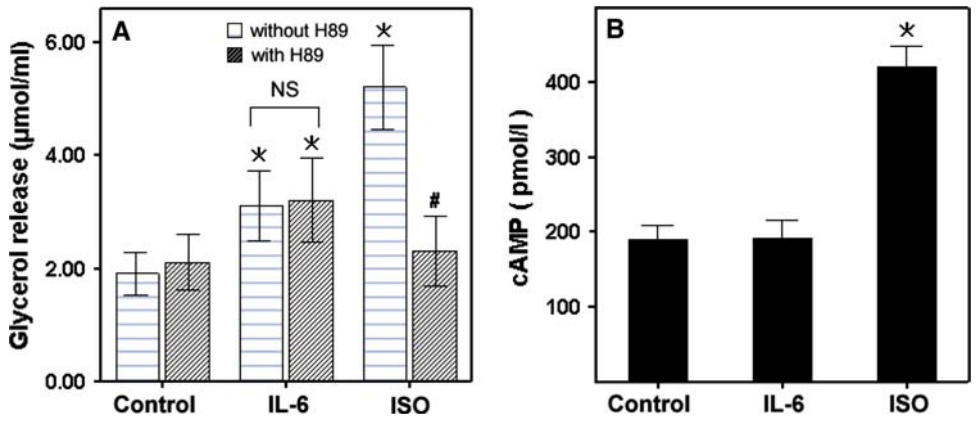


Fig. 3 Effect of PKA inhibitor on IL-6-induced lipolysis and intracellular cAMP levels. **a** Effect of PKA inhibitor on IL-6-induced lipolysis. Differentiated porcine adipocytes were pretreated with or without PKA inhibitor H89 (20 μM) for 24 h, and then incubated with or without 100 ng/ml IL-6 (for 48 h) or 10 μM ISO (for 2 h) in the continued presence or absence of the inhibitor before the glycerol concentration in the culture media was measured. The results are the means ± S.E. of triplicate measurements. *$P < 0.05$ compared with the control without inhibitor. $P < 0.05$ compared with ISO alone. NS means no significant difference between the two groups. **b** Effect of IL-6 on intracellular cAMP levels. After the differentiated porcine adipocytes were incubated with 100 ng/ml IL-6 (for 48 h) or 10 μM ISO (for 2 h), the intracellular cAMP content was measured. The results are the means ± S.E. of triplicate measurements. *$P < 0.05$ compared with the control without inhibitor

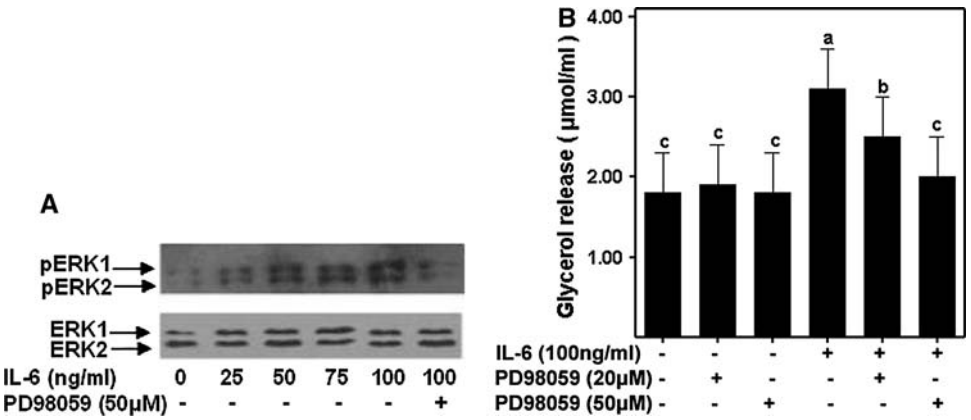


Fig. 4 Effect of ERK pathway on IL-6-induced lipolysis. **a** Effect of IL-6 on ERK phosphorylation. Differentiated porcine adipocytes were incubated with various concentrations of IL-6 (25–100 ng/ml) for 48 h. In addition, differentiated porcine adipocytes were incubated with IL-6 (100 ng/ml) in the presence of ERK inhibitor PD98059 (50 μM) for 48 h. Then, the cell extracts were analyzed by western blot with antibodies to phosphorylated ERK1/2 or total ERK1/2. **b** Effect of ERK inhibitor on IL-6-induced lipolysis. Differentiated porcine adipocytes were pretreated with or without various concentrations of PD98059 (20 or 50 μM) for 24 h, then incubated with or without IL-6 (100 ng/ml) in the continued absence (−) or presence (+) of the inhibitor for a further 48 h before the glycerol concentration in the media was measured. The results are the means ± S.E. of triplicate measurements. Values that share different superscripts are significantly different ($P < 0.05$)

increased the mRNA expression of PGC-1α, CPT-1, and UCP2 compared with the control ($P < 0.05$). The results indicated that IL-6 stimulated lipolysis in porcine adipocytes by promoting expression of genes relevant to FFA oxidation at least in part.

Discussion

IL-6 has traditionally been known as one of the most important mediators of fever and of the acute phase response, and may be secreted by immune cells during inflammatory conditions. However, recent research has focused on its metabolic roles in the etiology of obesity and type 2 diabetes due to its secretion from adipose tissue and contracting skeletal muscle [5, 21], furthermore, the elevated plasma concentrations of IL-6 in patients with obesity and type 2 diabetes is also an important cause [6, 7].

The present study was performed to explore the lipolytic role of IL-6 in porcine adipocytes and to clarify the underlying molecular pathways. The main finding was that IL-6 directly stimulated lipolysis in porcine adipocytes in a time- and dose-dependent manner and by activation of the ERK pathway, repression of perilipin A, and up-regulation

of PGC-1α expression. The results in porcine adipocytes provided further evidence that IL-6 was an important lipolytic mediator, which was consistent with some previous reports in human, rodents and 3T3-L1 adipocytes [8–11], indicating that pigs could act as an ideal medical model for studying lipid metabolism.

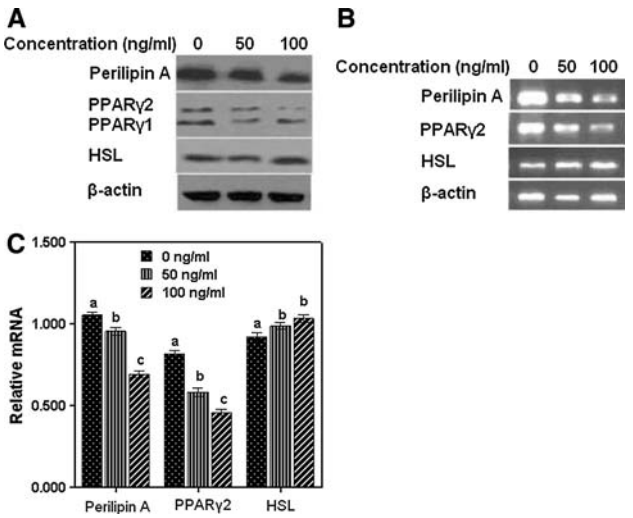
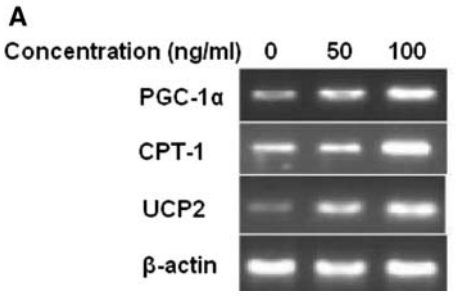


Fig. 5 Effect of IL-6 on related gene expression to lipolysis. Differentiated porcine adipocytes were incubated with various concentrations of IL-6 (0, 50, 100 ng/ml) for 48 h. Cell extracts were subjected to western blot or RT-PCR for perilipin A, PPARγ2, and HSL. **a** Effect of IL-6 on protein expression of perilipin A, PPARγ2, and HSL. **b** Representative ethidium bromide stained gels of target products as well as β-actin in the same sample. **c** Perilipin A, PPARγ2, and HSL mRNA abundance assigned as a ratio to β-actin mRNA. The results are expressed as the means ± S.E. of triplicate measurements. Values with different superscripts for the same gene are significantly different ($P < 0.05$)

In our study, porcine adipocytes expressed IL-6 receptor and gp130 (Fig. 2), a signal transducer of IL-6, indicating that IL-6 could directly stimulate lipolysis in porcine adipocytes. Furthermore, several kinds of lipolytic hormones such as TNF α, insulin, isoprenaline (ISO), growth hormone (GH), leptin, and IL-6 itself could elevate expression of IL-6 in human and rodent adipocytes [22], indicating that IL-6 could mediate the lipolysis of these lipolytic hormones at least in part. In addition, IL-6 could induce production of leptin in adipocytes [23, 24], indicating that IL-6 could also induce lipolysis indirectly.

Conventionally, hormone-stimulated lipolysis occurs by activation of cAMP-dependent PKA, which phosphorylates perilipin A, a key lipolytic modulator, and activates HSL and ATGL (adipose triglyceride lipase), two important lipases [17, 25, 26], and subsequently increases adipocyte lipolysis [13, 18, 19, 27]. Our data showed that IL-6-stimulated lipolysis is cAMP-independent and that IL-6 did not increase cAMP formation in porcine adipocytes (Fig. 3b). Furthermore, PKA inhibitor H89 did not block IL-6-induced lipolysis (Fig. 3a), showing that the IL-6-mediated signaling pathway was different from the conventional pathway leading to lipolysis involving activation of cAMP-dependent PKA. In addition, as shown in Fig. 5a, perilipin A migrated only as a 65-kDa band in SDS-PAGE rather than the electrophoretic shift from 65 to 67 kDa, also indicating that perilipin A was not phosphorylated by PKA. This result confirmed that IL-6 evoked a non-conventional lipolysis response in porcine adipocytes.

ERK1/2 belongs to a subfamily of MAPK (mitogen-activated protein kinase). There is evidence that activation of ERK pathway in adipocytes is linked to adipogenesis, lipolysis, and insulin resistance [16, 28, 29]. On the one



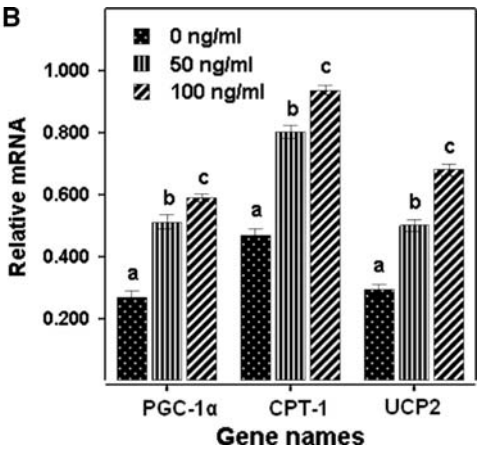


Fig. 6 Effect of IL-6 on related gene expression to FFA oxidation. Differentiated porcine adipocytes were incubated with various concentrations of IL-6 (0, 50, 100 ng/ml) for 48 h. Cell extracts were subjected to RT-PCR for PGC-1α, CPT-1 and UCP2 mRNA expression. **a** Representative ethidium bromide stained gels of target products as well as β-actin in the same sample. **b** PGC-1α, CPT-1 and UCP2 mRNA abundance assigned as a ratio to β-actin mRNA. The results are expressed as the means ± S.E. of triplicate measurements. Values with different superscripts for the same gene are significantly different ($P < 0.05$)

hand, ERK pathway activation during the early stage of differentiation promotes adipogenesis [29], but on the other hand, ERK pathway activation in differentiated adipocytes leads to insulin resistance [28, 30] and increased lipolysis [16]. ERK1/2 activity is regulated by the upstream kinase MEK1/2, which phosphorylates ERK1/2 on threonine and tyrosine residues [31, 32]. The specific MEK1/2 inhibitor, PD98059, inhibits MEK1/2 activation by binding to inactive MEK1/2 and preventing the phosphorylation by upstream kinases such as Raf-1 [33]; subsequently, ERK1/2 activity is inhibited. In our study, we observed a significant phosphorylation of ERK1/2 (Fig. 4a) in response to IL-6 treatment. The ERK1/2 inhibitor PD98059 blocked IL-6-induced lipolysis (Fig. 4b) and phosphorylation of ERK1/2 (Fig. 4a), demonstrating that ERK1/2 activation mediated IL-6-induced lipolysis in porcine adipocytes, which raises evidence that ERK1/2 activation may play a critical role in the adipocytokine-derived lipolytic signal in adipocytes.

In many cells, activation of ERK1/2 triggers its translocation into the nucleus, where it activates or represses a variety of transcription factors involved in proliferation and differentiation [34, 35]. For example, the ERK pathway has been shown to mediate phosphorylation of peroxisome proliferators-activated receptor γ, a major transcription factor in adipocytes, and reduce its transcriptional activity [36]. In our study, when ERK1/2 was chronically activated by IL-6, the protein and mRNA expressions of PPARγ2 were significantly down-regulated as well as its target gene perilipin A; however, the protein expression of HSL had no change although its mRNA expression was increased (Fig. 5a–c), indicating that the mechanisms of ERK1/2 action is more complicated than direct phosphorylation of HSL [16], and that alterations in gene and protein expression may be involved.

Perilipin A (the predominant perilipin subtype in adipocytes) is a key regulator of both basal and PKA-stimulated lipolysis. In vitro and in vivo studies demonstrate that reduced levels or absence of perilipin A results in increased basal lipolysis while substantially attenuating PKA-stimulated lipolysis. In addition, perilipin A is a target gene of PPARγ2, and there is peroxisome proliferator-activated receptor response element (PPRE) in the promoter of perilipin A [37]. So decreased expression of PPARγ2 in adipocytes will lead to repression of perilipin A; subsequently, the basal lipolysis will be enhanced. In our study, we found that the mRNA and protein expressions of perilipin A in porcine adipocytes were significantly reduced in response to IL-6 treatment (Fig. 5). Combined with the decreased expression of PPARγ2 (Fig. 5), we infer that IL-6 may repress perilipin A protein by down-regulating expression of PPARγ2. However, perilipin A protein also can be degraded through ubiquitin-proteasomes pathway [38]. Whether IL-6-induced lipolysis involves this pathway is still unsettled.

PGC-1α is a critical factor in mitochondrial biogenesis via co-activation nuclear respiratory factors (NRF) and mitochondrial transcriptional factor A (mtTFA) [39]. In adipocytes, increased PGC-1α promotes expression of CPT-1, and UCP2 [40–42], which involves FFA oxidation and energy expenditure [43]. In the present study, IL-6 significantly increased the expression of PGC-1α, CPT-1, and UCP2 mRNA (Fig. 6a, b), indicating that the related genes to FFA oxidation may contribute to IL-6-stimulated lipolysis. However, whether the changed gene expressions result from the phosphorylated ERK1/2 awaits further study.

Based on our findings, we suggest a model for IL-6-stimulated lipolysis in porcine adipocytes, which includes the activation of the ERK1/2 pathway, resulting in down-regulation of PPARγ2 expression with a subsequent decrease in perilipin A protein, leading to a modification of the lipid droplet surface and allowing lipase(s) to hydrolyze triacylglycerol. Meanwhile, the elevated expression of PGC-1α, CPT-1, and UCP2 may help to keep and promote IL-6-stimulated lipolysis. The model may represent only one of the pathways by which IL-6 stimulates lipolysis in porcine adipocytes, but certainly in our cell culture system, this is a major mechanism.

Materials and methods

Reagents

PD98059, H89, and the polyclonal anti-perilipin A antibody were purchased from Progen (Heidelberg, Germany), and the polyclonal antibodies of anti-PPARγ, anti-HSL, anti-β-actin, and anti-phosphorylated and total ERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human IL-6, glycerol assay kits, cAMP assay kit, and dimethyl sulfoxide (DMSO, cell culture grade) were purchased from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM)/F12, Type I collagenase, bovine serum albumin (BSA), Trizol Reagent, First Strand cDNA Synthesis Kit, and Taq DNA polymerase were purchased from Invitrogen (CA, USA). Penicillin/streptomycin, phosphate-buffered saline (PBS), and fetal bovine serum (FBS) were purchased from Gibco-Invitrogen. Electrophoresis reagents were from Bio-Rad Laboratories (Richmond, CA). Unless specified, other reagents were purchased from Sigma (St. Louis, MO).

Experimental animals

Three-day-old healthy male or female crossbred piglets (Duroc × Landrace × Large-White) were purchased from the experimental farm of Northwest A & F University (Yangling, Shaanxi, China). The laboratory procedures

used conformed to the guidelines of Northwest A & F University Animal Care Committee and the pigs were killed by electrocution.

Porcine adipocyte primary culture

The separation and primary culture of porcine preadipocytes was carried out using previously described methods [44]. Briefly, after adipose tissue was removed from porcine dorsal subcutaneous fat depot, the tissue was cut into approximately 1 mm^3 pieces and rinsed with PBS, followed by the digestion with 1 mg/ml type I collagenase in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (1:1 mixture of DMEM and Ham's F12) containing 100 mM HEPES and 20 mg/ml BSA, in a 37°C shaking water bath for 1 h. The digested tissue suspension was filtered through 200 μm nylon mesh and centrifuged at 800 × g for 5 min to separate the mature adipocytes and stromal-vascular fraction (SV fraction). The SV fraction was then incubated with erythrocyte lysis buffer (0.154 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA) at room temperature for 10 min. The collected pellets were used as preadipocytes [44]. The preadipocytes were washed twice with DMEM/Ham's F12 medium supplemented with 50 U/ml penicillin, and 50 U/ml streptomycin. Then the cells were re-suspended in DMEM/Ham's F12 medium containing 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 U/ml streptomycin. Preadipocytes were seeded in 60 mm diameter culture dish with the density of 5 × 10^4 cells/cm^2, and maintained at 37°C in 5% CO_2 humidified atmosphere. The medium was changed every 2 days until the cells were mostly differentiated. Typically, more than 80% of the preadipocytes may be differentiated into adipocytes by day 8, as determined using Oil Red O staining for lipid droplets in adipocytes [44].

Measurement of lipolysis

The glycerol content of the incubation medium was used as an index for lipolysis and was measured by a colorimetric method using glycerol assay kits according to the manufacturer's instruction. The results were corrected by cell numbers.

Western blot analysis

The cellular protein was extracted in lysis buffer (50 mmol/l Tris–HCl, 0.5% Triton X-100, 2 mmol/l EDTA, 150 mmol/l NaCl, pH 7.5) containing PMSF according to our established method [45]. The protein content was measured by the previous methods [46]. Samples with 50 μg total protein were electrophoresed through 12% SDS-polyacrylamide gel under reducing conditions, followed by electro-transfer onto nitrocellulose membranes. After being blocked in 5% defatted milk in TBST (Tris buffer system and Tween 20) for 60 min at room temperature, the membranes were reacted with specific antibodies of anti-perilipin A, anti-β-actin, anti-PPARγ, anti-HSL, anti-β-actin, or anti-phosphorylated and total ERK1/2 for 24 h at 4°C. This was followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 60 min at room temperature and then by revelation with chemiluminescence reagent.

RT-PCR analysis

Total RNA was extracted from adipocytes with TRizol Reagent (Invitrogen) according to manufacturer's instruction. The extracted RNA was dissolved in RNase-free water and the quality was checked by electrophoresis. First strand cDNA was synthesized with First Strand cDNA Synthesis Kit (Fermentas Life Science, Hanover, MD, USA), and the final volume was 20 μl including total RNA 5 μg, random hexamer primer 1 μl, 5 × reaction buffer 4 μl, RiboLock™ Ribonuclease Inhibitor (20 U/ml) 1 μl, 10 mmol/l dNTP mix 2 μl, RevertAid™ M-MuLV Reverse Transcriptase (200 U/μl) 1 μl, and the remaining volume was DEPC-treated water.

The expression level of each gene was determined by semi-q RT-PCR. The specific PCR primers were designed using Primer 5.0 Software (Table 1) based on gene bank information of porcine perilipin A, PPARγ2, HSL, PGC-1α, UCP2, CPT-1, IL-6R, gp130, and β-actin genes. A total of 25 μl PCR reaction system contained 15.3 μl sterile double distilled water, 10 × $(NH4)_2SO_4$ Buffer 2.5 μl, 2.5 mmol/l dNTP 2 μl, 10 μmol/l of specific primer I 1 μl, 10 μmol/l of specific primerII1 μl, 2.5 mmol/l $MgCl_2$ 2 μl, synthesized cDNA 1 μl, 0.5 U/μl Taq DNA Polymerase (Fermentas) 0.2 μl. PCR condition was as follows: a denaturation step at 95°C for 5 min, and then 27–30 cycles of amplification was performed in PTC-200 DNA engine. Each cycle consisted of a denaturation step at 95°C for 45 s, an annealing step at 49–58°C for 45 s and extension step at 72°C for 1 min. Finally, after a 10 min extension reaction at 72°C, the reaction system was stored at 4°C for 10 min. The amplified 2.5 μl products were separated by 1% agarose gel electrophoresis. Images of the semi-qRT-PCR stained with ethidium bromide were taken using Wealtec Gel Image Formation System and analyzed by Dophin-1D Gel Analysis software. β-actin was used as an internal control, and band optical density value of the genes was normalized to β-actin.

Measurement of the intracellular cAMP concentration

The intracellular cAMP concentrations in differentiated porcine adipocytes were measured using an enzyme

Table 1 The specific designed primers for related genes to lipolysis in polymerase chain reaction

Genes	Primers (5′– 3′)	Product sizes (bp)	Annealing temperature (°C)	GeneBank accession No.	Cycle times
IL-6R	S: 5′GGGTGCTCAGGAATCAGG 3′	411	53.5	NM_214403	28
	A: 5′TGTTGGCGACGCATAGGG 3′				
gp130	S: 5′ GTATTGGAGGGTCTTCGT 3′	301	49.8	EF151500	28
	A: 5′ TCTGGACTGGTTTCGTGT 3′				
Perilipin A	S: 5′ CCCTGGTGGCGTCTGTATG 3′	349	54.7	AY973170	28
	A: 5′ GCGGCATATTCAGCAGTGTC 3′				
PPARγ2	S: 5′ TGACCCAGAAAGCGATGC 3′	285	58	AF103946	28
	A: 5′ ATGGCACTTTGGTAGTCCTG 3′				
HSL	S: 5′ GTCTTTGCGGGTATTCGG 3′	498	54.4	NM214315	28
	A: 5′ GCCTGTTTCATTGCGTTTG 3′				
PGC-1α	S: 5′ GAGATTCCGTATCACCACC 3′	340	57.6	AB106108	30
	A: 5′ CTTTCAGACTCCCGCTTC 3′				
CPT-1	S: 5′ GGTGGTGTCAGCGTAGCA 3′	283	57.9	AF288789	28
	A: 5′ CCTTGTTGTCAGTTTGGGTAA 3′				
UCP2	S: 5′ CTTCTGCGGTTCCTCTGTGT3′	641	57.9	NM214289	30
	A: 5′ CATAGGTCACCAGCTCAGCA 3′				
β-actin	S: 5′ ACTGCCGCATCCTCTTCCTC 3′	399	55.5	AY550069	27
	A: 5′ CTCCTGCTTGCTGATCCACATC 3′				

immunoassay kit according to the manufacturer's instruction. The results were corrected by cell numbers.

Statistical analysis

The results were expressed as the means ± standard error (S.E.) of triplicate measurements. One-way ANOVA or the paired Student's *t* test was used for statistical analyses. *P* values <0.05 were considered statistically significant.

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