Inhibition of ERK1/2 downregulates triglyceride and palmitic acid accumulation in cashmere goat foetal fibroblasts

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ABSTRACT

An extracellular signal-regulated kinase (ERK) is a mitogen-activated protein kinase (MAPK) in mammalian cells that interact with the mechanistic target of rapamycin (mTOR) signalling pathway. ERK signalling integrates signals from growth factors, nutrients, and mitogens to regulate cell proliferation, differentiation, and the cell cycle. The purpose of this study was to examine the inhibitory effects of U0126, a specific MEK/ERK inhibitor, on ERK and mTOR signalling, Lipin 1 protein level, PPARγ activation, and triglyceride (TAG) and palmitic acid (PA) accumulation in Cashmere goat foetal fibroblasts (GFb). The proliferation of GFb was significantly impeded by U0126, the IC50 of which was 1.54 μM. U0126 prevented the phosphorylation of ERK1/2 (Thr202/Tyr204), S6 (Ser240/244), and 4EBP1 (Thr37/46), the latter 2 of which are mTOR downstream effectors. Moreover, the cytoplasmic level of Lipin 1 and the nuclear PPARγ protein abundance were attenuated by U0126 while the concentration of TAG and PA was significantly decreased (p < .05) in GFb. These data indicate that ERK1/2 have important functions in GFb proliferation, governing the activation of PPARγ and Lipin 1 level and the accumulation of TAG and PA.

Introduction

Mitogen-activated protein kinases (MAPKs) are Ser/Thr protein kinases that constitute a family of protein kinase cascades that regulate such processes as proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis in response to a variety of extracellular signals (Pearson et al. 2001; Johnson & Lapadat 2002). The prototypical MAPKs are the mammalian extracellular signal-regulated kinases (ERKs) 1 and 2 (Hill & Treisman 1995), which share 90% identity and have relative molecular weights of 44 kD and 42 kD, respectively (Boulton et al. 1990, 1991). ERK1/2 are activated by growth factors and mitogens, phosphorylating an array of cellular substrates, including nuclear transcription factors. ERK1/2 integrate signals from upstream Ras, Raf, and MEK (mitogen-activated protein kinase kinase) and interact with other protein kinases to form the ERK1/2 signalling pathway and regulate many processes, including cell proliferation, differentiation, and the cell cycle (Lin et al. 2017; War et al. 2015; Zhang et al. 2015).

The Ras/MAPK pathway governs mechanistic target of rapamycin (mTOR) signalling by targeting TSC2 (tuberous sclerosis complex 2), a negative regulator of mTORC1, thereby inactivating the TSC1-TSC2 complex and increasing mTORC1 signalling toward downstream effectors (Eblen et al. 2002). The induction of protein synthesis in mouse uterine epithelial cells by estradiol-17β is mediated by PKC-ERK1/2-mTOR signalling (Wang et al. 2015), and ERK1/2-dependent activation of mTORC1/p70S6 K regulates thrombin-induced human RPE cell proliferation (Parrales et al. 2013).

U0126 is a highly selective inhibitor of MEK1 and MEK2 and is a well-established inhibitor of the ERK pathway. Many cell types are sensitive to U0126, which inhibits cell proliferation and ERK1/2 activation (Du et al. 2013; Sandra et al. 2015; Liu et al. 2016). U0126 halts mTOR signalling and significantly reduces the activation of p70S6 K in glioblastoma (Sunayama et al. 2010). Estradiol inhibits osteoblast apoptosis by promoting autophagy through the ER-ERK-mTOR pathway (Yang et al. 2013). Conversely, inhibition of the ERK1/2 pathway by U0126 has little impact on mTOR signalling, but blockade of the mTOR pathway with rapamycin significantly increases ERK1/2 signalling in the cerebrospinal fluid-contacting nucleus (Li et al. 2015).

PPARs (peroxisome proliferator-activated receptors) are key transcription factors in lipid metabolism. The ERK/MAP kinase pathway stimulates osteoblastogenesis and inhibits adipogenesis through the phosphorylation of PPARγ (Ge et al. 2016). A recent study showed that cocoa tea significantly suppresses the expression of PPARγ to inhibit triglyceride accumulation in mature adipocytes through ERK signalling (Li et al. 2016). Advanced glycation end products (AGEs) repress PPARγ activity,
in turn decreasing lipid breakdown and mobilization (Mahali et al. 2014). The activation of mTORC1 upregulates PPARγ mRNA and protein in mouse embryo fibroblasts and 3T3-L1 pre-adipocytes (Zhang et al. 2009) and overexpressed PTEN (phosphatase and tensin homolog) inhibits expression of PPARγ (Wang et al. 2014). These data indicate that ERK/mTOR signalling is critical in regulating PPAR activation and, consequently, lipogenesis.

Lipin-1, a bifunctional protein, has important functions in the glycerol 3-phosphate pathway of triglyceride storage as a phosphatidate phosphatase (PAP) and a transcriptional coactivator/corepressor of metabolic nuclear receptors. The requirement of mTOR kinase activity for the effects of phosphorylation on the PAP activity of Lipin 1 is well established (Eaton et al. 2013; Brohée et al. 2015), but little is known about the relationship between Lipin 1 and ERK1/2 signalling.

Inner Mongolian Cashmere goat is a local variety of goat with the best cashmere and high-quality meat – intramuscular fat content is a key factor that influences meat quality. The impact of MAPKs signalling in cellular processes has extensively been addressed in human and mice but not in Cashmere goat, due to the lack of basic data in this animal. The role of ERK1/2 signalling in Cashmere goat foetal fibroblasts (GFbs) is unknown. To study the function of ERK signalling in cell proliferation and the accumulation of triglycerides (TAGs) and palmitic acid (PA) in Cashmere goat cells, we treated GFbs, a Cashmere goat cell model, with U0126 and observed its effects on cell proliferation and activation of ERK1/2 and mTOR signalling. Further, we examined the effects of U0126 on the expression of transcription factors and key enzymes that are related to TAG and PA synthesis in GFbs. We found that Lipin 1 level, PPARγ activation, and TAG and PA accumulation are regulated by ERK1/2 in GFbs.

**Materials and methods**

**Cell culture**

Inner Mongolian Cashmere GFbs were presented by Prof. Liu, State Key Laboratory of Reproductive Regulation & Breeding of Grassland Livestock, China. GFbs were obtained according to previously reported methods (Wang et al. 2012) and all animals and procedures used in this study were conducted according to the guidelines for the care and use of experimental animals established by the Inner Mongolia University Animal Care and Use Committee. GFbs were maintained as monolayer cultures in DMEM/F12 (Gibco, Paisley, PA49RF, Scotland, UK), supplemented with 10% foetal bovine serum (FBS) (Hyclone Laboratories, Inc. Logan, UT, USA), 100 U/mL penicillin G, and 100 mg/mL streptomycin (Sigma-Aldrich, Inc. St. Louis, MO, USA). Cell cultures were maintained and incubated at 37°C in humidified air with 5% CO₂. Morphology was examined by light microscopy.

**Reagents and antibodies**

U0126, a specific inhibitor of MEK (Sigma-Aldrich, Inc. St. Louis, MO, USA), was dissolved in DMSO (Sigma-Aldrich, Inc. St. Louis, MO, USA) to a stock concentration of 25 mM, stored at −20°C, and further diluted to the appropriate concentrations with culture media before use. The concentration of DMSO in the final solution did not exceed 0.5% (v/v) in all experiments. The following primary antibodies were purchased and used: anti-ERK1/2, anti-p-ERK1/2 (Thr202/Tyr204), anti-S6, anti-p-S6 (Ser240/244), anti-p-4EBP1 (Thr37/46) and anti-histone (Cell Signalling Technology, Inc., Beverly, MA, USA); anti-4EBP1 (Santa Cruz Biotechnology, Inc., CA, USA); anti-PPARγ and anti-Lipin 1 (Abcam, plc 330 Cambridge Science Park, Cambridge, UK); and anti-β-actin (Sigma-Aldrich, Inc. St. Louis, MO, USA). ECL Anti-Rabbit IgG-HRP and ECL Anti-Mouse IgG-HRP were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

**MTT assay and IC₅₀ calculation**

Exponentially growing cells were seeded into 96-well plates at 6 × 10³ cells per well 24 h before drug treatment. Then cells were incubated with U0126 at various concentrations (0.01 nM, 0.1 nM, 1.0 nM, 10 nM, 0.1 μM, 1.0 μM, 3.0 μM, 6.0 μM, 9.0 μM, and 12.0 μM) for 48 h to evaluate the inhibitory efficiency of U0126 on cell metabolic activity and formation accumulation in cells. The different volumes of the DMSO were added to the corresponding control group media, and the concentration of DMSO in the final solution did not exceed 0.5% (v/v). The medium with U0126 was absorbed, and fresh medium was added. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml, Sigma-Aldrich, Inc. St. Louis, MO, USA) was added to each well and incubated for 4 h at 37°C. The solution was absorbed, and formazan product was dissolved by adding 100 μl DMSO to each well and incubating it for 10 min at 37°C. MTT absorbance was measured at 490/630 nm with a spectrophotometer set (Thermo, Multiskan SX 353, USA). This assay was used to determine cell number in culture and IC₅₀ was calculated using a logit model, based on the data.

**Trypan blue exclusion assay**

The number of viable cells in treated groups with U0126 and control group (treatment with DMSO alone) was determined by trypan blue exclusion assay. GFbs were seeded into 24-well culture plates at 1 × 10⁵ per well and incubated for 24 h before drug treatment. Subconfluent cells were treated for 48 h with U0126 (0.3 μM, 3.0 μM), and the cells were harvested with trypsin on the indicated day after treatment and stained with trypan blue. Cell numbers were calculated using a hemocytometer.

**Western blot analysis**

GFbs were seeded into 6-well culture plates with medium at 5 × 10⁵ cells per well and incubated until 85% confluence. To determine the inhibitory effects of U0126 on the activation of ERK1/2 and its downstream targets S6 and 4EBP1 and the expression of PPARγ and Lipin 1, cells were treated with 1.5 μM U0126 for 1 h; untreated cells (treatment with DMSO alone) were used as a control.

To obtain total cell proteins, cells were washed 2 times with ice-cold PBS (pH 7.4), lysed in lysis buffer that contained 25 mM Tris-HCl (pH7.6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, protease inhibitor mixture, and...
phosphatase inhibitors (Sigma-Aldrich, Inc. St. Louis, MO, USA), and placed on ice for 10 min. Next, the cells were harvested by scraping and centrifuged at 12,000 rpm for 10 min at 4°C. The concentrations of protein lysate were measured by Bio-Rad protein determination method (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

To obtain cytoplasmic and nuclear protein extracts, treated cells were harvested with trypsin-EDTA, centrifuged at 2000 rpm for 5 min, washed by suspending the cell pellet in PBS, transferred to microcentrifuge tubes, and pelleted by centrifugation at 2000 rpm for 2–3 min. Cytoplasmic and nuclear proteins were extracted using the Thermo Scientific™ NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Thermo Fisher Scientific Inc. Meridian Road, Rockford, IL 61105, USA) per manufacturer. The concentrations of the lysate were measured by Coomassie Plus (Bradford) Assay (Thermo Fisher Scientific, Pittsburgh, PA, USA).

Equal amounts of protein (40 µg) were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to PVDF membranes, which were then immunoblotted with the designated primary antibodies. The membranes were treated with horse-radish peroxidase-conjugated goat anti-rabbit IgG or goat antimouse IgG (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and signals were detected using ECL (Thermo Fisher Scientific, Pittsburgh, PA, USA). The content of TAG (PA) was normalized by the amount of protein in lysates.

Enzyme-linked immunosorbent assay
GFbs were seeded into 6-well plates at 5x10^5 cells/well, incubated until 80% confluence, and treated with 1.5 μM U0126 for 24 h or 10 μM U0126 for 1 h, untreated cells (treatment with DMSO alone) were used as a control. Cell lysates were prepared by 5 freeze–thaw cycles and standardized with the same protein concentration in control groups and treatment groups by adjusting the volume of the protein lysate. Equal volume of protein lysates were measured for TAG and PA using enzyme-linked immunosorbent assay (ELISA) kits (Beijing winter song Boye Biotechnology Co. Ltd. Beijing, China) according to the manufacturer’s instructions. The absorbance was read at 450 nm and 570 nm on a Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Pittsburgh, PA, USA). The content of TAG (PA) was normalized by the amount of protein in lysates.

Statistical analysis
Descriptive statistics were generated for all quantitative data, which were expressed as mean ± SD. Normally distributed data were analysed utilizing standard parametric statistics including Student’s t test or one-way ANOVA following Tukey’s method. Each assay was performed in triplicate.

Results
U0126 inhibits proliferation of GFbs
To observe the effects of U0126 on cell proliferation of GFbs, subconfluent cells were treated with U0126 and harvested to calculate cell numbers by trypan blue exclusion assay. As shown in the growth curve, GFP growth was clearly inhibited from Days 1 to 9 after treatment with 0.3 μM and 3.0 μM of U0126 (Figure 1). The mean cell number of treatment groups at different time points from day 2 to day 9 was decreased significantly compared to control, respectively (p < .01). To determine the inhibitory efficiency of U0126 on GFbs growth and optimize its concentration for subsequent experiments, we calculated its half maximal inhibitory concentration (IC_{50}) against GFbs. GFbs were treated with various concentrations of U0126 (10^{-7}–12 μM) for 48 h, and the percentage of viable cells in control group and treatment groups were determined by MTT assay, and then the inhibitory efficiency was assessed through comparison of two groups. The IC_{50} of U0126 against GFbs was 1.54 μM (Figure 2).

U0126 inhibits phosphorylation of ERK1/2 and mTOR signalling
To determine the inhibitory effects of U0126 on ERK1/2 and mTORC1 signalling, we treated GFbs for 1 h with 1.5 μM U0126, which was a concentration that was close to the IC_{50} value. The protein level and phosphorylation levels of ERK1/2, S6, and 4EBP1 were measured by western blot. The phosphorylation of ERK1/2 (Thr202/Tyr204), S6 (Ser240/244), and 4EBP1 (Thr37/46) was significantly inhibited (Figure 3). S6 and 4EBP1 are two downstream effectors of mTORC1, these data suggest that the activation of ERK/mTOR signalling is attenuated by the ERK1/2 inhibitor U0126.

U0126 inhibits cytoplasmic lipin 1 level and nuclear PPARY protein abundance in GFbs
To examine the effects of U0126 on Lipin 1 level and the activation of PPARY, we measured Lipin 1 and PPARY levels in the
Figure 2. Inhibitory efficiency of U0126 on percentage of viable GFb cells. GFbs were treated with various concentrations of U0126 (0.01 nM–12 µM) for 48 h and subjected to MTT assay. Each column is given as the percentage of live cells compared with the control (cells treated with an equal volume of vehicle (absolute DMSO), which was set at 100%. Each assay was performed in triplicate (NS, not significant compared to control group) (**p < .01).

Figure 3. U0126 inhibits the activities of downstream ERK1/2 and mTORC1 signalling in GFbs. (a) The phosphorylation of ERK1/2 (Thr202/Tyr204), 4EBP1 (Thr37/46), and S6 (Ser240/244) is inhibited by U0126. (b–d) The resolved bands were quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). Each assay was performed in triplicate (*p < .05).
cytoplasmic protein content and the accumulation in the nucleus by western blot. The cytoplasmic Lipin 1 level was downregulated by U0126, but its accumulation in the nucleus was unchanged significantly versus PPARγ accumulates in the cytoplasm but decreases in the nucleus in treated cells with 1.5 μM U0126 for 1 h. β-actin serve as the loading control of cytoplasm protein and histone serve as same role in nucleus protein. (b–e) The resolved bands were quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). Each assay was performed in triplicate (*p < .05) (NS, not significant compared to control group).

Figure 4. U0126 reduces the cytoplasmic level of Lipin 1 and the nuclear PPARγ abundance in GFbs. (a) The cytoplasmic Lipin 1 level was downregulated by U0126, but its accumulation in the nucleus was unchanged significantly versus PPARγ accumulates in the cytoplasm but decreases in the nucleus in treated cells with 1.5 μM U0126 for 1 h. β-actin serve as the loading control of cytoplasm protein and histone serve as same role in nucleus protein. (b–e) The resolved bands were quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). Each assay was performed in triplicate (*p < .05) (NS, not significant compared to control group).
U0126 decreased triglyceride and palmitic acid concentration in GFbs

To determine whether ERK1/2 regulates TAG and PA accumulation in GFbs, we examined the effects of U0126 on this process in GFbs. Cells were treated with 1.5 μM U0126 for 24 h and with 10 μM U0126 for 1 h, and TAG and PA in cell lysates were measured by ELISA. The content of TAG (PA) was normalized by the amount of protein in lysates. U0126 decreased the concentration of TAG at 1.5 μM for 24 h non-significantly ($p > .05$) (Figure 5(a)), but significantly at 10 μM for 1 h ($p < .05$) (Figure 5(b)). PA concentration was also significantly reduced by U0126 (Figure 6) ($p < .01$), indicating that TAG and PA accumulation are regulated by ERK1/2 signalling in GFbs.

Discussion

The highly conserved ras-MAPK signalling pathway channels a wide variety of extracellular signals to govern cellular processes in response to extracellular mitogens, hormones, and neurotransmitters. ERK1 and ERK2 are key transducers of upstream signals, and the Ras-Raf-MEK-ERK cascade is an important pathway that regulates many cellular functions, including cell proliferation, survival, differentiation, angiogenesis, and migration (Lei et al. 2014). ERK1/2 signalling controls transcription by targeting several regulators of gene expression, such as transcription factors and histone proteins (Ciccarelli & Giustetto 2014) – inactivation of ERK1/2 signalling inhibits cell proliferation.

Figure 5. U0126 decreased TAG concentration in GFbs. (a) TAG in cell lysate was detected by ELISA after cells were treated with 1.5 μM U0126 for 24 h ($p > .05$); (b) TAG in cell lysate was detected by ELISA after cells were treated with 10 μM U0126 for 1 h ($p < .05$). Cells treated with an equal volume of vehicle (absolute DMSO) were used as a control. U0126 significantly decreased the concentration of TAG in cells. Each assay was performed in triplicate ($p < .05$) (NS, not significant compared to control group).

Figure 6. PA concentration is significantly decreased by U0126. (a) PA in cell lysates was measured by ELISA after cells were treated with 1.5 μM U0126 for 24 h; (b) PA in cell lysates was determined by ELISA after cells were treated with 10 μM U0126 for 1 h. Cells treated with an equal volume of vehicle (absolute DMSO) were used as a control. U0126 significantly decreased the concentration of PA in both groups ($p < .01$). Each assay was performed in triplicate ($**p < .01$).
In our study, we determined the IC$_{50}$ of U0126 against GFbs was 1.54 μM, and a concentration of 1.5 μM U0126 was used to pretreat GFbs for 1 h and the activation of the ERK1/2 was inhibited. Meanwhile, cells were treated with 1.5 μM U0126 for 24 h, the content of TAG in cells was decreased without significant difference ($p > .05$) while PA concentration was significantly reduced ($p < .01$). These data indicate that the phosphorylation of ERK1/2 can be inhibited by U0126 in a low concentration and short time manner versus long time was needed for low concentration to reduce accumulation of TAG and PA in GFbs. On the other hand, we considered the effects of U0126 on different cells, including endometrial stromal cells (Matsuzaki et al. 2017), cancer cells (Ong et al. 2015) and mesenchymal stem cells (Xu et al. 2015), we choose 10 μM treated for 1 h as a group which is high concentration for short time treatment. The data indicate that accumulation of TAG and PA can be attenuated significantly ($p < .01$) in GFbs.

Whether an interaction exists between Lipin-1, PPARγ, and ERK1/2 signalling is unknown. Zhang et al. (2012) reported that phosphatidate species accumulate and ERK signalling is activated in adipose tissue in Lipin-1-deficient mice. Lipin-1 PAP activity modulates phosphatidate levels to promote PPARγ expression during adipogenesis (Zhang et al. 2012).

Conversely, a recent report suggests that Lipin-1 that is induced by PPAR agonists decreases the levels of PPAR and ERK1/2 phosphorylation in 3T3-L1 cells and that PPAR agonists induce adipocyte differentiation by modulating the expression of Lipin-1 (Kim et al. 2016). In addition, ERK1/2 cooperates with mTOR signalling in cell proliferation and metabolism as a result of their crosstalk, based on direct or indirect interaction. ERK1 and ERK2 interact with Raptor in cells and mediate its phosphorylation in vivo and in vitro (Carriere et al. 2011). We propose that the ERK1/2 pathway is critical in the activation of transcription factor PPARγ and the expression of key enzyme Lipin 1, and mTOR signalling may be involved in TAG and PA accumulation in GFbs.

In this study, we used the ERK-specific inhibitor U0126 to treat GFbs and found that ERK1/2 activation was suppressed while the phosphorylation of S6 (Ser240/244) and 4EBP1 (Thr37/46), two downstream effectors of mTORC1, was inhibited. Cytoplasmic Lipin 1 level and the nuclear PPARγ protein abundance were downregulated by U0126. Moreover, U0126 impeded TAG and PA concentration in GFbs. These data indicate that ERK/mTOR signalling is related to TAG and PA accumulation in GFbs, the inhibition of which is presumed to be attributed to decreased cytoplasmic Lipin 1 level and PPARγ accumulation in the nucleus.

Inner Mongolian Cashmere goat is a local goat variety that has evolved through long-term natural and artificial selection, with some particularities with regard to using and metabolizing fatty acids to produce lipids. This study has demonstrated that U0126 inhibits the activation of ERK1/2 and mTORC1 signalling. ERK1/2 signalling has an important function in TAG and PA accumulation of Cashmere GFbs.

**Conclusions**

We provide novel evidence that ERK1/2 signalling is critical in lipid metabolism in GFbs. U0126, a specific inhibitor of ERK, inhibits the activation of ERK and mTORC1 signalling, affecting the cytoplasmic level of Lipin 1 and the nuclear PPARγ protein abundance and decreasing TAG and PA concentration in GFbs. We propose that ERK1/2 is a key regulator of the expression level of Lipin 1, the activation of PPARγ, and TAG and PA accumulation in GFbs.

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**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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