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Insulin and insulin-like growth factor-1 can modulate the phosphoinositide-3-kinase/Akt/FoxO1 pathway in SZ95 sebocytes *in vitro*

Yasaman Mirdamadi [a, 1], Anja Thielitz [a, 1], Antje Wiede [a], Alexander Goihl [b], Eleni Papakonstantinou [a], Roland Hartig [b], Christos C. Zouboulis [c], Dirk Reinhold [b], Luca Simeoni [b], Ursula Bommhardt [b], Sven Quist [a], Harald Gollnick [a, *]

[a] Department of Dermatology and Venereology, Otto-von-Guericke-University Magdeburg, Magdeburg, Germany
[b] Institute of Molecular and Clinical Immunology, Otto-von-Guericke-University Magdeburg, Magdeburg, Germany
[c] Departments of Dermatology, Venereology, Allergology and Immunology, Dessau Medical Center, Dessau, Germany

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ABSTRACT

A recent hypothesis suggests that a high glycaemic load diet-associated increase of insulin-like growth factor-1 (IGF-1) and insulin may promote acne by reducing nuclear localization of the forkhead box-O1 (FoxO1) transcription factor via activation of the phosphoinositide-3-kinase (PI3K)/Akt pathway. Using SZ95 sebocytes as a model, we investigated the effect of the most important insulinotropic western dietary factors, IGF-1 and insulin on acne. SZ95 sebocytes were stimulated with different concentrations of IGF-1 and insulin (0.001, 0.01, 0.1 and 1 μM) for 15 to 120 min ± PI3K inhibitor LY294002 (50 μM). Cytoplasmic and nuclear protein expression of p-Akt and p-FoxO1 as well as FoxO transcriptional activity was analysed. In addition, the proliferation and differentiation of sebocytes and their TLR2/4 expression were determined. We found that high concentrations of IGF-1 and insulin differentially stimulate the PI3K/Akt/FoxO1 pathway by an early up-regulation of cytoplasmic p-Akt and delayed up-regulation of p-FoxO1 resulting in FoxO1 shift to the cytoplasm and the reduction of FoxO transcriptional activity, physiological serum concentration had no effect. IGF-1 at concentrations of 0.1 and 1 μM significantly reduced proliferation but increased differentiation of sebocytes to a greater extent than insulin (0.1 and 1 μM), but up-regulated TLR2/4 expression to comparable extent. These data provide the first *in vitro* evidence that FoxO1 principally might be involved in the regulation of growth-factor-stimulatory effects on sebaceous lipogenesis and inflammation in the pathological condition of acne. However, the *in vivo* significance under physiological conditions remains to be elucidated.

1. Introduction

Acne vulgaris is a chronic inflammatory skin disorder of the pilosebaceous unit (Gollnick et al., 2003; Karciauskiene et al., 2014; Thiboutot et al., 2009). Androgen-dependent sebum production, follicular hyperkeratinization, inflammation and dysbalanced adaptive immune response represent main factors in acne pathogenesis (Kurokawa et al., 2009; Taylor et al., 2011), while the exact pro-inflammatory role of *Propionibacterium acnes* (*P. acnes*) is under continuous debate (Kistowska et al., 2013; Shaheen and Gonzalez, 2011). Acne arises mostly during teenage years, when a transient increase in insulin resistance is a physiological phenomenon. A close relation of the growth hormone (GH)/insulin-like growth factor 1 (IGF-1) axis to insulin resistance was found (Moran et al.,

Abbreviations: ACS, Acyl-CoA synthetase; ANOVA, analysis of variance; AR, androgen receptor; DAPI, 4, 6-diamidino-2-phenylindole, dihydro-chloride; ECL, Enhanced chemiluminescence; FAS, Fatty acid synthase; FoxO1, forkhead box-O1; GH, growth hormone; HMGCR, HMG-CoA reductase; IGF-1, insulin-like growth factor-1; IGFBP-1, IGF binding protein-1; LXR, liver X receptor; *P. acnes*, *Propionibacterium acnes*; PBS, Phosphate buffered saline; PDGF, platelet-driven growth factor; PI3K, phosphoinositide-3-kinase; PFA, paraformaldehyde; PPAR-γ, peroxisome proliferator-activated receptors; RXR, retinoid X receptor; SCD, stearoyl-CoA desaturase; SQD, Squalene epoxidase; SREBP-1, sterol regulatory element binding protein-1; TLR, Toll-like receptor.

* Corresponding author. Department of Dermatology and Venereology, Otto-von Guericke University, Leipziger Str. 44, 39120 Magdeburg, Germany.
E-mail address: harald.gollnick@med.ovgu.de (H. Gollnick).

[1] These authors contributed equally to this paper.

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2002). Recent reports and prospective as well as retrospective studies on dietary factors shed a new light on the pathogenetic factors involved in the course of acne and suggest that a high glycemic load diet and potentially high milk consumption (Bowe et al., 2010; Cordain et al., 2002; Norat et al., 2006; Qin et al., 2009) might exaggerate the physiological "overstimulation" of this endocrine axis by increasing serum concentrations of insulin, free IGF-1 and consecutively androgens, while simultaneously reducing insulin-like growth factor-binding protein 3 (IGFBP-3) (Cordain et al., 2003; Melnik and Schmitz, 2009; Smith et al., 2007a,b). Several interventional studies demonstrated that a low-glycemic-load diet improved acne (Kwon et al., 2012) and insulin sensitivity (Smith et al., 2007a,b), while a deficiency of IGF-1 prevents acne development (Ben-Amitai and Laron, 2011). According to a recent hypothesis, the main targets by which dietary metabolic signals are sensed is forkhead box class O1 transcription factor (FoxO1) (Melnik and Zouboulis, 2013). FoxO transcription factors are an important subgroup of the Fox family that play a pivotal role in cell cycle, cell differentiation and other cellular functions (Huang and Tindall, 2007). Growth factors and other cell stimuli activate the phosphoinositide-3-kinase (PI3K)/Akt pathway resulting in phosphorylation of FoxO proteins. P-FoxO is translocated from the nucleus to the cytoplasm, degraded and hence FoxO-dependent gene expression is inhibited (Huang and Tindall, 2007; Obsil and Obsilova, 2008). Interestingly, IGFBP-1 expression is FoxO-controlled (Kim and Fazleabas, 2004). Recently, it has been hypothesised that FoxO1 translocation to the cytoplasm induces androgen-receptor (AR)-mediated signal transduction and influences cell proliferation, sebaceous lipogenesis and Toll-like receptor 2 (TLR2)-dependent inflammatory cytokines (Melnik, 2010a,b), but *in vitro* and *in vivo* evidence has not been shown. Understanding those key regulatory molecules that may be involved in the clinical course of acne in response to growth factors could help to identify novel treatment strategies.

The aim of the present study was to investigate the regulation of the PI3K/Akt/FoxO by those most important insulinotropic western dietary factors in SZ95 sebocytes *in vitro*. Our results indicate that IGF-1 and insulin activate p-FoxO1 through the PI3K/Akt pathway via nuclear mobilization of FoxO1 to the cytoplasm and thereby increase the differentiation and up-regulation of TLR2/4 in SZ95 sebocytes, while proliferation is suppressed. Our findings support a possible role of the FoxO1 transcription factor in acne pathogenesis.

2. Materials and methods

2.1. Cell culture

Immortalized SZ95 human sebocytes (Zouboulis et al., 1999) were cultured in Sebomed Basal Medium (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal bovine serum (Biochrom, Berlin, Germany), 1% antibiotics (Biochrom, Berlin, Germany) and 5 ng/mL human epidermal growth factor (PromoCell, Heidelberg, Germany) and grown to confluence. Cell viability was measured by lactate dehydrogenase cytotoxicity detection kit (Roche Diagnostics, Manheim, Germany) and trypan blue staining (Sigma–Aldrich, Steinheim, Germany).

2.2. Western blot

SZ95 sebocytes were plated at a density of 6×10^5 cells in 25 cm^2 cell culture flasks (Nunc, Wiesbaden, Germany) and cultivated in serum-free complete medium (Sebomed Complete Medium, Biochrom). This medium was checked for insulin and IGF-1 content; insulin content was less than 7×10^{-6} μM and IGF-1 less than 0.003 μM. On the second day, sebocytes were washed with phosphate buffered saline (PBS) (Biochrom, Berlin, Germany) and stimulated with IGF-1 (BioVision, California, USA), insulin (Sigma–Aldrich, Steinheim, Germany) or GH (Sigma–Aldrich, Germany) (0.001, 0.01, 0.1 and 1 μM) for 15 to 120 min. For pre-treatment with the LY294002 (Cell Signalling Technology, USA), sebocytes were incubated for 30 min with 50 μM LY294002 before the growth factors were added. Nuclear and cytoplasmic protein was isolated using the NE-PER Kit (Thermo Scientific, Rockford, IL USA) following manufacturer's instruction. Twenty-five micrograms of protein was run on a 10% Tris-glycine SDS-polyacrylamide gel. Protein was transferred to a nitrocellulose membrane and incubated with the following 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), Lamin B (Cell Signaling #12586, 1:1000) (Cell Signaling Technology, USA) or β-actin (SIGMA #A5441, 1:5000) (Sigma, USA). Membranes were incubated with peroxidase-conjugated secondary antibodies (Peroxidase-conjugated AffiniPure Anti-Rabbit IgG, Jackson ImmunoResearch, USA #74425, 1:10000) (Goat anti mouse IgG (H/L): HRP, A Bio-Rad, USA #0300-0108P, 1:10000) and visualized by enhanced chemiluminescence (ECL).

2.3. Proliferation assay

SZ95 sebocytes (1×10^5cells/ml) were seeded in triplicates in 96-well plates (Nunc, Wiesbaden, Germany) for 24 to 76 h, or in 6-well plates (Corning, USA) for 168 h in serum-free complete medium. After overnight, IGF-1, insulin or GH (0.001, 0.01, 0.1 and 1 μM) with or without 50 μM LY294002 were added and sebocytes were grown for 24–168 h. Each 72 h the medium was replaced and growth factors were added. Before cell harvest, cells were labelled with [^{3}H] thymidine (0.2 μCi/well; ICN, Meckenheim, Germany) for 16 h and then trypsinized (Biochrom, Berlin, Germany). Incorporated radioactivity was harvested on glass fiber filters determined by liquid scintillation counting.

2.4. Oil red O staining

SZ95 sebocytes were plated in 2-chamber slides (Nunc, USA) at 3×10^5cells/ml and complete medium was added. After overnight, IGF-1, insulin or GH (0.001, 0.01, 0.1 and 1 μM) with or without 50 μM LY294002 and linoleic acid as a positive control was added for 72 h. Cells were rinsed 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 filtered. Cells were stained for 20 min, and then washed in 60% isopropanol and dH_2O. Hematoxylin (Thermo Scientific, USA) was added for counterstaining.

2.5. Lipid quantification

Stained cells were washed twice with dH_2O. After Oil Red O elution, Lipid accumulation in cell colonies was quantified by a light microscopy (Leica, Wetzlar, Germany) equipped with colour camera (Pixelink, Ottawa, Ontario) at 63x magnification. Images were captured and cells and lipid droplets were counted in six randomly chosen areas.

2.6. Transfection and luciferase assay

Sebocytes were cultured in 25 cm^2 cell culture flasks and transfected at 80% confluence with Cignal FoxO Reporter (QIAGEN, USA) using Lipofectamin 2000 (Invitrogen) transfection reagent. Six hours later, sebocytes were trypsinized and cultivated in 96-well plates. After overnight, cells were stimulated with 1 and 0.1 μM