Metformin prevents the psoriasis under hyperlipidemia via activation of the FOXO3

Chengxin Li (chengxinderm@163.com)

The First Medical Center of Chinese PLA General Hospital

Li Zhang

The First Medical Center of Chinese PLA General Hospital

Xiaoling Liu

The First Medical Center of Chinese PLA General Hospital

Min Huang

The First Medical Center of Chinese PLA General Hospital

Rui Wang

The First Medical Center of Chinese PLA General Hospital

Wenwei Zhu

The First Medical Center of Chinese PLA General Hospital

Yu Li

The First Medical Center of Chinese PLA General Hospital

Lin Shen

The First Medical Center of Chinese PLA General Hospital

Fang Xie

The First Medical Center of Chinese PLA General Hospital

Wenjuan Wang

The First Medical Center of Chinese PLA General Hospital

Cuihao Song

The First Medical Center of Chinese PLA General Hospital

Zijie Tang

The First Medical Center of Chinese PLA General Hospital

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Abstract

Background: Psoriasis patients have obvious lipid metabolism disorders. The hyperlipidemia aggravates the development of psoriasis. In this study, we investigated that under the hyperlipidemia conditions, whether metformin could alleviate psoriasis via a Forkhead box O 3 (FOXO3)-driven pathway, as well as the mechanisms underlying.

Methods: Wild type or FOXO3 knockdown HaCat cells were treated with free fatty acids (FFA) for 10 days and then co-treated with metformin for another 4 days. Triglyceride (TG) level, cell viability, proliferation, apoptosis, antioxidant enzymes, ROS levels, as well as the transcription activity of FOXO3 were analyzed.

Results: Our data showed that FFA treatment promoted the proliferation and inhibited the apoptosis in HaCat cells. However, metformin could reversed all of these changes. Metformin treatment significantly increased the expressions and the activities of superoxide dismutase (SOD) and catalase (CAT), and reduced the reactive oxygen species (ROS) level, while FFA inhibited the antioxidant enzymes and increased the ROS level in HaCat cells. Metformin significantly promoted the autophagy and increase FOXO3 protein level in the nucleus under hyperlipidemia conditions, which was significantly inhibited by FFA treatment. However, all of the effects from metformin were partially blocked by FOXO3 knockdown.

Conclusion: This study demonstrated that under the hyperlipidemia conditions, metformin has significant antiproliferation and proapoptosis effects by reducing ROS level as well as increasing autophagy. Both of these were responsible for the alleviation of psoriasis. All of these effects from metformin were through FOXO3-dependent pathway.

Background

Psoriasis is a common chronic inflammatory skin disease that affects approximately 3% of the population worldwide. The phenotype of psoriasis is characterized by the development of erythematous papules and overlying scaly plaques [1]. Psoriasis is associated with a variety of factors, including innate and adaptive immune responses, genetic factors, environmental factors and metabolic disorders [2]. Beyond the skin, psoriasis is often associated with comorbidities such as metabolic syndrome [3]. A retrospective study has shown that the incidence of metabolic syndrome in patients with psoriasis is much higher than that in healthy people. Psoriasis is a chronic systemic inflammatory skin disease associated with dyslipidemia [4]. Significantly abnormal lipid metabolism is shown by patients with psoriasis. Elevated cholesterol, triglyceride, and low-density lipoprotein (LDL) levels can accelerate the adhesion and aggregation of platelets as factors affecting psoriasis [5, 6]. As the underlying mechanism is still unclear, to date, no effective drug for treating psoriasis is available.

Excessive and long-term lipid accumulation can induce oxidative stress in the cell. It has been demonstrated oxidative stress contributes to the development of psoriasis [7]. Impaired antioxidant system, together with excessive reactive oxygen species (ROS) production, is involved in the pathogenesis of psoriasis. External detrimental agents such as cigarette smoking, air pollution, physical

damages as well as biological agents (virus, bacteria etc.) can trigger a keratinocytes damages by an overproduction of ROS [8]. Several studies have found there is an increased ROS production in psoriatic skin [7]. Furthermore, it has been demonstrated that Hydrogen peroxide (H_2O_2) could promote cell proliferation in the tissue regeneration as well as embryonic development [9]. A series of antioxidative enzymes including superoxide dismutase (SOD) and catalase (CAT), comprise the antioxidant defense system, which scavenges excessive ROS to maintain the normal function of the cell. We speculated that in psoriatic skin, the expressions of several antioxidative enzymes decrease, which would be responsible for the increased ROS level. As a consequence, increased ROS may aggravate the psoriasis through stimulating keratinocytes proliferation.

It is well accepted that long-term hyperlipidemia inhibits the autophagy in a series of metabolic diseases. Autophagy is a self-digestion process that occurs in all cells. Basal level of autophagy in a cell helps to maintain its homeostatic state and normal function under stressful conditions. It has widely accepted that autophagy is up-regulated during the early stage of non-alcoholic fatty liver disease (NAFLD) as an attempt to prevent lipid accumulation. However, excessive lipid accumulation in the liver for a long time would block the autophagy process [10]. It has been well established that many therapeutic strategies induce apoptosis through promoting autophagy in tumor cells. Therefore, we speculated that increasing autophagy in free fatty acids (FFA) treated keratinocytes would induce apoptosis and ameliorate psoriasis effectively.

Forkhead box O 3 (FOXO3) is a crucial transcription factor in various biological processes, including development, proliferation, apoptosis, metabolism, and differentiation, by regulating a wide spectrum of genes [11]. It inhibits cell proliferation through transcription of multiple cell cycle kinase inhibitors like p16, p21 and p27. Under more oxidizing conditions, to counteract elevated ROS production in the cell, FOXO3 mediates the transcription of antioxidant genes including CAT and SOD2 [12]. In addition, FOXO3 initiates several autophagy pathways to repress cell proliferation. In response to the accumulation of stress, FOXO3 may activate both the ubiquitin-proteasome pathway and the transcription of autophagy-related genes such as those encoding LC3 and BNIP3, inducing the formation of autophagy [13]. As these, we speculated that FOXO3 would be a drug target for regulating cell proliferation and apoptosis in psoriasis.

Metformin is utilized as a first-line antidiabetic agent for the treatment of type 2 diabetes mellitus (T2DM), as it has been shown to improve metabolic homeostasis including hyperglycemia and dyslipidemia. Previous studies have demonstrated that the long-term use of metformin is associated with a reduced risk of psoriasis [14, 15]. However, further studies are required to identify the potential mechanism.

In our current study, we demonstrated for the first time that under the hyperlipidemia conditions, metformin suppressed cell proliferation and induced apoptosis in FFA treated human keratinocyte via FOXO3-dependent pathway. Metformin activated the FOXO3 to upregulate expressions of antioxidative enzymes SOD2 and CAT to reduce ROS. Consequently, it would block the proliferation caused by ROS.

Meanwhile, FOXO3 activation induced the apoptosis by increasing autophagy. Both of these were responsible for the alleviation of psoriasis under hyperlipidemia.

Methods

Cell Culture

HaCat cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Invitrogen, NY, USA) and 1% penicillin/streptomycin (Gibco, Invitrogen, NY, USA) (defined as full medium in this study) in a 5% CO₂ atmosphere at 37°C. The mixture of oleate (Sigma-Aldrich, MO, USA) and palmitate (Sigma-Aldrich, MO, USA) was used as FFA to induce the lipid accumulation in the cells [16]. In brief, 0.5 M bovine serum albumin (BSA) (Roche, Basel, Switzerland) solution was prepared by dissolving it in 150 mM NaCl solution. Oleate and palmitate were dissolved in 0.5 M BSA to make a 4 mM total FFA mixture (Oleate: palmitate 2:1 molar ration), and the products were filtered. The stock solution was conveniently diluted in culture medium (1:10) to obtain the 400 µM final concentration. After the cells were treated with FFA for 10 days, the metformin was added to the intervention groups to co-treat the cells with FFA for additional 4 days.

Cell Viability Assay

HaCat cells were seeded into 96-well plates at 5×10^3 cells/well and incubated for increasing durations (0, 12, 24, 48, and 72 hours) before adding 10 µL of Cell Counting Kit-8 (CCK8) (Topscience, Shanghai, China) solution per well for 1 hour. Absorbance (450 nm) was measured using a quantitative automatic microplate reader.

Apoptosis Assay

Apoptosis was measured with a apoptosis kit (Beyotime Biotechnology, Shanghai, China). HaCat cells were detached with 0.25% trypsin/0.01% EDTA (Gibco, Invitrogen, NY, USA) and washed with cold 1x PBS for one time. Cells were then resuspended in 1· annexin-binding buffer to $1 \cdot 10^6$ cells/ml, stained with 100 ml binding buffer containing 5 ml Annexin V for 15 min and 1 ml of 100 mg/ml PI for additional 5 min at room temperature. Cells were analyzed using BD Accuri C6 (BD biosciences, CA, USA).

SOD Activity and CAT Activity Assay

SOD activity and CAT activity were determined by Total Superoxide Dismutase Assay Kit and Catalase Assay Kit respectively (Beyotime Biotechnology, Shanghai, China). The experimental procedures were strictly according to the instruction from the vendor.

Western Blotting

The western blot analysis was performed using specific primary antibodies. Anti-SOD2 and Anti-CAT antibodies were from Cell Signaling Technology (Beverly, MA, USA). Anti-LC3 antibody was from Novus Biologicals (Centennial, CO, USA). Anti-P62 antibody was from Abcam (Abcam, Cambridge, UK). Anti-

Tubulin antibody was from Bioworld Technology (Louis Park, MN, USA). The membranes were incubated with primary antibodies for at 4°C and species-specific secondary antibodies for 1 h at room temperature. RIPA lysis buffer was used to prepare cell lysates. Nuclear extracts was prepared with NE-PER nuclear and cytoplasmic extraction reagents (Thermo, MA, USA). 10–20 µg protein was loaded and separated on SDS-PAGE gels. Fractionated proteins were then transferred to nitrocellulose membranes, blocked in 5% nonfat milk for 2 h, and probed overnight with primary antibodies. Immunoblots were washed three times (5 min each) with TBS containing 0.1% Tween 20 and then incubated with horseradish peroxidase conjugated secondary antibody for 2 h. The densitometry of all the bands was analyzed by Image J and normalized to Tubulin.

Quantitative RT-PCR

Total RNA was isolated from cells using TRIzol (Thermo, MA, USA) following the manufacturer's instructions. Then 1µg of RNA was reverse-transcribed with iScript™ cDNA Synthesis Kit (BioRad, CA, USA). The following PCR primers were used: human p16, 5- *GA C* (forward) and 5- GCATGGTTACTGCCTCTGGT-3 (reverse); human p21, 5-CATTTGGTGGACCCAAAGAC-3 (forward) and 5-TTGCAGGTCGCTTCCTTATT-3 (reverse); human actin, 5-GAGCGCGGCTACAGCTT-3 (forward) and 5-TCCTTAATGTCACGCACGATTT-3 (reverse). Quantitative RT-PCR analysis was performed with the Power SYBR™ Green PCR Master Mix (Thermo, MA, USA) and the CFX Connect Realtime System (BioRad, CA, USA). Relative gene expression was obtained after normalization to actin expression. Fold differences in comparisons were expressed as relative mRNA levels using the $2^{-\Delta\Delta Ct}$ method.

Intracellular ROS

Intracellular ROS was measured by flow cytometry using DCFH-DA (Sigma-Aldrich, MO, USA) as the fluorescent probe. Briefly, after the treatment, the cells were washed once with PBS and then incubated with 10µM DCFH-DA in fresh medium for 40 min in the cell culture incubator. After the incubation, the cells were trypsinized and collected, and then washed three times with PBS before flow cytometry analysis (Excitation 488 nm/Emission 525 nm for DCFH-DA).

Autophagic Flux Quantification

Autophagic flux in RFP-GFP-LC3 adeno virus (Orbitalgene, Xi'an, China) infected HaCat cells was performed using an imaging-based assay as previously reported [17]. Briefly, 30–60 cells for each condition were quantified. The red only was considered as autolysosome, while yellow was early autophagosome alone (Red and Green = yellow). Because of the acidic pH, the GFP fluorescence was diminished while RFP still remains stable. The conversion of yellow puncta to red puncta provided a readout for autophagic flux. The puncta in cells were analyzed with a confocal laser scanning microscope (LSM800, Carl Zeiss), using a 40× oil immersion objective. The yellow puncta and red only puncta were quantified with the Image J program.

shRNA Lentivirus Knockdown

The FOXO3 shRNA and scramble shRNA lentiviruses were obtained from Orbitalgene Co. Ltd (Orbitalgene, Xi'an, China). The shRNA targeting the FOXO3 coding sequence was as follow: FOXO3-shRNA (NM_001455.4) : 5'- CCGGCACCATGAATCTGAATG – 3', Scramble-shRNA : CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG. Scramble shRNA was treated as a negative control group. HaCat cells were infected with FOXO3 shRNA and scramble shRNA lentivirus and the cells were selected with 1 µg/ml puromycin according to the manufacturer's protocol. Stably FOXO3 knockdown cell line was confirmed by western blotting.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (version 6.01). Data are presented as means ± SEM. One-way or two-way ANOVA were performed and significance was accepted at $P < 0.05$.

Results

Metformin inhibited FFA-treated human keratinocyte proliferation.

Human HaCat cells were treated with FFA for 10 days and then cotreated with different concentrations of metformin for another 4 days. FFA treatment increased the TG level in HaCat cells, which suggested that excessive lipid accumulation (Fig. 1a). Data from CCK8 assay showed that FFA significantly increased cell viability, while metformin hindered cell growth in a dose-dependent manner (Fig. 1b). qPCR data showed that metformin upregulated expressions of p16 and p21 which could arrest the cell cycle in the G1 phase and suppress cell proliferation (Fig. 1c and d). Apoptosis was assessed by flowcytometry. Our data showed that FFA treatment significantly promoted cell proliferation and inhibited cell apoptosis, while it was reversed by metformin (Fig. 1e).

Metformin alleviated oxidative stress in FFA-treated human keratinocyte.

In FFA-treated HaCaT cells, FFA treatment significantly decreased the SOD2 and CAT protein levels as well as enzyme activities of total SOD and CAT. Here, metformin (at 40 mM) upregulated the protein levels as well as activities of these antioxidant enzymes (Fig. 2a, b, and c). Furthermore, flow cytometry of DCFH-DA revealed that the intracellular ROS level of FFA-treated HaCaT cells was significantly increased, and this was effectively reversed by metformin at 40 mM (Fig. 2d). All of these data suggested that decreasing intracellular ROS level could be important mechanism for metformin inhibiting proliferation in FFA-treated HaCaT cells.

Metformin promoted autophagy in FFA-treated human keratinocyte.

Next, we sought to determine whether metformin promoted keratinocyte autophagy. Western blot data showed that FFA treatment significantly decreased the protein levels of LC3II/LC3I, increased the P62, suggesting the autophagy was suppressed by FFA. However, the inhibition of autophagy by FFA was reversed by metformin at 40 mM concentration (Fig. 3a). To confirm this, FFA treated cells were transfected with a tandem fluorescent tagged RFP-GFP-LC3 adenovirus to examine the autophagy flux. Our data showed that FFA treatment significantly blocked the autophagy flux, illustrated by more early autophagosome (yellow puncta on colocalization) with less auto-lysosome (red only puncta), whereas metformin treatment showed less early autophagosome with more autolysosome, (Fig. 3b). These results demonstrated metformin reversed FFA-caused suppression of autophagy.

Activation of FOXO3 contributed to the antiproliferative effects of metformin through reducing ROS in FFA-treated human keratinocyte.

In this study, compared with BSA treated group, FFA treatment inhibited the nuclear translocation of FOXO3. However, metformin remarkably reversed this effect, which meant metformin could increase activity of FOXO3 (Fig. 4a). To further confirm whether FOXO3 mediated the antiproliferative roles of metformin in FFA-treated human keratinocyte, we speculated FOXO3 shRNA lentivirus to knockdown FOXO3 in HaCat cells as confirmed by western blot (Fig. 4b). In scramble control group, consistent with above data, metformin inhibited cell viability, upregulated p16 and p21 gene expressions, whereas FOXO3 knockdown significantly attenuated these effects (Fig. 4c,d and e). To analyze the role of FOXO3 in FFA-induced ROS generation, we firstly detected the cellular ROS level. The generation of ROS was significantly suppressed by metformin, while FOXO3 knockdown blocked this effect (Fig. 4f). Then we measured the activities of antioxidant enzyme SOD and CAT as well as the expressions of SOD2 and CAT after FOXO3 knockdown. Results showed FOXO3 knockdown reversed the effects of metformin on increasing activities and protein levels of these two antioxidant enzyme (Fig. 4g, h and i). All of above implied that in FFA-treated human keratinocyte, metformin inhibited proliferation potentially through promoting FOXO3 activity to reduce ROS level.

FOXO3 mediated the proapoptosis of metformin through inducing autophagy in FFA-treated human keratinocyte.

We determined if FOXO3 was involved in the effects of metformin on proapoptosis in FFA-treated human keratinocyte. In scramble control group, metformin induced apoptosis in FFA-treated HaCat cells, while this effect was blocked by FOXO3 knockdown (Fig. 5a). Furthermore, metformin induced the autophagy

in FFA treated HaCat cells, assessed by protein levels of LC3II/LC3I and P62. However, these effects were weakened by FOXO3 knockdown (Fig. 5b). These results suggested that metformin increased FOXO3 activity to promote apoptosis through inducing autophagy in FFA-treated keratinocyte, which might contribute to its inhibitory role in psoriasis under hyperlipidemia.

Discussion

Psoriasis is a multifactorial skin disease that inconveniences many patients. As abnormally excessive and rapid growth of the keratinocytes accounts for the skin lesion of psoriasis, inhibition of these cellular events is the principal method of treating psoriasis [18]. Despite remarkable advances in understanding the pathogenesis of psoriasis, the chronological order of keratinocyte hyperproliferation has not been completely elucidated. Normally, skin cells are replaced every 28 to 30 days; however, in patients with psoriasis, skin cells are replaced every 3 to 5 days. These alterations are believed to originate from the excessive proliferation of keratinocytes [19]. Therefore, the strategies for proliferation inhibition would be effective treatment of psoriasis.

Psoriasis patients have been reported to frequently exhibit dyslipidemia. Several studies have demonstrated a linear correlation between body mass index (BMI) and risk of incident psoriasis existed [5, 20]. Other clinical researches have also demonstrated the existence of lipid metabolism disorders in patients with psoriasis, which is more likely to be complicated with fatty liver [21, 22]. Hence, dyslipidemia could be the important risk factor for the development of psoriasis. However, the underlying mechanism of lipid inducing psoriasis is still unknown [23, 24]. In the present study, we treated the HaCat cells with FFA to create an hyperlipidemia microenvironment *in vitro*. We found that ROS, induced by excessive lipid accumulation, could be the key factor to promote the proliferation of keratinocytes in psoriasis. Currently, several studies have been proved that ROS is an key factor for cell proliferation in different cell and animal models [25]. Therefore, understanding the mechanism of regulating ROS generation in psoriasis under hyperlipidemia conditions may be an important hint to cure this intricate disease.

Many studies have demonstrated that metformin could ameliorate psoriasis through repressing inflammation via different pathways in the keratinocytes. In our study, we have for the first time shown that metformin significantly decreased ROS level and promoted autophagy in FFA treated keratinocytes, which contributed to the antiproliferation. Increased ROS production and decreased antioxidant system functions have been demonstrated to be associated with the pathogenesis of psoriasis [7, 26]. One study found ROS at high levels promotes the function of Treg and so prevent the psoriatic dermatitis induced by imiquimod [27]. Another study has shown that metformin increases ROS production and then promote apoptosis [28]. In all of these studies, ROS is considered as the factor to aggravate inflammation. By contrast, in the present study, metformin reduced ROS level to inhibit proliferation of HaCat cells, as ROS could be the key signal factor to promote cell proliferation. Like the dual function in cancer, ROS can also act as a regulator or a suppressor of immune-mediated diseases. At high level, ROS would induce apoptosis and inhibit proliferation, while low level of ROS would only be as an regulator to induce

proliferation. FFA treatment is a mild stress which stimulated a low level of ROS production. At this condition, metformin plays a major role in repressing ROS to inhibit proliferation.

In addition, we found autophagy was decreased in the HaCat cells treated with FFA. Normal levels of autophagy have been shown to be involved in various types of physiological activities. Defects in autophagy lead to inflammatory cytokine production and cell proliferation in keratinocytes, thus inducing the occurrence of psoriasis. PSORI-CM02 depresses the proliferation of TNF- α -stimulated HaCat cells via induction of autophagy. The classical treatments for psoriasis such as retinoids, vitamin D analogues and ultraviolet B therapy can induce autophagy, which suggests that the clinical benefits of these drugs may be related to autophagy activation [29, 30]. Excessive lipid accumulation inhibits autophagy in different cells. Consistent with these studies, in the present study, metformin promoted the autophagy to induce apoptosis in FFA treated HaCat cells.

FOXO transcription factor family has emerged as a central player in cells proliferation and antioxidant defense. Researches have shown that the phosphorylation of FOXO proteins can regulate cell survival by manipulating their target genes and some of the target genes may play an important role in the suppressing cell proliferation [31]. Within the nucleus, FOXO inhibits proliferation most likely by inducing the expression of genes that are critical for cell growth like p16, p21 as well as p27. Activation of FOXO blocks cellular proliferation and drives cells into a quiescent state [11]. FOXO3 also triggers the expression of several antioxidant enzymes, such as SOD2 and CAT, to cope with oxidative stress [32]. There are several studies investigate the relationship between FOXO and the proliferation of psoriatic keratinocyte, and find out that the gene expression and the activity of FOXO3 are both significantly decreased in psoriatic lesions compared with that uninvolved psoriatic lesions and normal skin [33]. In common with these findings, we found FFA treatment significantly decreased the FOXO3 level in nucleases, while metformin upregulated its activity, increased the expression of p21, SOD2 as well as SOD. FOXO3 knockdown partially blocked these effects from metformin. It suggested under hyperlipidemia conditions, metformin induced autophagy and reduced ROS to suppress keratinocyte proliferation via promoting FOXO3 activity.

Conclusion

In summary, the current findings provided the first evidence for the mechanism of metformin action in psoriasis under hyperlipidemia conditions. This study provided the first results to demonstrate that through increasing FOXO3 activity, metformin inhibited FFA-treated human keratinocyte proliferation by reducing ROS level. Meanwhile, metformin induced the apoptosis by increasing autophagy. Both of these were responsible for the alleviation of psoriasis under hyperlipidemia. Our data suggested lipid metabolic disorder could aggravate psoriasis, and metformin might be a promising medication in the treatment of psoriasis through activating FOXO3.

Abbreviations

FOXO3: Forkhead box O 3; FFA: Free fatty acids; ROS: Reactive oxygen species; DCFH-DA: 2,7-dichlorofluorescein-diacetate; SOD: Superoxide dismutase; CAT: Catalase; LDL: Low-density lipoprotein; H₂O₂: Hydrogen peroxide; NAFLD: Nonalcoholic fatty liver disease; T2DM: Type 2 diabetes mellitus; CCK8: Cell counting kit-8; TG: Triglyceride; qPCR: Quantitative RT-PCR; BMI: Body mass index

Declarations

Authors' contributions

L.Z. and C.-X.L., created the outline and drafted the manuscript, designed the research, data acquisition, analysis, and interpretation of data. L.Z., X.-L.L, M.H., R.W., W.-W.Z., Y.L., L.S., F.X., W.-J.W., and C.-H.S. performed the experiments, data acquisition, analysis, and interpretation of data and manuscript drafting. Z.T. contributed to statistical analysis. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The data collected for the study are available from the corresponding author upon request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Metformin prohibited FFA-treated human keratinocyte proliferation. HaCat cells were treated with 400 mM FFA for 10 days, and then the intervention groups were co-treated with metformin at different concentrations and FFA for additional 4 days. (a) Metformin inhibited TG level of HaCat cells. (b) Metformin inhibited HaCat cell proliferation analyzed by CCK8. Metformin (40mM) increased the mRNA levels of p16 (c) and p21 (d). (e) Metformin (40mM) promoted HaCat cells apoptosis. Representative FACS analysis (left panel) and the ratios (right panel) of annexin V⁺ PI⁻ HaCat cells. Data are presented as the mean $\pm$ SD and are representative of three independent experiments. * $p < 0.05$.

Figure 2

Metformin alleviated oxidative stress in FFA-treated human keratinocyte. HaCat cells were treated with 400 mM FFA for 10 days, and then the intervention groups were co-treated with metformin (40mM) and FFA for additional 4 days. (a) Immunoblot of SOD2 and CAT in HaCat cells. Total (b) CAT activity and (c) SOD activity in HaCat cells. (d) Total ROS levels in the HaCat cells. Data are presented as the mean $\pm$ SD and are representative of three independent experiments. * $p < 0.05$.

Figure 3

Metformin promoted autophagy in FFA-treated human keratinocyte. HaCat cells were treated with 400 mM FFA for 10 days, and then the intervention groups were co-treated with metformin (40mM) and FFA for additional 4 days. (a) Immunoblot of LC3 and P62 in HaCat cells. (b) Representative confocal images of HaCat cells expressing GFP-RFP- LC3 and quantitation of early autophagosome puncta and autolysosome puncta following FFA and metformin treatment. Yellow showed co-localization of GFP and RFP, indicating early autophagosomes. Red only showed autolysosomes, Scale: 20 μ m. Data are presented as the mean $\pm$ SD and are representative of three independent experiments. $*p < 0.05$.

Figure 4

FOXO3 was involve in the reduction of ROS by metformin in FFA-treated human keratinocyte. HaCat cells were treated with 400 mM FFA for 10 days, and then the intervention groups were co-treated with metformin (40mM) and FFA for additional 4 days. (a) Immunoblot of FOXO3 in the nucleus of HaCat cells. HaCat cells were transfected with FOXO3 shRNA or scramble control (SC) by lentivirus. (b) Immunoblot of FOXO3 in the HaCat cells. HaCat cells were pretransfected with FOXO3 shRNA or SC by lentivirus, then treated with 400 mM FFA for 10 days. After that, the intervention groups were co-treated with metformin (40mM) and FFA for additional 4 days. (c) Proliferation of the HaCat cells detected by CCK8. mRNA levels of p16 (d) and p21 (e) in the HaCat cells. ROS levels (f), total SOD activities (g) and CAT activities (h) in HaCat cells. (i) Immunoblot of SOD2 and CAT in HaCat cells. A : BSA; B : FFA; C: FFA+Metformin (40mM). Data are presented as the mean $\pm$ SD and are representative of three independent experiments. $*p < 0.05$.

a **b** **Figure 5**

FOXO3 mediated the effect of pro-autophagy by metformin in FFA-treated human keratinocyte. HaCat cells were pretransfected with FOXO3 shRNA or scramble control (SC) by lentivirus, then treated with 400 mM FFA for 10 days. After that, the intervention groups were co-treated with metformin (40mM) and FFA for additional 4 days. (a) Cell apoptosis was detected by FACS analysis. (b) Immunoblot of LC3 and P62 in HaCat cells. A : BSA; B : FFA; C: FFA+Metformin (40mM). Data are presented as the mean $\pm$ SD and are representative of three independent experiments. * $p < 0.05$.