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Effects of Isotretinoin on the Phosphoinositide-3-kinase/Akt/FoxO1 Pathway and Molecular Functions of SZ95 Sebocytes *In vitro*

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Abstract

Objective: For more than 3 decades, isotretinoin [13-cis retinoic acid (13-cis RA)] is established as the most potent oral treatment for severe acne. However, the molecular mechanisms of isotretinoin action have not been fully elucidated. Recently, we showed that insulin and insulin-like growth factor 1 (IGF-1) increase differentiation and decrease proliferation of SZ95 sebocytes *in vitro via* activation of the phosphoinositide 3-kinase (PI3K)/Akt/forkhead box-O1 (FoxO1) pathway. Furthermore, this pathway is activated in patient acne biopsies *in vivo*. Using SZ95 sebocytes as an *in vitro* model, the aim of this study was to investigate the effect of isotretinoin on PI3K pathway activation.

Methods: SZ95 sebocytes were treated under light protection with 0.1 µM isotretinoin in the presence or absence of 1.0 and 0.1 µM IGF-1 or insulin or 50 µM LY294002 inhibitor in a time- dependent manner. The expression of p-Akt and its downstream target p-FoxO1 was analyzed by Western blot. FoxO transcriptional activity was measured by dual luciferase assay. Nuclear and cytoplasmic mobilization of FoxO1, p-FoxO1, Akt, and p-Akt were also determined by immunofluorescence microscopy. Proliferation of sebocytes was measured by [^{3}H]-thymidine incorporation and sebocyte differentiation by oil red O staining.

Results: We show that isotretinoin activates the PI3K/Akt pathway and decreases the nuclear content of FoxO1 and its transcriptional activity in sebocytes *in vitro* and in sebaceous gland biopsies from an isotretinoin-treated acne patient. Isotretinoin reduces the proliferation of IGF-1- or insulin-stimulated sebocytes more effectively than IGF-1 and insulin alone. However, it normalizes the increased lipid accumulation in IGF-1- or insulin-stimulated sebocytes *via* a PI3K-independent pathway.

Conclusion: Our data from the SZ95 sebocyte *in vitro* model suggest that the potent therapeutic effect of isotretinoin in acne is not mediated *via* the PI3K/Akt/FoxO1 pathway.

Keywords: Isotretinoin; PI3K pathway; FoxO1; Sebocytes, IGF-1; Insulin

Abbreviations: ANOVA: One-Way Analysis of Variance; ATRA: All-Trans-Retinoic Acid; DAPI: 4, 6-Diamidino-2-Phenylindole, Dihydrochloride; FoxO1: Forkhead Box-O1; IGF-1: Insulin-like Growth Factor 1; p-FoxO: Phospho-Forkhead Box-O; PI3K: PhosphoInositide 3-kinase; RAR: Retinoic Acid Receptor.

Introduction

Acne vulgaris is the most frequent chronic inflammatory skin disorder. Acne mostly occurrs during teenage years, when insulin resistance is increased [1]. Since approval by the US Food and Drug Administration (FDA) in 1982, isotretinoin [13-cis retinoic acid (13-cis RA)] is considered as a most efficacious drug against acne [2]. Isotretinoin is a natural metabolite of vitamin A. It directly acts on the major acne pathogenic factors including suppression of lipogenesis, reducing proliferation and differentiation, normalizing follicular hyperkeratinization, and reducing inflammation and (indirectly) the prevalence of Propionibacterium acnes [2-9]. Isotretinoin modulates cell cycle, differentiation, cell survival, and apoptosis [10,11].

Sebocytes are sebaceous gland epithelial cells located in the basement of the glands. In 1999, Zouboulis et al. cultivated human facial sebocytes from an 87-year old woman and transfected the sebocytes with the Simian Virus-40 large T antigen for immortalization. These cells were called SZ95 and show the main features of normal human sebocytes. SZ95 sebocytes are widely used in acne investigations. It has been demonstrated that isotretinoin modulates cell proliferation and apoptosis in a retinoid receptor-independent manner while its effect on sebocyte differentiation is mediated *via* retinoic acid receptor (RAR) and retinoid X receptor [11]. Isotretinoin suppresses sebocyte proliferation and lipogenesis [12-14]. Although isotretinoin has been used in clinical practice for decades, the molecular mechanisms of its action are still incompletely understood. Recently, it has been hypothesized that isotretinoin might mediate its therapeutic effects *via* up-regulation of nuclear forkhead box-O (FoxO1) transcription factor and thereby counteracts some effects of acne-stimulating growth factors [15]. FoxO transcription factors are a pivotal subgroup of the Fox family that have an important role in apoptosis, cell cycle, cell differentiation, and other cellular functions [16].

High glycemic load diet and high milk consumption increase insulin and insulin-like growth factor 1 (IGF-1) serum levels while reducing insulin-like growth factor-binding protein 3 and, thereby, can aggravate acne [17-19]. Recently, we showed that high doses of insulin and IGF-1 activate the phosphoinositide 3-kinase (PI3K)/Akt/FoxO1

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pathway, suppress proliferation but enhance differentiation of SZ95 sebocytes [20]. The aim of our present study was to investigate possible effects of isotretinoin on the PI3K/Akt pathway and FoxO1 expression. In addition, isotretinoin's effect on proliferation and lipogenesis of untreated as well as IGF-1- or insulin-stimulated sebocytes was explored *in vitro*. We used isotretinoin at the concentration achievable in serum (0.1 μM) which has been found to be the best concentration to simulate the *in vivo* condition [12,14,21]. In our experimental setting, 0.1 μM isotretinoin activated the PI3K/Akt/FoxO1 pathway. In addition, isotretinoin further suppressed SZ95 sebocyte proliferation and normalized IGF-1- or insulin-mediated lipogenesis induction.

Materials and Methods

Cell culture

SV40 immortalized SZ95 human sebocytes [22] were cultured in Sebomed Basal Medium (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal bovine serum (Biochrom), 1% Penicillin/Streptomycin antibiotics (Biochrom) and 5 ng/mL human epidermal growth factor (EGF) (PromoCell, Heidelberg, Germany) and were grown to confluence. For treatments, this medium was replaced with serum-free complete medium (Sebomed Complete Medium, Biochrom) which comprises Sebomed Basal Medium supplemented with 2.5 mM L-glutamine (GlutaMAX™, Gibco, Grand Island, New York), 5 ng/mL EGF, 0.15 μM linoleic acid (LA) (Sigma-Aldrich, USA), 0.5 gram bovine serum albumin (BSA) (Sigma-Aldrich), 1 gram sodium bicarbonate (Sigma-Aldrich), 25 mg bovine pituitary extract (Gibco, New York) and 1% antibiotics. The serum-free complete medium was analyzed for insulin and IGF-1 content; insulin content was less than 7 x 10^{-6} μM and IGF-1 content less than 0.003 μM. Cell viability was analyzed by lactate dehydrogenase cytotoxicity detection kit (Roche Diagnostics, Manheim, Germany), an appropriate method for detection of necrosis in sebocytes, and trypan blue staining (Sigma-Aldrich, Steinheim, Germany).

Western blot

SZ95 sebocytes were seeded at a density of 6 x 10^5 cells in 25 cm^2 cell culture flasks (Nunc, Wiesbaden, Germany) and cultivated for two days in serum-free complete medium. After two days, cells were washed with phosphate buffered saline (PBS) (Biochrom) and sebocytes were stimulated with 0.1 μM isotretinoin (Sigma-Aldrich, Deisenhofen, Germany) in the dark in the presence or absence of IGF-1 (BioVision, California, USA) and insulin (Sigma-Aldrich, Steinheim, Germany) for 60 and 90 minutes. For pre-treatment with PI3K inhibitor, sebocytes were incubated for 30 minutes with 50 μM LY294002 (Cell Signaling Technology, USA). Cells were lysed with RIPA buffer (Sigma-Aldrich, Missouri, USA) and 25 micrograms of protein was run on a 10% Tris-glycine SDS-polyacrylamide gel. Protein was transferred to a nitrocellulose membrane and incubated with the primary antibodies p-Akt (Cell Signaling #9271, 1:1000), p-FoxO1 (Cell Signaling #9464, 1:1000), Akt (Cell Signaling #9272, 1:1000), FoxO1 (Cell Signaling #2880, 1:1000), and β-actin (SIGMA #A5441, 1:5000) (Sigma, USA). Blots were re-incubated with peroxidase-conjugated AffiniPure Anti-Rabbit IgG, (Jackson ImmunoResearch, USA #74425, 1:10000) or Goat anti mouse IgG (H/L): HRP, (A Bio-Rad, USA #0300-0108P, 1:10000) secondary antibody. Blots were developed with SuperSignal West Pico enhanced Chemiluminescent (ECL) Substrate (Thermo Scientific, USA) and exposed to film. Films of blots were analyzed and quantified by densitometry with Kodak 1D Image Analysis Software, Version 3.6 after background reduction.

[^{3}H]-thymidine incorporation assay

SZ95 sebocytes (1 x 10^5 cells/ ml) were plated in triplicates in 96-well plates (Nunc, Wiesbaden, Germany) for 24-72 hours, or in 6-well plates (Corning, USA) for 168 hours in serum-free complete medium. On the following day, 0.1 μM isotretinoin was added in the dark in the presence or the absence of 50 μM LY294002, IGF-1 or insulin and cells were incubated for 24-168 hours. Each 72 hours the factor-supplemented medium was renewed. Prior to the end of the treatment period, cells were labelled with [^{3}H]-thymidine (0.2 μCi/ well; ICN, Meckenheim, Germany). After 16 hours, the medium was removed and cells were washed with PBS. 5% Trichloroacetic acid was added. Afterwards, cells were trypsinized (Biochrom), the incorporated radioactivity was harvested on glass fiber filters and analyzed by liquid scintillation counting with a 1450 MicroBeta TriLux Microplate Scintillation and Luminescence Counter (PerkinElmer).

Oil red O staining

SZ95 sebocytes were plated in 2-chamber slides (Nunc, USA) at 3 x 10^5 cells/ ml and grown in complete medium. After overnight incubation, 50 μM linoleic acid (LA) or 0.1 μM isotretinoin was added in the dark with or without 50 μM LY294002, IGF-1 or insulin. 72 hours later, cells were washed with PBS and fixed with 10% formaldehyde. Oil red O solution was prepared by mixing 0.6% oil red O (Sigma-Aldrich, Steinheim, Germany) in 99% isopropanol and dH_2O and then filtration. Cells were stained with this solution for 20 minutes, rinsed in 60% isopropanol, and dH_2O. Hematoxylin (Thermo Scientific, USA) was added for counterstaining.

Lipid quantification

Stained cells were rinsed twice with dH_2O. After oil red O elution, lipid accumulation in cell colonies was evaluated by an upright light microscopy (Leica DM1000) (Leica, Wetzlar, Germany) using a color camera (Pixelink, Ottawa, Ontario) at 63 x magnification. Images were captured and cells and lipid droplets were counted in six randomly chosen areas.

Transfection and luciferase assay

SZ95 sebocytes at 80% confluence were transfected with Cignal FoxO Reporter (QIAGEN, USA) using Lipofectamin 2000 (Invitrogen) transfection reagent. Six hours later, cells were trypsinized and cultivated in 96-well plates. After overnight incubation, cells were stimulated with 0.1 μM isotretinoin in the dark with or without 50 μM LY294002, IGF-1, and insulin for 30, 60, and 90 minutes. Afterwards, medium was removed and cells were rinsed with PBS. Lysis buffer (Promega, USA) was added for 15 minutes and luciferase assay reagent (Promega, USA) was dispensed. Firefly luciferase activity was measured with a luminescence plate reader. Afterwards, Stop & Glo® reagent (Promega, USA) was added and Renilla luciferase activity was evaluated with a luminescence plate reader. The ratio of Firefly to Renilla-luciferase was determined.

Immunofluorescence cell staining

SZ95 sebocytes were cultivated on 13 mm coverslips (Marienfeld, Germany) in serum-free complete medium. After overnight, sebocytes were stimulated with 0.1 μM isotretinoin in the dark in the presence or the absence of 50 μM LY294002 and 1 μM IGF-1 or insulin for 60 and 90 minutes and then fixed with 4% paraformaldehyde. Cell membranes were permeabilized with 0.2% Triton X-100. Cells were blocked with PBS containing 4% FBS and incubated with anti-p-Akt (1:25), anti-FoxO1 (1:50), and anti-p-FoxO1 (1:50) for 1 hour. Sebocytes were then

incubated with FITC-conjugated anti-rabbit IgG antibody (1:1000, Invitrogen, USA). 4, 6-diamidino-2-phenylindole-dihydro-chloride (DAPI, 1:1000, Invitrogen) was used for nuclei staining. Sebocytes were analyzed with a confocal fluorescence microscope (Leica TCS SP2, Germany) and images were captured and analyzed with Metaview software.

Fluorescent IHC staining

Tissue specimens were gained from one acne patient that was treated with isotretinoin (10 mg/day at minimum) for at least three weeks and from a patient with sebaceous hyperplasia as a control group after signed informed consent according to the recommendations of the local ethics committee and the German Medical Council for diagnostic tissue used in research. Tissues were fixed in 10% formaldehyde and embedded in paraffin. Paraffin blocks were sectioned and 3 µM tissue sections were mounted on glass slides (Thermo Scientific). Sections were de-paraffinized and rehydrated. Sodium citrate pH 6 was used to retrieve the antigen. To block non-specific binding of primary antibodies to the tissue, samples were incubated with PBS containing 4% FBS, 0.25% fish gelatin, and 0.1% Triton X-100 before FoxO1, p-FoxO1, p-Akt, and Akt antibodies were added. After three hours and washing, samples were incubated with anti-Rabbit IgG H&L (Alexa Fluor® 555) (Abcam, USA) for 30 minutes. The samples were analyzed by fluorescence microscopy (ZEiSS Axioskop 2 mot plus, Germany) and images were captured (Visiview Software, Visitron, Germany, Munich).

Statistics

All experiments were repeated at least three times. Statistical significance was evaluated by Student's t-test and one-way analysis of variance (ANOVA). Significance was adjusted at $p^{*}<0.05$.

Results

Isotretinoin activates the PI3K/Akt pathway in SZ95 sebocytes

To test the effect of isotretinoin on PI3K/Akt pathway activation, SZ95 sebocytes were treated with 0.1 µM isotretinoin for 60 and 90 minutes. Western blot results from whole cell protein extracts showed that the PI3K pathway is activated by 0.1 µM isotretinoin as p-Akt and p-FoxO1 expression was significantly up-regulated at 60 and 90 minutes. Pre-incubation with the 50 µM PI3K inhibitor LY294002 blocked the up-regulation of p-FoxO1 and p-Akt at 60 and 90 minutes (Figures 1 and 2).

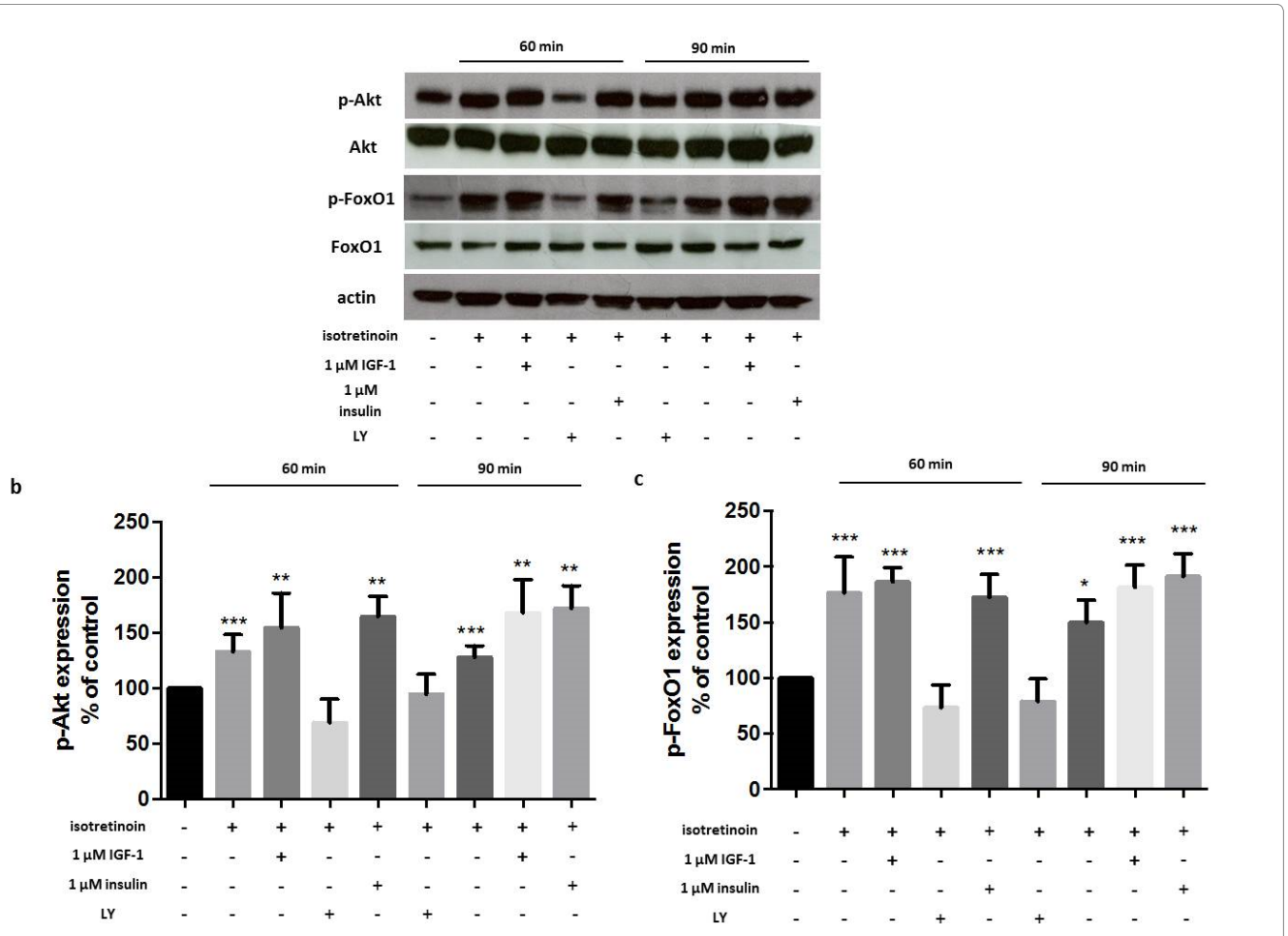

Figure 1. Expression of p-Akt and p-FoxO1 is up-regulated in SZ95 sebocytes after isotretinoin treatment and is further up-regulated in combination with 1 µM IGF-1 or insulin. SZ95 sebocytes were stimulated with 0.1 µM isotretinoin in the presence or the absence of 1 µM IGF-1, 1 µM insulin, and the PI3K inhibitor LY294002 (50 µM) for 60 and 90 minutes. Total protein fractions were analyzed by Western blot. (a) Representative Western blots probed for p-Akt, Akt, p-FoxO1, FoxO1, and β-actin. (b and c) Densitometric quantification of blots normalized to β-actin. Densitometric data were calculated as a percentage of the value in untreated cells. Data represent the means+SEMs (n=3) (one-way ANOVA) ($^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$).

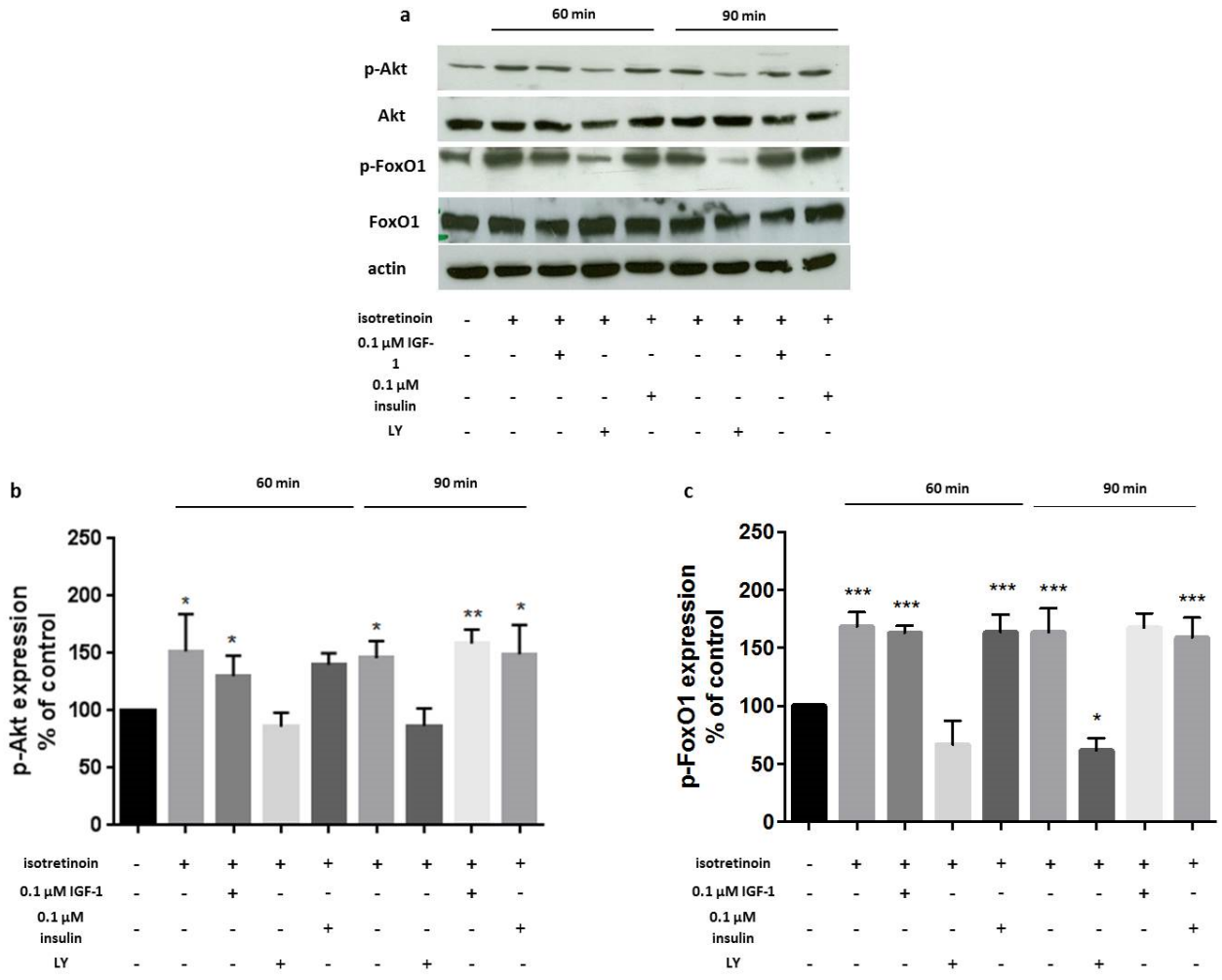

Figure 2. Expression of p-Akt and p-FoxO1 is up-regulated in SZ95 sebocytes and 0.1 µM IGF-1- or insulin-stimulated sebocytes after treatment with 0.1 µM isotretinoin. SZ95 sebocytes were stimulated with 0.1 µM isotretinoin in the presence or the absence of 0.1 µM IGF-1, 0.1 µM insulin, and PI3K inhibitor LY294002 (50 µM) for 60 and 90 minutes. Total protein fractions were extracted and corresponding Western blots performed. (a) Representative blots probed for p-Akt, Akt, p-FoxO1, FoxO1, and β-actin. (b and c) Densitometric quantification of blots normalized to β-actin. Densitometric data were calculated as a percentage of the value in untreated cells. Data represent the means+SEMs (n=3) (one-way ANOVA) (*p<0.05, **p<0.01 and ***p<0.001).

Results from our previous study [20] showed that 1 and 0.1 µM IGF-1 and insulin activate the PI3K pathway with up-regulation of p-Akt and p-FoxO1 in SZ95 sebocytes. In this experimental setting, we treated 1 µM IGF-1- or insulin-stimulated sebocytes with 0.1 µM isotretinoin for 60 and 90 minutes. The expression of p-Akt and p-FoxO1 was increased in 1 µM IGF-1- as well as 1 µM insulin-stimulated sebocytes after isotretinoin treatment compared to untreated sebocytes (Figure 1a-c). The expression of p-Akt (at 60 and 90 minutes) and p-FoxO1 (at 90 minutes) was even higher in cells co-treated with 1 µM IGF-1 or 1 µM insulin compared to cells treated with isotretinoin alone (Figure 1a-c).

Furthermore, we treated sebocytes with 0.1 µM isotretinoin and a ten-fold lower concentration of IGF-1 or insulin (0.1 µM). Western blot results showed an increased expression of p-Akt and p-FoxO1 in 0.1 µM IGF-1- or insulin-stimulated sebocytes after isotretinoin treatment compared to untreated sebocytes at 60 and 90 minutes (Figure 2a-c). Expression of p-Akt at 90 minutes was even higher in 0.1 µM IGF-1- or insulin-stimulated sebocytes upon isotretinoin treatment compared to isotretinoin alone (Figure 2a-c). These results demonstrate that in the *in vitro* experimental setting 0.1 µM isotretinoin alone activates the PI3K/Akt pathway and up-regulates p-FoxO1 expression in SZ95 sebocytes, which is even stronger in combination with 1 µM IGF-1 or insulin.

Isotretinoin treatment increases the expression of cytoplasmic p-Akt and p-FoxO1 in sebocytes

Next, we investigated the expression of p-Akt, FoxO1, and p-FoxO1 in untreated sebocytes and IGF-1- or insulin-stimulated sebocytes after isotretinoin treatment by immunofluorescent staining. The results obtained by immunofluorescence microscopy showed that 0.1 µM isotretinoin treatment leads to a down-regulation of nuclear FoxO1 at 60 and 90 minutes, whereas the expression of cytoplasmic p-Akt and p-FoxO1 is up-regulated (Figure 3). Pre-incubation of sebocytes with 50 µM LY294002 blocked p-Akt and p-FoxO1 up-regulation and inhibited export of nuclear FoxO1 compared to sole isotretinoin treatment (Figure 3). We previously reported that IGF-1 and insulin

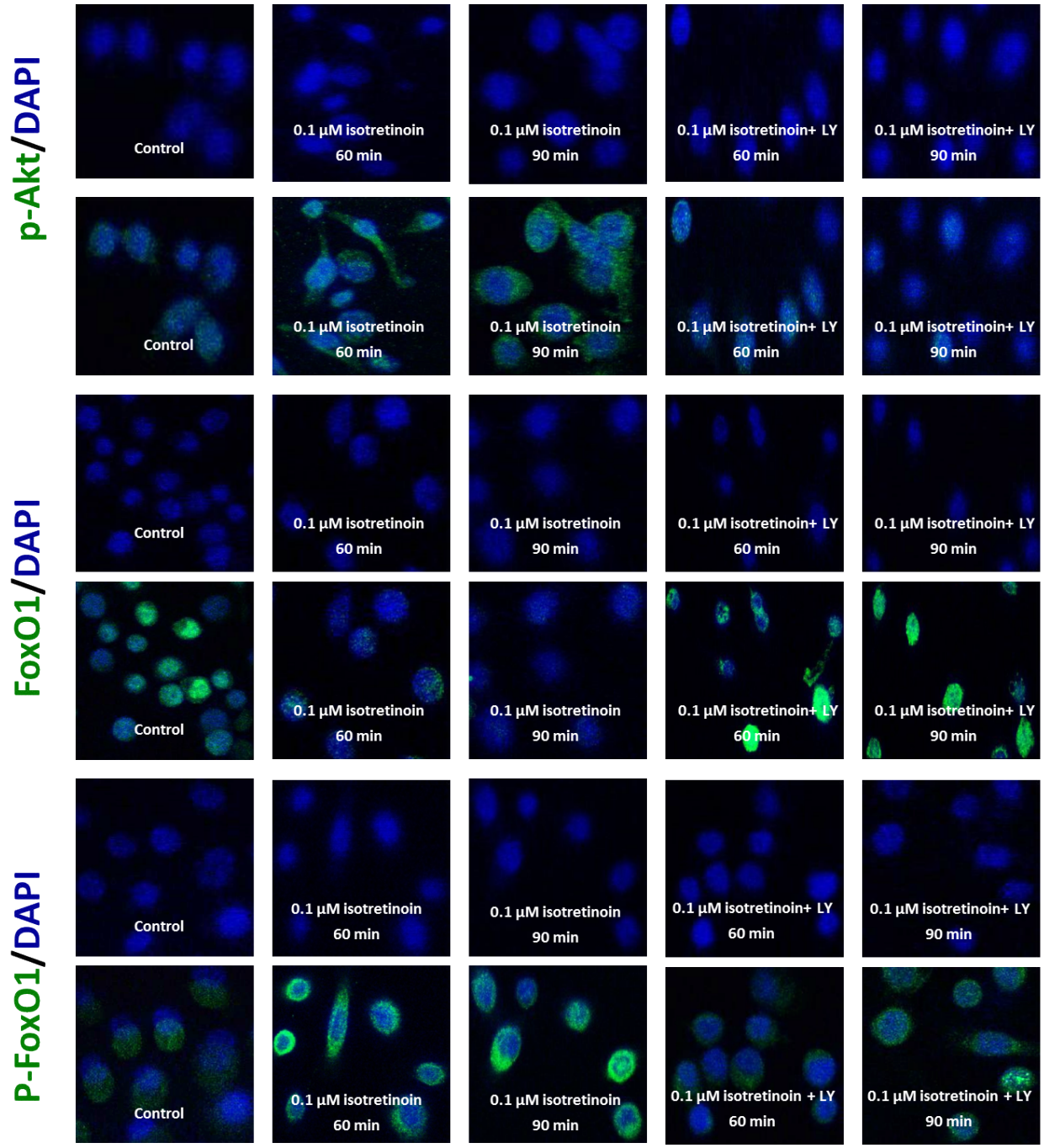

Figure 3. 0.1 µM isotretinoin up-regulates the expression of p-Akt and p-FoxO1 in SZ95 sebocytes. Sebocytes were treated with 0.1 µM isotretinoin for 60 and 90 minutes in the presence or the absence of 50 µM PI3K inhibitor LY294002. Immunofluorescence staining was performed for p-Akt, FoxO1, p-FoxO1 (FITC) (pseudo-colored in green), and DAPI (pseudo-colored in blue).

increase cytoplasmic p-Akt and p-FoxO1 expression and decrease the expression of nuclear FoxO1 [20]. The present co-treatment studies with isotretinoin using immunofluorescence staining also showed a down-regulation of nuclear FoxO1 in IGF-1- or insulin-cotreated SZ95 sebocytes and an up-regulation of cytoplasmic p-Akt and p-FoxO1 (Figure 4). Taken together, isotretinoin in a therapeutic serum concentration down-regulates the nuclear content of FoxO1 in sebocytes and up-regulates the expression of cytoplasmic p-Akt and p-FoxO1.

Isotretinoin treatment reduces FoxO transcriptional activity in SZ95 sebocytes

A firefly luciferase FoxO reporter assay was used to measure the effect of isotretinoin on FoxO transcriptional activity in SZ95 sebocytes. Sebocytes were transiently transfected with a FoxO reporter construct and then treated with 0.1 µM isotretinoin alone or in combination with 1 and 0.1 µM IGF-1 or insulin. FoxO transcriptional activity was measured at 30, 60, and 90 minutes. Data showed that

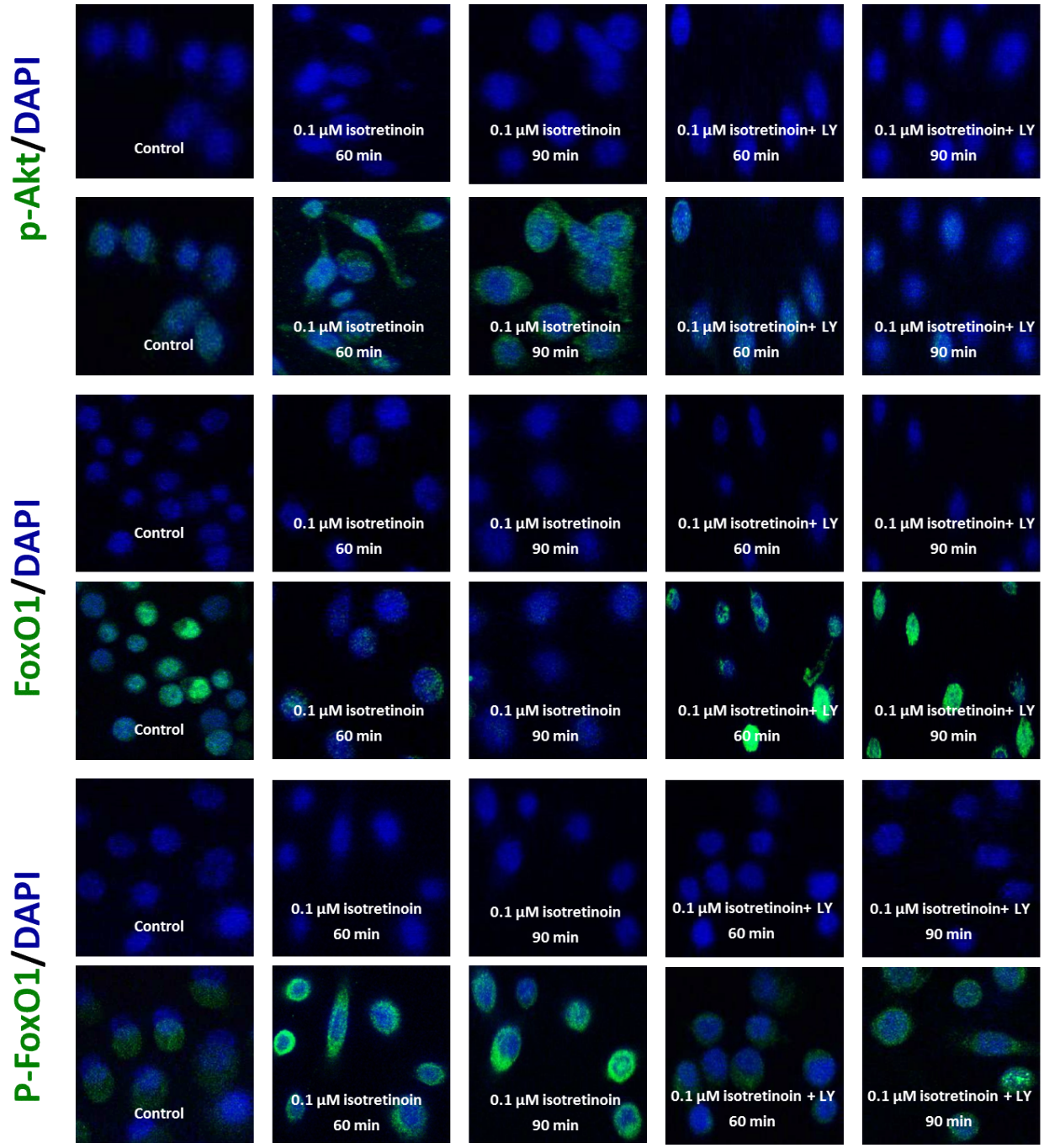

Figure 4. Expression of p-Akt and p-FoxO1 is up-regulated in 1 µM IGF-1- or insulin-stimulated sebocytes after 0.1 µM isotretinoin treatment. Sebocytes were treated with 0.1 µM isotretinoin in the presence of 1 µM IGF-1 or insulin for 60 and 90 minutes. Immunofluorescence staining was performed for p-Akt, FoxO1, p-FoxO1 (FITC) (pseudo-colored in green), and DAPI (pseudo-colored in blue).

FoxO transcriptional activity is down-regulated in response to 0.1 µM isotretinoin treatment in non-stimulated sebocytes after 30, 60, and 90 minutes. The highest reduction of FoxO transcriptional activity (20%) was detected 90 minutes after isotretinoin treatment (Figure 5a and b). We previously showed that 1 and 0.1 µM IGF-1 or insulin down-regulate FoxO transcriptional activity after 30, 60, and 90 minutes in SZ95 sebocytes. The highest significant reduction was observed 60 minutes after 1 µM IGF-1 (25%) and 1 µM insulin (21%) stimulation [20]. In our current study, we found that FoxO transcriptional activity is more (7%) suppressed in presence of isotretinoin in IGF-1- or insulin-stimulated sebocytes than isotretinoin alone (Figures 5a and b). Thus, similar to insulin or IGF-1, 0.1 µM isotretinoin leads to a reduction of FoxO transcriptional activity in SZ95 sebocytes *via* activation of the PI3K pathway.

Expression of p-Akt, Akt, p-FoxO1, and FoxO1 in sebaceous glands obtained from an acne patient treated with isotretinoin

Next, we investigated the expression of p-Akt, Akt, p-FoxO1, and

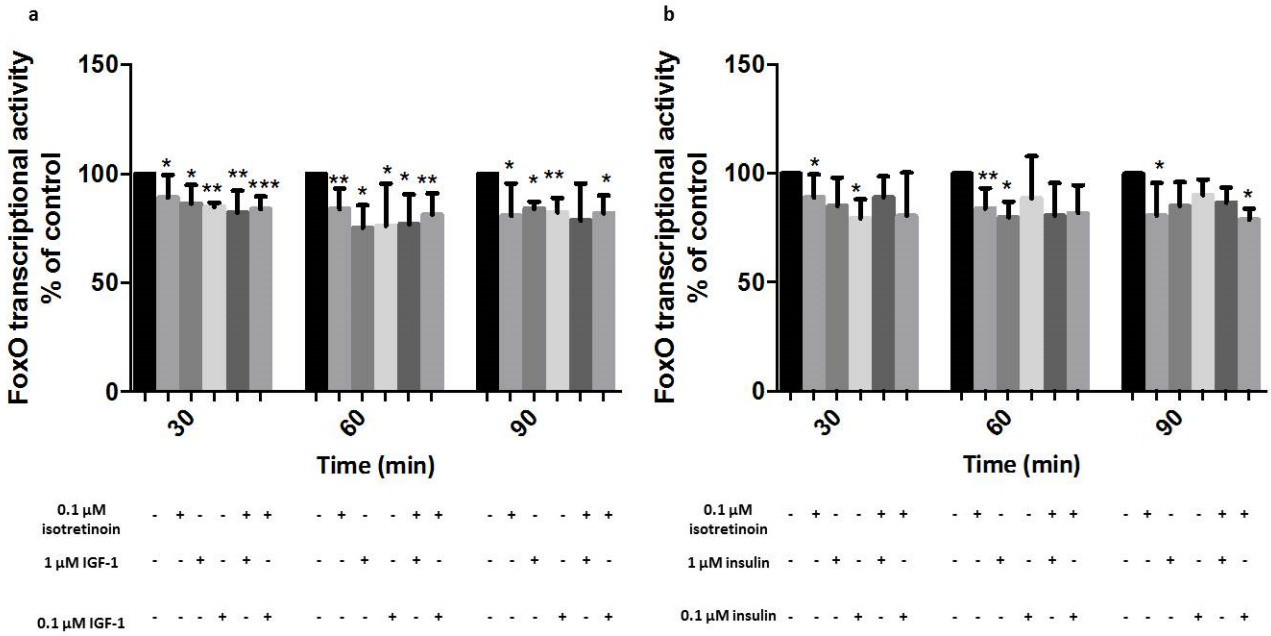

Figure 5. FoxO transcriptional activity is down-regulated in SZ95 sebocytes and IGF-1- or insulin-stimulated sebocytes after stimulation with 0.1 µM isotretinoin. FoxO transcriptional activity in SZ95 sebocytes was analyzed by luciferase assay after stimulation with 0.1 µM isotretinoin and (a) 1 and 0.1 µM IGF-1 and a combination thereof or stimulation with (b) 1 and 0.1 µM insulin and a combination thereof for 30, 60, and 90 minutes. Data were calculated as a percentage of the value in untreated cells. Data represent the means+SEMs of four independent experiments; (* p<0.05, ** p<0.01 and *** p<0.001, Student's t-test).

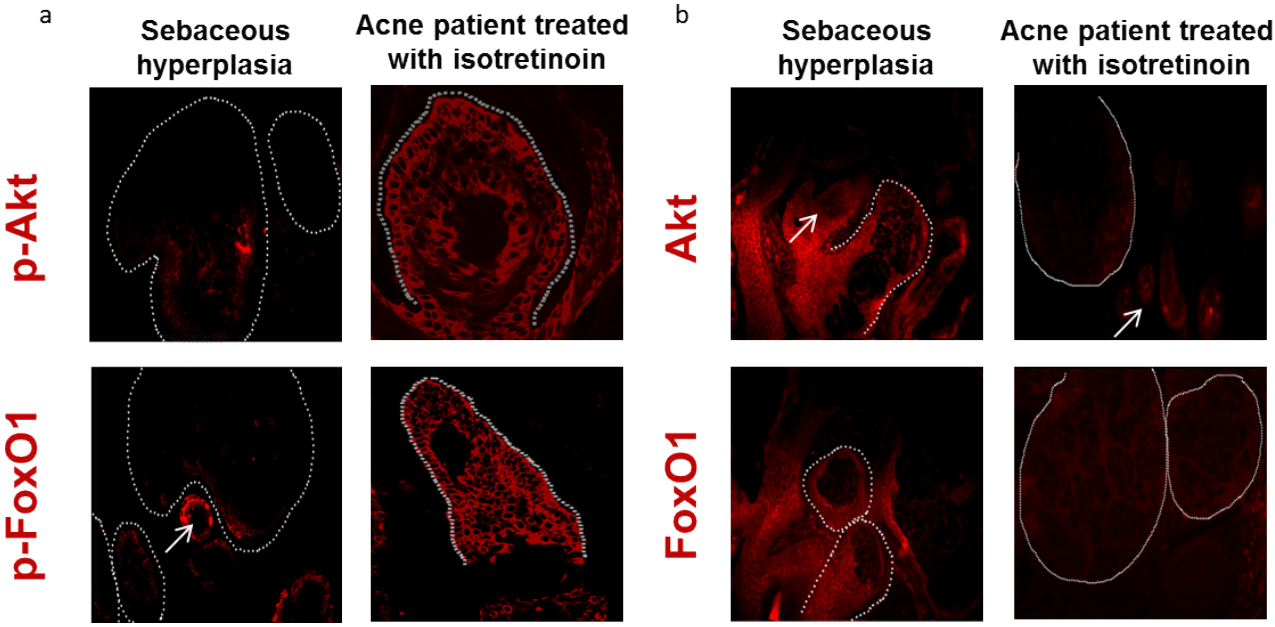

Figure 6. p-Akt and p-FoxO1 expression is increased in sebaceous gland obtained from a representative acne patient treated with isotretinoin compared to sebaceous gland from a hyperplasia patient. Immunofluorescence staining of paraffin skin sections obtained from an acne patient treated with isotretinoin for several weeks and a patient with sebaceous hyperplasia were done for (a) p-Akt and p-FoxO1 (Rhodamine) (pseudo-coloured in red) and (b) Akt and FoxO1 (Rhodamine) (pseudo-coloured in red). Sebaceous glands are marked by dotted lines and arrows show hair follicles.

FoxO1 in human specimens *ex vivo* using sebaceous glands obtained from a patient with sebaceous hyperplasia and an acne patient who was treated with isotretinoin (10 mg/d at a minimum) for at least three weeks. Fluorescent IHC staining showed that the expression of p-Akt and p-FoxO1 is higher in the sebaceous gland of the acne patient compared to a patient with sebaceous gland hyperplasia of the elderly (Figure 6a). The expression of Akt and FoxO1 was lower in skin biopsies of the isotretinoin-treated acne compared to the sebaceous

gland obtained from the patient with sebaceous gland hyperplasia (Figure 6b). These results demonstrate that isotretinoin treatment *in vivo* induces phosphorylation of Akt and FoxO1, which correlates with the *in vitro* results.

Isotretinoin treatment suppresses DNA synthesis in untreated and IGF-1- or insulin-stimulated sebocytes

SZ95 sebocytes were treated with 0.1 µM isotretinoin for 24 to 168 hours. [^{3}H]-thymidine incorporation assays showed that 0.1 µM isotretinoin reduces DNA synthesis in viable SZ95 sebocytes at 24, 48, 72, and 168 hours. At 168 hours, DNA synthesis was maximally suppressed by 42% (Figure 7a). IGF-1 and insulin were found to suppress proliferation of SZ95 sebocytes *via* the PI3K pathway with a maximum suppression of 59% and 44%, respectively, after 168 hours [20]. Thus, we also studied the effect of combined isotretinoin and IGF-1 or insulin treatment on sebocyte proliferation at 168 hours. Treatment of cells with isotretinoin plus either IGF-1 or insulin led to a greater inhibition of proliferation than observed for each individual reagent. 0.1 µM isotretinoin lowered proliferation of 1 µM IGF-1-stimulated sebocytes by 1.9-fold (79%) and it reduced proliferation of 1 µM insulin-stimulated sebocytes by 1.7-fold (71%) compared to isotretinoin alone. In addition, the combination of 0.1 µM isotretinoin and 1 µM IGF-1 and the combination of 0.1 µM isotretinoin and insulin reduced sebocyte DNA synthesis 2- and 1.9-fold more than 1 µM IGF-1 and 1 µM insulin alone, respectively (Figures 7b and c). Pre-incubation with the 50 µM LY inhibitor effectively reduced SZ95 sebocyte proliferation with a maximum suppression of 85% at 168 hours. LY inhibitor together with isotretinoin also decreased sebocyte DNA synthesis with a maximum suppression of 73%. LY could not restore the isotretinoin-mediated suppression of proliferation (Figure 7b and c). Lactate dehydrogenases assay showed that the viability of these sebocytes was not decreased compared to unstimulated cells (data not shown). Taken together, 0.1 µM isotretinoin in combination with IGF-1 or insulin shows an additive effect on the suppression of SZ95 sebocyte proliferation. The suppressive effect of isotretinoin on sebocyte proliferation, however, seems to be mediated *via* PI3K-independent mechanisms.

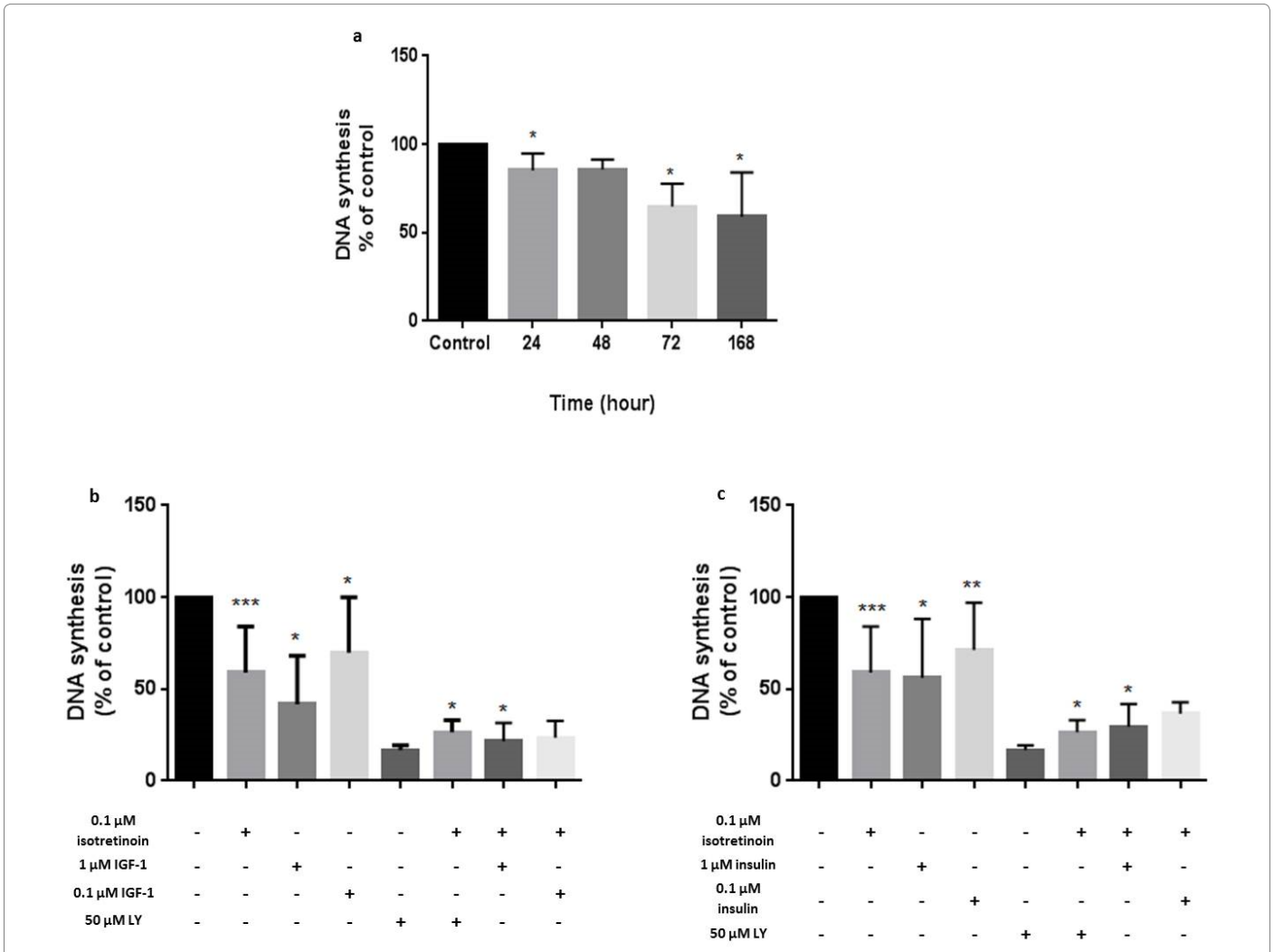

Figure 7. Isotretinoin suppresses proliferation of SZ95 sebocytes and further reduces proliferation of IGF-1- or insulin-stimulated sebocytes. Sebocytes proliferation was measured by [3H]-thymidine incorporation assay following stimulation with (a) 0.1 µM isotretinoin for the indicated time points (b) 0.1 µM isotretinoin in the presence or the absence of 1 and 0.1 µM IGF-1 and 50 µM LY inhibitor (c) 0.1 µM isotretinoin in the presence or absence of 1 and 0.1 µM insulin and 50 µM LY inhibitor for 168 hours. The DNA synthesis values are expressed as a percentage of untreated cells and represent means + SEMs (n=6) (* p<0.05, ** p<0.01 and *** p<0.001, Student's t-test).

Isotretinoin treatment decreases lipid accumulation in untreated and IGF-1- or insulin-stimulated sebocytes

To investigate the effect of isotretinoin on sebaceous lipogenesis, SZ95 sebocytes were stimulated with 0.1 μM isotretinoin and intracellular lipid droplet formation was analyzed by oil red O staining. 0.1 μM isotretinoin slightly reduced SZ95 sebocyte lipid production with a maximum reduction of 28% at 72 hours (Figure 8a, b, and i). Our previous study demonstrated that 1 μM and 0.1 μM IGF-1 or 1 μM insulin increase lipogenesis of SZ95 sebocytes with a maximum increase of 136%, 254%, and 64%, respectively at 72 hours [20]. Subsequently, we treated IGF-1- or insulin-stimulated sebocytes with isotretinoin. 0.1 μM isotretinoin inhibited the increase of lipogenesis in 1 μM and 0.1 μM IGF-1- and 1 μM insulin-stimulated sebocytes (Figure

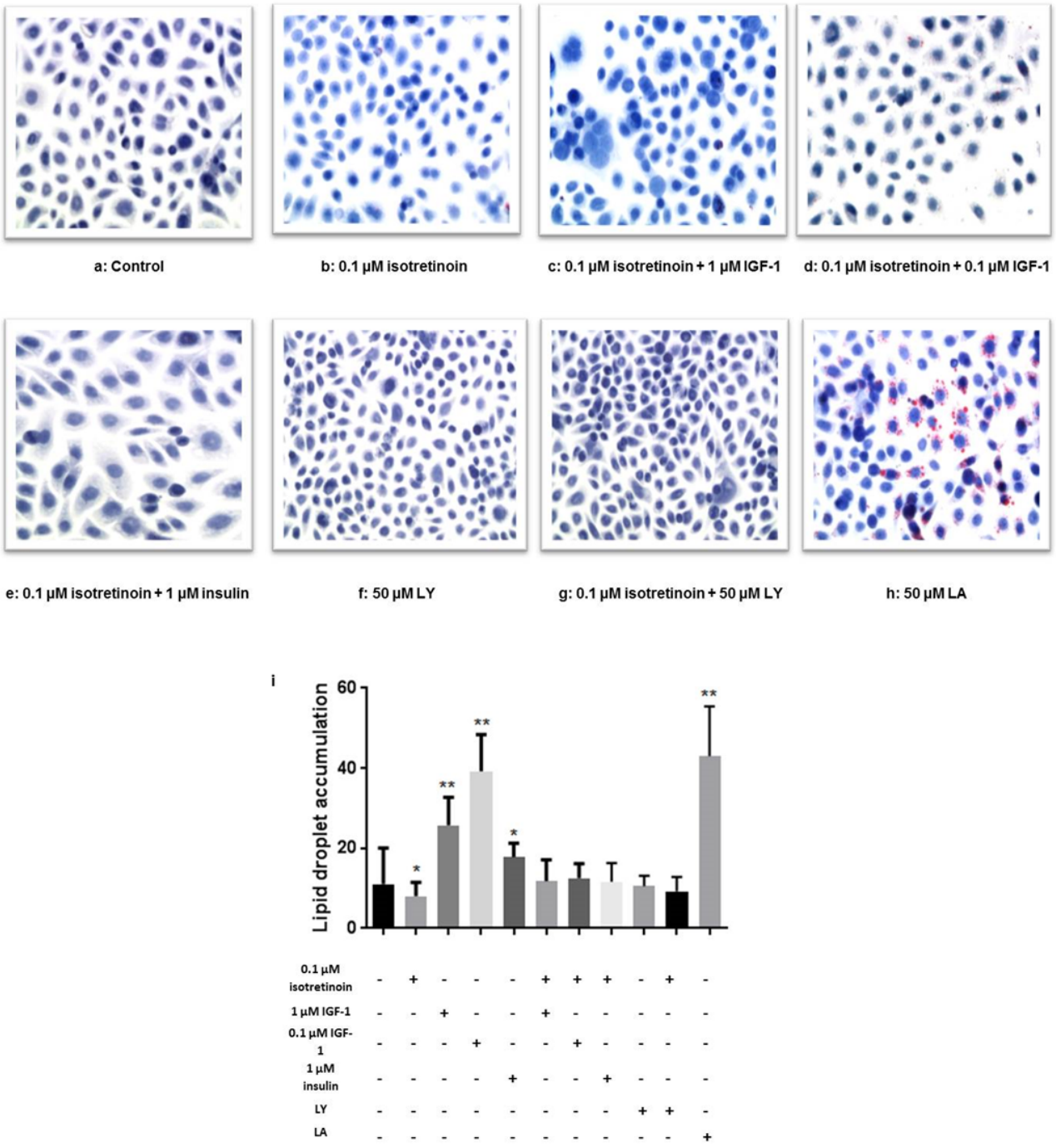

Figure 8. Isotretinoin suppresses lipogenesis in SZ95 sebocytes. Sebocytes were cultured (a) without treatment, with (b) 0.1 μM isotretinoin only, or (c-h) with 0.1 μM isotretinoin and 1 μM IGF-1, 0.1 μM IGF-1, 1 μM insulin, 50 μM LY inhibitor, 50 μM linoleic acid (LA) alone or in combinations as indicated (c-h) for 72 h followed by oil red O staining. Data in (i) represent the means + SEMs (n=4) (* $p<0.05$ and ** $p<0.01$, Student's t-test).

8c-e). To investigate the involvement of PI3K signaling on lipogenesis in isotretinoin-treated sebocytes, sebocytes were pre-incubated with 50 μM LY inhibitor and then treated with isotretinoin for 72 hours. LY did not significantly affect the isotretinoin-induced reduction of lipogenesis (Figure 8f-i). These data indicate that 0.1 μM isotretinoin decreases lipogenesis in SZ95 sebocytes via PI3K-independent mechanisms (Figure 8i). Importantly, isotretinoin abrogates the increased lipid production induced by IGF-1 or insulin.

Discussion

Although isotretinoin is considered as the most efficacious drug against acne and widely applied [2,23-28], the molecular mechanisms of its action are still unresolved. A recent speculative hypothesis suggested that isotretinoin exerts its action by up-regulation of FoxO1 transcription factors. This hypothesis, however, has never been experimentally proven *in vivo* or in sebocyte cultures [29].

RA signaling is complex and has a role in the early activation of several signaling pathways. It induces cell differentiation and cell growth arrest [30]. The PI3K pathway, like RA signaling, also plays an essential role in cell differentiation and proliferation [31,32]. Consequently, it is coherent to suppose that the PI3K pathway contributes to the RA actions. Recently, research in cancer focused more on retinoid actions mediated *via* RAR-independent pathways such as extracellular signal-regulated kinases, PI3K, and stress-activated protein kinases/jun amino-terminal kinases pathways [33-37]. Both, natural and synthetic RAR-selective agonists including, all-trans-retinoic acid (ATRA), isotretinoin as well as pan-RAR agonists (9-cis-RA and LG 100567), bind to RARs and activate several signaling cascades such as the PI3K pathway [36]. It has been reported that 1 μM RA activates the PI3K pathway with p-Akt up-regulation within 5 to 120 minutes in neuroblastoma cells and within 5 to 30 minutes in NIH-3T3 cells [36]. No study to date has investigated the action of ATRA and isotretinoin on this pathway in sebocytes. We investigated PI3K/Akt pathway activation in SZ95 sebocytes upon 0.1 μM isotretinoin treatment *in vitro* and *in vivo* and its effect on sebocyte proliferation and lipogenesis. To the best of our knowledge, no study to date has investigated the expression of FoxO1 and FoxO transcriptional activity in response to isotretinoin. It has been reported that 1 μM RA activates the PI3K pathway with p-Akt up-regulation within 5 to 120 minutes in neuroblastoma cells and within 5 to 30 minutes in NIH-3T3 cells [36]. Our results show that 0.1 μM isotretinoin activates the PI3K pathway in SZ95 sebocytes with up-regulation of cytoplasmic p-Akt and p-FoxO1 at 60 and 90 minutes. In addition, expression of nuclear FoxO1 and FoxO transcriptional activity was significantly down-regulated after isotretinoin treatment. Thus, isotretinoin activates the PI3K/Akt/FoxO1 pathway in SZ95 sebocytes.

The relevance of the results *in vivo* was underlined by the induced expression of p-Akt and p-FoxO1 in sebaceous glands obtained from an acne patient treated with isotretinoin for at least three weeks while in sebaceous glands from biopsies of a patient with sebaceous hyperplasia this could not be observed. These findings reveal that the PI3K/Akt/FoxO1 pathway is not only activated in SZ95 sebocytes after isotretinoin treatment *in vitro* but also in sebaceous glands of an isotretinoin-treated acne patient.

Acne mostly arises during adolescence [38-40] when androgens (dehydroepiandrosterone), first from the adrenal glands and later by the testosterone producing glands, IGF-1 and insulin levels come to their peak [41]. There is discussion that rising consumption of dietary and hyperglycemic food further increases the natural age-related serum levels of insulin and IGF-1 and thereby may contribute to the pathogenetic factors in acne as hypothesized by Melnik [18]. We previously reported that insulin and IGF-1 up-regulate the expression of p-Akt and p-FoxO1 in SZ95 sebocytes and may contribute to the development of acne *via* activation of the PI3K pathway [20]. In this study we further analyzed how isotretinoin acts on IGF-1- or insulin-stimulated sebocytes. Isotretinoin further up-regulated the expression of cytoplasmic p-Akt and p-FoxO1 in 1 μM IGF-1- or insulin-stimulated sebocytes and nuclear FoxO1 expression and FoxO transcriptional activity was suppressed after isotretinoin co-treatment. These findings suggest that isotretinoin and IGF-1/insulin may regulate the PI3K/Akt/FoxO1 pathway in a similar manner.

It was reported that isotretinoin suppresses proliferation of SZ95 sebocytes at day 7 and 14 with concentrations (0.1-0.001 μM) achievable in serum [42-44] and at higher levels (10 and 1 μM) [12,21,45,46]. Also, in another cell line (SEB-1 sebocytes) 0.1, 0.5, and 1 μM isotretinoin reduced cellular proliferation and activated apoptosis after 48 and 72 hours. Isotretinoin is known from those experiments to mediate suppression of proliferation by increasing p21 and decreasing cyclin D1 expression in SEB-1 sebocytes [14]. It up-regulates tumor suppressor genes after one week in sebaceous glands of acne patients [47]. Furthermore, it has been demonstrated that neutrophil gelatinase-associated lipocalin, tazarotene-induced gene-1, and TIG3 can modulate the apoptotic effect of isotretinoin in human sebocytes and sebaceous glands of acne patients [48-50]. These findings suggest that isotretinoin suppresses sebocyte proliferation *via* RAR-independent mechanisms. Experiments with inhibitors of dipeptidyl peptidase IV and amino-peptidase N from our group showed an inhibitory effect on proliferation and differentiation in the SZ95 culture model [13,51]. Our results confirmed that 0.1 μM isotretinoin reduces sebocyte proliferation at different time points between 24 to 168 hours in culture with a maximum suppression at 168 hours. LY is a growth inhibitor and induces apoptosis. LY induced inhibition of the PI3K pathway and reduced sebocyte proliferation. In addition, the LY inhibitor together with isotretinoin significantly suppressed proliferation of SZ95 sebocytes compared to untreated sebocytes. The results showed that the PI3K pathway inhibitor does not restore the suppression of proliferation after isotretinoin treatment and reduce sebocyte proliferation more than isotretinoin alone. From these data it can be assumed that isotretinoin reduces sebocyte proliferation *via* PI3K-independent mechanisms.

In our previous study, we observed that IGF-1 and insulin decrease SZ95 sebocyte proliferation at 168 hours [20]. Our present results showed that 0.1 μM isotretinoin suppressed DNA synthesis of IGF-1- or insulin-stimulated sebocytes more effectively than IGF-1 or insulin alone at 168 hours.

It is well established that isotretinoin has an inhibitory effect on sebocyte lipogenesis *via* RAR-dependent mechanisms [11,12,52]. Gene expression analysis and histometric analyses demonstrated that isotretinoin decreases sebaceous glands' volume and down-regulates the expression of genes involved in lipid production and the cholesterol synthesis pathways, such as mitochondrial fatty acid β-oxidation, fatty acid synthesis, cholesterol biosynthesis, and biosynthesis of steroids, and can suppress lipogenesis [8,14,47]. Using oil red O staining to determine sebocyte lipogenesis in response to isotretinoin treatment, we found that 0.1 μM isotretinoin slightly decreases sebocyte lipogenesis *in vitro*. A pre-incubation with LY did not significantly inhibit the isotretinoin-induced reduction of lipogenesis, suggesting that this pathway is not involved in isotretinoin-mediated sebosuppression. Our previous data demonstrated that 1 and 0.1 μM IGF-1 and 1 μM insulin

increase sebocyte lipogenesis [20]. Most importantly, in this study we found that isotretinoin inhibits the increase of lipid production induced by 1 and 0.1 μM IGF-1 and 1 μM insulin in SZ95 sebocytes and restores lipid production to levels found in untreated sebocytes. These findings demonstrate that isotretinoin treatment can normalize lipid production which is increased after stimulation with IGF-1 and insulin.

Altogether, this study reveals that isotretinoin used in serum achievable concentration activates the PI3K/Akt pathway, increases cytoplasmic p-FoxO1 and reduces FoxO transcriptional activity in SZ95 sebocytes in a culture model. These data allow the conclusion that the strong effects of this naturally occurring retinoid on acne and the suppression of proliferation and lipogenesis must be mediated by PI3K-independent pathways.

Conflict of Interest

The authors state no conflict of interest.

Acknowledgments

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Disclosure statement

The authors have nothing to disclose.

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