IGF-1 Induces SREBP-1 Expression and Lipogenesis in SEB-1 Sebocytes via Activation of the Phosphoinositide 3-Kinase/Akt Pathway

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Understanding the factors that regulate sebum production is important in identifying therapeutic targets for acne therapy. Insulin and IGF-1 stimulate sebaceous gland lipogenesis. IGF-1 increases expression of sterol response element-binding protein-1 (SREBP-1), a transcription factor that regulates numerous genes involved in lipid biosynthesis. SREBP-1 expression, in turn, stimulates lipogenesis in sebocytes. The goal of this study was to identify the intracellular signaling pathway(s) that transduces the lipogenic signal initiated by IGF-1. Sebocytes were treated with IGF-1 and assayed for activation of the phosphoinositide 3-kinase (PI3-K) pathway and of the three major arms of the mitogen-activated protein kinase (MAPK) pathway (MAPK/extracellular signal-regulated kinase (ERK), p38 MAPK, and stress-activated protein kinase/c-Jun-N terminal kinase). IGF-1 activated the MAPK/ERK and PI-3K pathways. Using specific inhibitors of each pathway, we found that the increase in expression of SREBP-1 induced by IGF-1 was blocked in the presence of the PI3-K inhibitor but not in the presence of the MAPK/ERK inhibitor. Furthermore, inhibition of the PI3-K pathway also blocked the IGF-1-induced transcription of SREBP target genes and sebocyte lipogenesis. These data indicate that IGF-1 transmits its lipogenic signal in sebocytes through activation of Akt. Specific targeted interruption of this pathway in the sebaceous gland could be a desirable approach to reducing sebum production and improving acne.

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INTRODUCTION

Sebum production is a critical factor in the pathogenesis of acne, although the molecular signals involved in sebum production are largely unknown. To design rational therapies for acne, it is essential to understand the signaling pathways that drive seborrhea in acne patients. Previous work has shown that IGF-1 levels reach their peak in adolescents during the growth spurt and then decline, which coincides with the incidence of acne in many individuals (Deplewski and Rosenfield, 1999). The same group has also shown that IGF-1 stimulates sebaceous gland lipogenesis (Deplewski and Rosenfield, 2000). Lipogenesis is also stimulated by IGF-1 in sebaceous glands grown in organ culture (Downie *et al.*, 2002).

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Abbreviations: ERK, extracellular signal-regulated kinase; JNK, c-Jun-N terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PI3-K, phosphoinositide 3-kinase; SAPK, stress-activated protein kinase; SREBP, sterol response element-binding protein

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Recent reports indicate a correlation between severity of acne in women and serum IGF-1 levels (Cappel *et al.*, 2005). Furthermore, studies show that IGF-1 increases lipogenesis in the SEB-1 sebocyte model and this increase in lipogenesis is accompanied by an increase in expression of sterol response element-binding protein-1 (SREBP-1) mRNA and protein (Smith *et al.*, 2006). Dubbed, ''master regulators of lipid homeostasis'', SREBPs have been shown to be regulated by androgens in the hamster ear sebaceous model (Rosignoli *et al.*, 2003; Eberle *et al.*, 2004). In other tissues and cell lines, there have been numerous reports demonstrating an increase in SREBP mRNA and protein in response to a variety of stimuli (Kotzka *et al.*, 1998; Foretz *et al.*, 1999; Chang *et al.*, 2005). There is little doubt that an increase in SREBP protein increases lipogenesis, particularly in the liver (Goldstein *et al.*, 2006). Here, we sought to extend these findings to the sebaceous gland.

SREBPs bind sterol response elements, which are nucleotide sequences found in the promoter regions of several lipogenic genes in the cholesterol and fatty acid biosynthesis pathways. There are three members in the SREBP family: SREBP-1a, SREBP-1c, and SREBP-2. SREBP-1a and 1c are transcribed by alternative start sequences, and the transcripts are each spliced to form an identical protein from the second exon onward. The longer first exon found in SREBP-1a makes it the more potent activator of transcription, and more prone to activate transcription of genes typically controlled by

SREBP-2. There is functional overlap between SREBP-1 and SREBP-2, but SREBP-1 typically controls genes in the fatty acid biosynthesis pathway, whereas SREBP-2 controls transcription of genes associated with cholesterol biosynthesis (Shimano *et al*., 1997, 1999).

The goal of this study is to determine the molecular signaling pathways by which IGF-1 stimulation of SEB-1 sebocytes increases SREBP-1 mRNA and protein. It has been shown in other cell types that IGF-1 activates both the stress-activated protein kinase/c-Jun-N terminal kinase (SAPK/JNK) and the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) cascades, in addition to the phosphoinositide 3-kinase (PI3-K) pathway. Furthermore, all three of these pathways have been implicated in activation of SREBP in at least one model system. It is likely that the molecular pathways governing the SREBPs may be tissue/cell type specific. In this paper, we report that IGF-1 activates the PI3-K and MAPK/ERK pathways in SEB-1 sebocytes, but not the SAPK/JNK or MAPK/p38 pathways. Moreover, pharmacological antagonism of the MAPK/ERK pathway has no effect upon the induction of SREBP-1 mRNA, protein, or lipogenesis by IGF-1. Most importantly, induction of the PI3-K pathway by IGF-1 mediates the increase in SREBP-1 mRNA, protein, and lipogenesis in SEB-1 sebocytes. Finally, inhibition of the PI3-K pathway with the inhibitor LY294002 completely blocks the increase in lipogenesis in SEB-1 cells seen in response to IGF-1. These data demonstrate that inhibition of PI3-K signaling in the sebaceous gland decreases lipogenesis and, as such, this approach may represent a novel strategy in the treatment of acne.

RESULTS

The MEK inhibitor, PD98059, blocks the activation of MAPK/ERK signaling by IGF-1, yet has no effect on the induction of SREBP-1 by IGF-1

The MAPK/ERK pathway is activated when MAPK/ERK kinase (MEK) phosphorylates p44 MAPK or p42 MAPK (ERK 1 and 2, respectively). To determine if IGF-1 mediates its effect on SREBP-1 expression in sebocytes through the MAPK/ERK pathway, SEB-1 cells were treated with IGF-1 (20 ng ml^{-1}) in the presence and absence of the MEK inhibitor PD98059 (50 μM) and the effect on downstream mediators was examined by Western blot. IGF-1 induced a robust increase in phosphorylated p44/p42 protein in SEB-1 sebocytes after 24 hours (Figure 1a). Pretreatment of SEB-1 cells with 50 μM of the MEK inhibitor PD98059 for 30 minutes prior to the addition of IGF-1 blocked activation of this pathway (Figure 1a), indicating that IGF-1 activates the MAPK/ERK pathway in SEB-1 sebocytes. No cytotoxicity was observed with 50 μM PD98059 treatment and cell counts at 24 hours showed no difference from DMSO-treated cells (data not shown).

To determine if the MAPK/ERK pathway plays a role in the induction of SREBP-1 by IGF-1, we treated SEB-1 cells with PD98059 (50 μM) and/or IGF-1 (20 ng ml^{-1}). Addition of the MEK inhibitor failed to block the induction of SREBP-1 mRNA (Figure 1b) or SREBP-1 protein (Figure 1c) by IGF-1, indicating that IGF-1-induced expression of SREBP-1 is not mediated by the MAPK/ERK pathway.

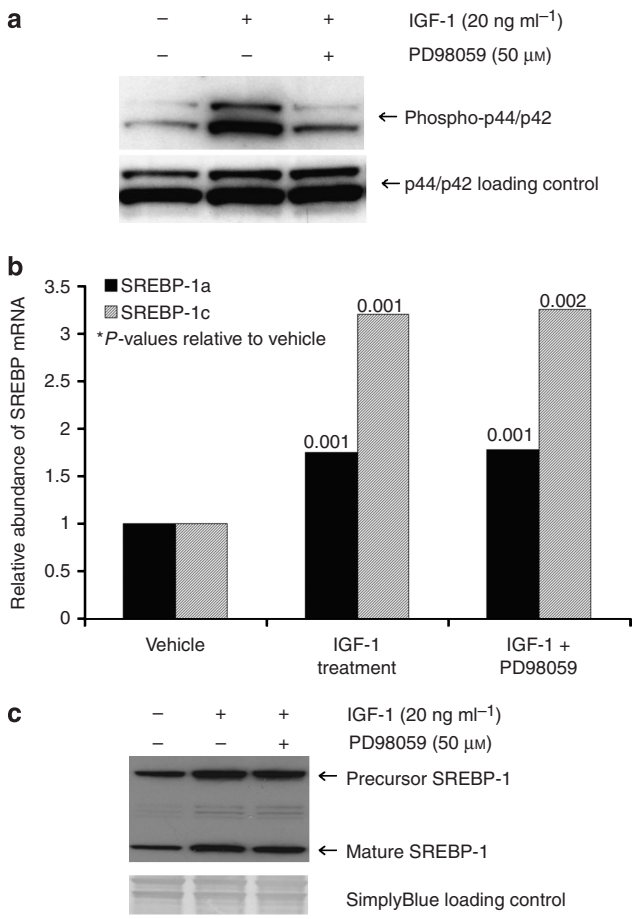

Figure 1. The MAPK/ERK pathway is activated by IGF-1 but does affect SREBP-1 expression in SEB-1 cells. (**a**) Western blot reveals that p44/p42 (ERK1/2) is phosphorylated in SEB-1 cells treated with 20 ng ml^{-1} IGF-1 and that this stimulation can be blocked by 50 μM PD98059. The phospho-p44/p42 blot was stripped and re-probed with an antibody that recognizes total p44/p42 regardless of phosphorylation status to serve as a loading control. These data indicate that IGF-1 activates the MAPK/ERK pathway and that this activation is blocked in the presence of a MEK inhibitor. A representative of five blots is shown. (**b**) SEB-1 cells were treated with MEK inhibitor PD98059 (50 μM) and/or IGF-1 (20 ng ml^{-1}) and QPCR was performed to quantify SREBP-1a and -1c mRNA. Data are representative of six samples per treatment group as analyzed by the REST-XL program. A *P*-value <0.05 was considered statistically significant. Using the REST program, it is not possible to generate SD or SE unless the same reverse transcription reaction is run repeatedly, thus, *P*-values are used to gauge variation. These data indicate that inhibition of the MAPK/ERK pathway does not abrogate the induction of SREBP-1 mRNA by IGF-1. (**c**) Western blot was used to confirm that the MEK inhibitor PD98059 did not block the induction of SREBP-1 protein by IGF-1 in SEB-1 cells. Cells were treated as described in (**b**). After being probed for SREBP-1, the membrane was stained with Simply Blue to ensure equal protein loading. A representative of five blots is shown.

IGF-1 does not activate signaling through the p38 MAPK or the SAPK/JNK pathways in SEB-1 sebocytes at 24 hours

In addition to signaling in cell growth and differentiation, MAPKs are also involved in signaling pathways involving inflammation and apoptosis. Both the p38 MAPK and the SAPK/JNK are involved in these alternative pathways. To determine if the p38 MAPK or the SAPK/JNK pathways transduce the signal by IGF to increase lipogenesis, Western

blots were performed under the same experimental conditions to determine the phosphorylation status of p38 MAPK and SAPK/JNK in SEB-1 sebocytes stimulated with IGF-1. We did not observe activation of either p38 or SAPK/JNK in response to IGF-1, indicating that these pathways are not critical for IGF-1-induced lipogenesis in sebocytes (Figure 2).

The PI3-K inhibitor, LY294002, blocks the increase in transcription, translation, and processing of SREBP-1 that is induced by IGF-1

Akt is a critical member of the PI3-K pathway. As such, phosphorylation of Akt is a measure of activation of the PI3-K pathway. To determine if the PI3-K pathway transduces the signal by IGF to increase lipogenesis, Western blots for pAkt and SREBP-1 were performed in the presence of the PI3-K inhibitor, LY294002, with and without IGF-1. SEB-1 sebocytes were found to have very low amounts of phosphorylated Akt when maintained in medium without serum. However, the addition of IGF-1 (20 ng ml^{-1}) to this same medium induced a robust increase in phosphorylated Akt at 24 hours (Figure 3a). Importantly, we demonstrate that this induction of phosphorylated Akt was blocked quite potently by the addition of 50 μM of the PI3-K inhibitor, LY294002 (Figure 3a). No cytotoxicity was observed with 50 μM LY294002 treatment. Minimal decreases in viable count were observed after 24 hours of treatments as cells treated with LY294002, both with or without IGF-1 stimulation had approximately 10% fewer viable cells than the vehicle-treated cells (data not shown).

We then treated SEB-1 cells with LY294002 and/or IGF-1 to determine if Akt activation was essential for the induction of SREBP-1 mRNA and protein by IGF-1 that was observed in

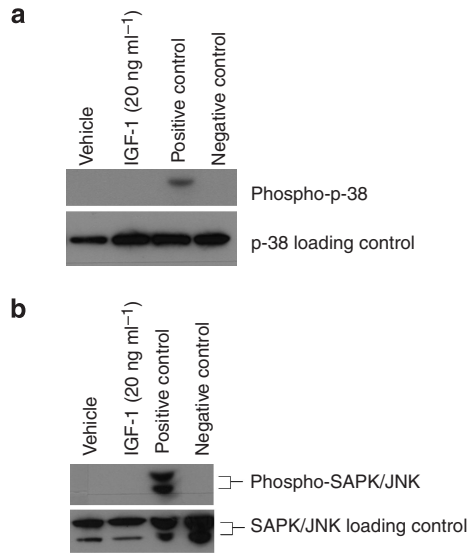

Figure 2. IGF-1 does not activate p38 MAPK or SAPK/JNK pathways in SEB-1 cells. (**a**) A Western blot probing for phosphorylated MAPK/p38 reveals that SEB-1 sebocytes do not phosphorylate p38 in response to IGF-1. C-6 glioma cells treated with anisomycin serve as a positive control for phosphorylated p38, whereas unstimulated C-6 glioma cells represent the negative control. The phospho-p38 blot was stripped and re-probed with an antibody that recognizes total p38 as a loading control. This experiment was repeated with no phosphorylation of p38 observed when the film was overexposed. (**b**) A western blot probing for phosphorylated SAPK/JNK reveals that SEB-1 sebocytes do not phosphorylate SAPK/JNK in response to IGF-1. Total cell extracts from 293 cells treated with UV light (Cell Signaling Technology) serve as a positive control for phosphorylated JNK, whereas untreated 293 extracts serve as the negative control. The phospho-SAPK/JNK was stripped and re-probed with an antibody that recognizes total SAPK/JNK as a loading control. This experiment was repeated with no phosphorylation of SAPK/JNK observed when the film was overexposed.

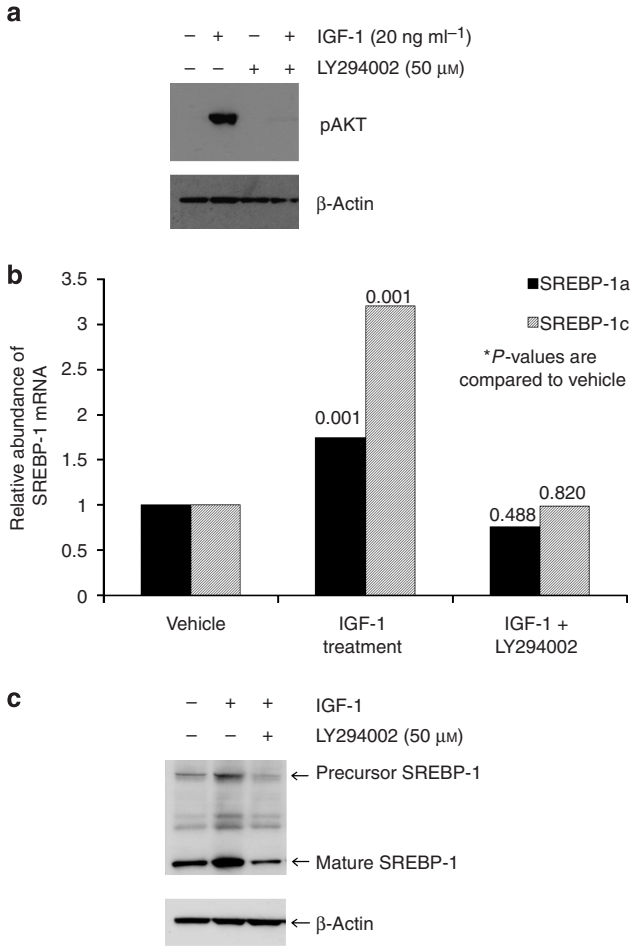

Figure 3. IGF-1 activates the PI3-K/Akt pathway, which mediates the increased expression of SREBP mRNA and protein in SEB-1 sebocytes. (**a**) Western blot reveals that Akt is phosphorylated by IGF-1 at 24 hours and that this induction is blocked when cells are treated with the PI3-K inhibitor LY294002 for 30 minutes prior to the addition of IGF-1. A representative of five blots is shown. (**b**) SEB-1 cells were treated with 50 μM of the PI3-K inhibitor LY294002 and/or 20 ng ml^{-1} IGF-1 for 24 hours and QPCR was performed to quantify transcription of SREBP-1a and -1c. Data are representative of six samples per treatment group as analyzed by the REST-XL program. Using the REST program, it is not possible to generate SD or SE unless the same reverse transcription reaction is run repeatedly, thus *P*-values are used to gauge variation. These data indicate that inhibition of the PI3-K pathway with LY294002 abrogates the IGF-1-induced increase in expression of mRNA for both SREBP-1a and -1c. (**c**) A western blot probing for SREBP-1 was performed in SEB-1 cells in the presence or absence of PI3-K inhibitor LY294002 and IGF-1 (20 ng ml^{-1}). β-Actin was used as the loading control. Data indicate that the precursor and mature forms of the SREBP-1 protein are not induced by IGF-1 in the presence of the PI-3K inhibitor, which supports the QPCR data above. A representative of five blots is shown.

previous studies (Smith *et al.*, 2006). We found that inhibition of the PI3-K/Akt pathway by LY294002 blocked the induction of SREBP-1 mRNA and protein by IGF-1 (Figure 3b and c).

IGF-1 induces lipogenesis in SEB-1 sebocytes via the PI3-K pathway

As both the PI3-K and MAPK/ERK pathways are activated in SEB-1 cells by IGF-1, we next wanted to dissect the contribution of each of these signaling pathways to the increase in lipogenesis noted in response to IGF-1 treatment. We know that the MEK inhibitor PD98059 has no effect on SREBP activation by IGF-1. Keeping in line with our central hypothesis that SREBPs are important mediators of lipid metabolism in SEB-1 cells, we hypothesized that treatment of SEB-1 cells with an MAPK inhibitor would reduce lipogenesis to a much lesser extent than treatment with a PI3-K inhibitor. The ^{14}C acetate incorporation assay was performed on SEB-1 cells treated with IGF-1 alone, or with the addition of PD98059 or LY294002 in conjunction with IGF-1. IGF-1 increased lipogenesis as we have shown previously (Smith *et al.*, 2006). Additionally, treatment with MAPK inhibitor PD98059 had no effect on this induction of lipogenesis (Figure 4). However, when cells were treated with LY294002, IGF-1 failed to induce lipogenesis, as cells in this treatment group produce a lipid profile nearly identical to the group that received no IGF. From this, we conclude that IGF-1 induces lipogenesis in SEB-1 cells via the PI3-K pathway (Figure 4).

Numerically minor, yet statistically significant, decreases in rates of lipogenesis were observed for nearly all lipids in cells treated with either PD98059 or LY294002 in the absence of IGF-1 relative to the DMSO-treated control. The only exception were both the PD98059- and LY294002-treated wax ester class, which were unchanged; and that the cells treated with LY294002 actually had a statistically significant increase in cholesterol oleate relative to the unstimulated DMSO-treated control group. There were no morphological changes to the cells. We would attribute these minor changes in rate of lipogenesis to a slight decrease in proliferation associated with inhibition of either the PI3-K or MAPK pathway that is difficult to detect at 24 hours.

Analysis of transcriptional targets of SREBPs

We next sought to determine if the increase in nuclear SREBP protein was accompanied by a corresponding increase in the mRNA levels of SREBP transcriptional targets. Following 14 hours of IGF-1 treatment (20 ng ml^{-1}), SEB-1 RNA was isolated and QPCR was performed to determine the relative amounts of fatty acid synthase, acyl-CoA synthetase, stearoyl-CoA desaturase, squalene epoxidase, and HMG-CoA reductase. Transcription of each of these genes has been shown to be activated by SREBP-1 in other models (Shimano *et al.*, 1997; Ikeda *et al.*, 2001; Sakakura *et al.*, 2001; Sone *et al.*, 2002; Chang *et al.*, 2005; Murphy *et al.*, 2006) and each plays a role in lipogenesis. Here, we show that IGF-1 increases mRNA of each of these five genes, and further, this

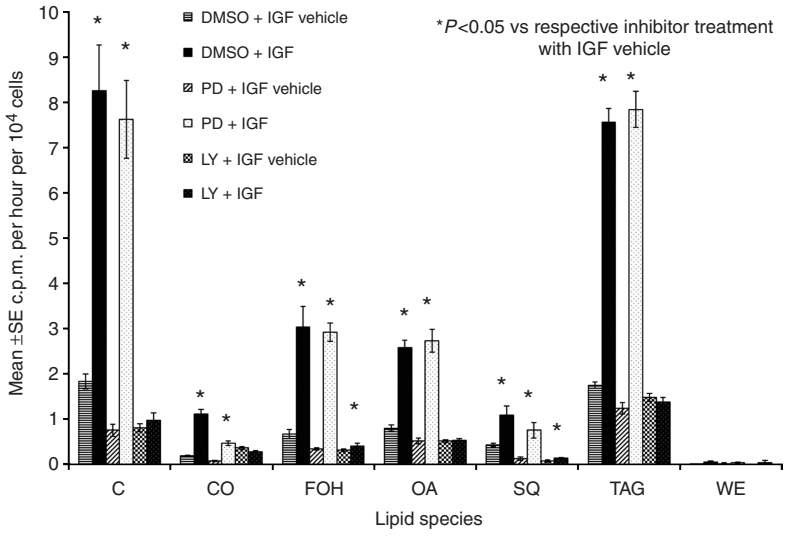

Figure 4. IGF-1 induces lipogenesis in SEB-1 sebocytes through activation of the PI3-K pathway. The ^{14}C incorporation assay was performed on SEB-1 cells treated with 50 μM of a pharmacological inhibitor of PI3-K (LY294002) or MAPK/ERK (PD98059) and/or IGF-1 (20 ng ml^{-1}). IGF-1 induces a robust increase in lipogenesis (horizontal striped bars vs solid black bars). Interestingly, addition of 50 μM PD98059 to cells treated with IGF-1 has no effect on the IGF-1-induced lipogenesis (diagonally striped bars vs white bars with black dots). Most importantly, the addition of 50 μM PI3-K inhibitor LY294002 blocks any induction of lipogenesis when cells are stimulated with IGF-1 (checkered bars vs black bars with white dots). All samples within an experiment were prepared in triplicate, and each experiment was performed at least three separate times. Data were analyzed by Student's *t*-test and considered significant if a *P*-value of <0.05 was observed compared to the sample treated with the same inhibitor (DMSO-vehicle, LY294002, or PD98059) that did not receive IGF-1 treatment. Additionally, treatment with PD or LY caused a statistically significant decrease in C, FOH, OA, SQ, and TAG relative to the unstimulated, untreated control cells (diagonally striped or checkered vs horizontal stripe bars). C, cholesterol; CO, cholesterol oleate; FOH, fatty alcohols; OA, oleic acid; SQ, squalene; TAG, triglycerides; WE, wax esters.

effect was attenuated in the presence of the PI3-K inhibitor LY294002 (Figure 5).

DISCUSSION

It is well established in the literature that expression of SREBP-1 is increased in a variety of cell lines in response to a litany of growth factors (Nadeau *et al.*, 2004). Previous work in the SEB-1 sebaceous cell model system has shown that IGF-1 increases both expression of SREBP-1 protein and lipogenesis (Smith *et al.*, 2006). Recent reports indicate that SREBP-1 and other lipogenic factors are also expressed in sebaceous glands and the SZ95 sebocyte cell line (Harrison *et al.*, 2007). There are conflicting reports as to which molecular pathway(s) is involved in transmitting the growth factor signal that eventually leads to induction of the SREBPs in other cell systems (Nadeau *et al.*, 2004). In this study, we sought to determine which pathway(s) is critical for the induction of SREBP-1 protein by IGF-1 in SEB-1 sebocytes, and furthermore, to determine which pathway(s) is important for the increase in lipogenesis observed when SEB-1 cells are stimulated with IGF-1. An understanding of the signaling mechanisms that regulate the production of sebaceous lipids can lead to the identification of potential therapeutic targets to reduce sebum production and improve acne. To this end, we studied the role of the PI3-K pathway and the three major arms of the MAPK pathway (p38, SAPK/JNK, and p44/p42) in mediating the lipogenic signal induced by IGF-1.

There are cell-type specific pathways that mediate signals from growth factor receptors and that regulate the expression of SREBP and lipogenesis (Nadeau *et al.*, 2004). The p38 MAPK and the SAPK/JNK pathways are typically associated with cell stress and are variably activated by growth factors. In fact, activation of the IGF-1 receptor can actually downregulate both pathways. Conversely, in the H292 pulmonary epithelial cancer cell line, keratinocyte growth factor (KGF) activates the SAPK/JNK pathway to increase SREBP protein and lipogenesis (Chang *et al.*, 2005). Our data indicate that neither the p38 MAPK nor the SAPK/JNK pathways are activated in SEB-1 cells in response to IGF-1 stimulation, and thus would not be involved in the activation of the SREBPs.

Numerous reports, particularly by groups using skeletal myoctyes, have found the SREBPs to be activated by the p42/p44 (ERK1/2) arm of the MAPK pathway in response to insulin (Kotzka *et al.*, 2000; Nadeau *et al.*, 2004). When myocytes were treated with insulin in the presence of PD98059, an inhibitor of the p42/p44 MAPK pathway, the increase in expression of SREBP-1 was blocked. However, in adipocytes, the converse was true wherein the induction of SREBP-1 by insulin was blocked in the presence of the PI3-K inhibitor, LY294002, but not in the presence of the MAPK/ERK inhibitor, PD98059. As expected, SEB-1 sebocytes stimulated with IGF-1 display a robust activation of the p44/p42 MAPK/ERK pathway (Figure 1) and also activation of the PI3-K pathway (Figure 3). As activation of SREBP proteins has been shown to increase transcription of several lipogenic genes (Rawson, 2003), we hypothesized that one or both pathways are responsible for the increase in SREBP and ultimately, the increase in lipogenesis that we have observed previously with IGF treatment (Smith *et al.*, 2006).

As far as SREBP activation is concerned, we found that inhibition of the p44/p42 MAPK pathway with PD98059 had no effect on SREBP-1a or SREBP-1c mRNA, the translation of SREBP-1 protein, or the processing of the protein into the mature form in IGF-1 stimulated cells (Figure 1b and c). Here, we establish that MAPK activation is not necessary for IGF-1-induced activation of SREBPs in SEB-1 sebocytes. Importantly, inhibition of the PI3-K pathway with LY294002 blocked transcription of SREBP-1a and 1c mRNA, translation of SREBP-1 protein, and also decreased the amount of the mature SREBP-1 protein found in the nucleus of IGF-1 stimulated SEB-1 cells (Figure 3). This clearly implicates the PI3-K/Akt pathway as the means by which IGF-1 mediates the increase in SREBP-1 activity in SEB-1 sebocytes.

Recent reports indicate that activation of Akt is involved in the transport of the SREBP cleavage activating protein/SREBP complex from the endoplasmic reticulum to the Golgi (Du *et al.*, 2006). This is a major regulatory step in SREBP activity. Our data indicate that IGF-1 increases the amount of cleaved (mature) SREBP protein and that this increase in mature protein can be blocked in the presence of the PI3-K inhibitor (Figure 3c). This suggests that, in addition to possible transcriptional/translational control, Akt activation may also affect SREBP processing in SEB-1 sebocytes. Additional experiments are needed to test this hypothesis.

Akt activation has been shown to increase the expression of lipogenic genes (Porstmann *et al.*, 2005). Microarray

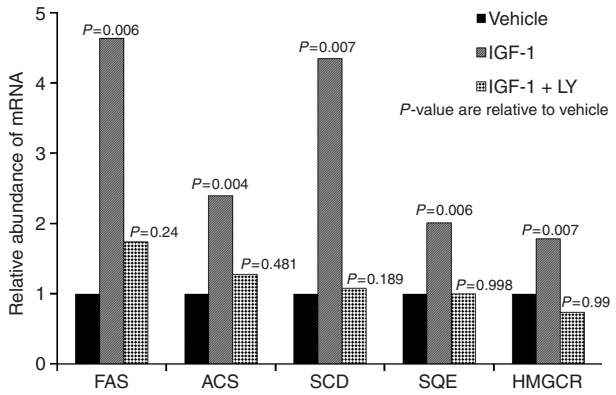

Figure 5. Inhibition of the PI3-K pathway in SEB-1 sebocytes abrogates the induction of SREBP target genes by IGF-1. SEB-1 cells were treated with IGF-1 (20 ng ml^{-1}) in the presence or absence of 50 μm of the PI3-K inhibitor LY294002, and QPCR was performed to ascertain the mRNA levels of several transcriptional targets of SREBPs. IGF-1 induces a robust increase in the expression of SREBP target genes that is blocked in the presence of the PI3-K inhibitor (which blocks SREBP induction). Fatty acid synthase, acyl-CoA synthetase, and stearoyl-CoA desaturase are traditional targets of SREBP-1, as they are involved in fatty acid biosynthesis. Squalene epoxidase and HMG-CoA reductase are involved in cholesterol biosynthesis. Data are representative of five samples per treatment group as analyzed by the REST program. Using the REST program, it is not possible to generate SD or SE unless the same reverse transcription reaction is run repeatedly, thus *P*-values are used to gauge variation. FAS, fatty acid synthase; ACS, acyl-CoA synthetase; SCD, stearoyl-CoA desaturase; SQE, squalene epoxidase; HMGCR, HMG-CoA reductase.

analysis of gene expression was compared between retinal pigment epithelial cells that express a constitutively activated Akt construct and wild-type cells. Data indicated increased expression of numerous genes involved in sterol and lipid metabolism in the cells expressing activated Akt (Porstmann *et al.*, 2005). Additional experiments revealed that these gene changes were mediated by an increase in the expression of SREBP-1a and SREBP-1 c (Porstmann *et al.*, 2005). These data are supported by our observation that inhibition of Akt activation blocks the increase in mRNA expression of lipogenic genes induced by SREBP-1 (Figure 5).

After establishing that activation of the PI3-K pathway by IGF-1 leads to an increase in mRNA for SREBP-1 and its target genes, we sought to determine the role of the PI3-K pathway in lipogenesis in SEB-1 sebocytes. Lipid production in sebocytes is a key predictive end point for drugs that may be of benefit in acne. Sebum, the lipid product of sebocytes, consists primarily of cholesterol, triglycerides, and wax esters. Here, we show that treatment with the PI3-K inhibitor LY294002 blocks the robust induction of lipogenesis noted in response to IGF-1 treatment for all lipids assayed. In contrast, although IGF-1 activated the MAPK/ERK pathway in SEB-1, inhibition of this pathway had no effect on the IGF-1-induced lipogenesis in SEB1 cells (Figure 4). These data provide a rationale for investigation of members of the PI3-K pathway as drug targets to decrease sebum production and improve acne. Topical application of an inhibitor to a specific PI3-K isoform would be desirable, as the broad inhibition of PI3-K by an oral agent renders it undesirable as a systemic therapy.

The PI3-K enzyme consists of a p110 and a p85 subunit, each of which has several isoforms. The p110α isoform is activated in response to insulin and mediates the metabolic effects of insulin, including glucose uptake (Knight *et al.*, 2006). Whether this isoform mediates the same effects in sebocytes remains to be determined. Research is underway to develop small molecule drugs to target several p110 isoforms. As it has been recently learned that p110α is frequently mutated in human tumors, this class will come to the forefront of cancer research (Samuels *et al.*, 2004). Progress in this area could be utilized to develop a topical inhibitor of PI3-K to reduce sebum production. Although the pathogenesis of many diseases may be attributable to PI3-K signaling, acne lends itself to the treatment with small molecules inhibitors due to the accessibility of the sebaceous gland by topical application, particularly lipophilic molecules.

Building on our previous work that showed that IGF-1 induces lipogenesis in SEB-1 sebocytes, here we have shown that (1) the PI3-K molecular signaling pathway transduces the IGF-1 signal, resulting in an increase in lipogenesis, (2) the PI3-K pathway is essential for IGF-1 induced lipogenesis, and (3) the increase in SREBP-1 nuclear protein is accompanied by an increase in the transcription of several lipogenic genes downstream of SREBP-1. As the p110α subunit of the PI3-K molecule has recently been shown to mediate the insulin signal, it is plausible that the small molecules designed to inhibit this isoform of the protein could be useful in reducing sebaceous gland lipogenesis and improving acne.

MATERIALS AND METHODS

Cell culture and treatments

SEB-1 (passage 22–27) SV40 immortalized human sebocytes were grown to confluence in all experiments unless stated otherwise and were cultured in standard medium consisting of DMEM with 5.5 mM glucose/Ham's F-12 3:1, fetal bovine serum 2.5%, adenine 1.8×10^{-4} M, hydrocortisone 0.4 μg ml^{-1}, insulin 10 ng ml^{-1}, epidermal growth factor 3 ng ml^{-1}, and cholera toxin 1.2×10^{-10} M (Thiboutot *et al.*, 2003).

For treatments with IGF-1, SEB-1 cells were plated at 7.6×10^5 in a 100 mm dish, or 1.3×10^5 in a 35 mm dish and grown for 6 days. On the sixth day, medium was removed; cells were washed twice with phosphate-buffered saline, and given DMEM containing 5.5 mM glucose. IGF-1 was added 1:1,000 in 0.1 M acetic acid plus 0.1% BSA for 24 hours. MEK inhibitor PD98059 (50 μM) was used as a pharmacological inhibitor of the MAPK/ERK pathway, and cells were pretreated with the compound 30 minutes prior to stimulation with 20 ng ml^{-1} IGF-1. Likewise, the cell permeable PI3-K inhibitor, LY294002, at a dose of 50 μM was also administered 30 minutes prior to IGF-1 treatment. Both compounds were suspended in DMSO and the final concentration of DMSO in the media was 0.1%. Dose response experiments (10, 20, and 50 μM) with each inhibitor were conducted to determine the optimal concentration that inhibited phosphorylation of Akt (LY294002) or ERK (PD98059). For each inhibitor, optimal inhibition was obtained with the 50 μM concentration as has been commonly reported in other cell systems.

Lipogenesis assay

The incorporation of ^{14}C-acetate into lipids was used as a measure of lipogenesis as described previously (Smith *et al.*, 2006). Briefly, SEB-1 cells were grown to confluence in a 35 mm dish and treated as described above. Cells were trypsinized and resuspended in a solution containing 1 μCi ^{14}C-acetate (New England Nuclear, Boston, MA) in DMEM, and incubated for 2 hours at 37°C with agitation. Following the incubation, lipids were extracted twice with ethyl ether and non-radioactive carrier lipids. The solvents were evaporated under a stream of nitrogen gas, and lipids were taken up in petroleum ether and separated by thin layer chromatography. Lipid spots were visualized, excised, and radioactivity in each spot was quantified in a liquid scintillation counter. All samples within an experiment were prepared in triplicate, and each experiment was performed at least three separate times. Data were analyzed by ANOVA and considered significant if a P-value of <0.05 was observed compared to the vehicle-treated control group.

Western blot

Protein was extracted using the Ne-Per kit (Pierce, Rockford, IL). Both the precursor and mature SREBP-1 protein was found in the nuclear lysate as described previously (Smith *et al.*, 2006). This appears to be unique to the SEB-1 cells, as the endoplasmic reticulum is solubilized by the cytoplasmic detergents in the Ne-Per kit. Western blot for the endoplasmic reticulum protein GRP-78 revealed that this marker was present in both the nuclear and cytoplasmic fraction (data not shown). Thus, all blots shown using SEB-1 extracts probed for SREBP-1 are the nuclear fractions. Twenty-five micrograms of protein was run on a 4–12% Bis-Tris NuPage polyacrylamide gel (Invitrogen, Carlsbad, CA). Protein was then transferred to a polyvinylidene fluoride membrane and probed using

standard methods. The SREBP-1 (K-10) antibody was purchased from Santa Cruz (Santa Cruz, CA). In samples that were probed for phospho-JNK, JNK, phospho-p38, p38, phospho-ERK, ERK, or phospho-Akt, cytoplasmic protein lysates were used and the antibody was purchased from Cell Signaling Technology (Boston, MA). Membranes were probed with an anti-actin antibody or stained with SimplyBlue (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions to ensure even protein loading.

C-6 glioma cells treated with anisomycin were used as a positive control for phosphorylated p38, whereas unstimulated C-6 glioma cells were the negative control. Total cell extracts from 293 cells treated with UV light served as a positive control for phosphorylated JNK, whereas normal 293 extracts served as the negative control. All control cell extracts were purchased from Cell Signaling Technology.

Quantitative reverse transcription-PCR

For QPCR, cells were grown in standard medium for 6 days. On the sixth day, cells were washed twice with phosphate-buffered saline and pre-treated 30 minutes with the appropriate inhibitor/vehicles prior to 14 hour treatment with 20 ng ml^{-1} IGF-1 in serum-free DMEM. Total RNA was isolated and complementary DNA was generated from 4.2 µg RNA/reaction primed with oligo-dT using the SuperScript First-Strand Synthesis System for reverse transcription-PCR (Invitrogen). QPCR was performed using the Brilliant SYBR Green QPCR Core Reagent Kit in an Mx4000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA). TATA-binding protein was used as a reference gene. The following primer sequences were used: TATA-binding protein upstream (GenBank number NM_003194) 5′-cacggcactgattttcagttct, TATA-binding protein downstream 5′-ttcttgctgccagtctggact, SREBP-1a upstream (GenBank number NM_004176) 5′-gctgctgaccgacatcgaa, SREBP-1c upstream (GenBank number NM_001005291 5′-ggagccatggattgcacttt, and SREBP-1a, c downstream 5′-tcaaataggccagggaagtca. Primer pairs for fatty acid synthase (product number Hs00188012_m1), acyl-CoA synthetase short-chain family member 2 (Hs002318766_m1), stearoyl-CoA desaturase (delta-9-desaturase) (Hs00748952_s1), squalene epoxidase (Hs00162288_m1), and HMG-CoA reductase (Hs00168352_m1) were purchased from ABI (Foster City, CA). Data were analyzed using the REST-XL© program (Pfaffl *et al.*, 2002). A P-value <0.05 was considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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