ORIGINAL ARTICLE

Insulin-Like Growth Factor-1 Induces Lipid Production in Human SEB-1 Sebocytes Via Sterol Response Element-Binding Protein-1

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An understanding of the molecular signaling involved in sebaceous gland lipid production is needed to develop therapeutic targets to improve acne. Treatment with methylisobutylxanthine, dexamethasone, and a high dose of insulin (MDI) has been shown to differentiate 3T3-L1 preadipocytes into adipocytes, a differentiation marked by an increase in lipid production. The present study has the following aims: (1) Since high doses of insulin, as found in MDI, will activate the IGF-1 receptor, we sought to determine if IGF-1 is capable of reproducing the lipogenic effect seen with MDI treatment, and (2) to determine if the sterol response element-binding protein-1 (SREBP-1) pathway mediates the increase in lipogenesis. Here we report that MDI increases lipogenesis and that this effect can be attributed wholly to the high-dose insulin in SEB-1 cells. Further, we show that a physiologically relevant dose of IGF-1 or high-dose (1 μM) insulin induces an increase in SREBP-1 mRNA, protein, and total lipid production; while 100 nM insulin induces lipogenesis yet the SREBP protein levels remain unchanged. These data indicate that activation of the IGF-1 receptor increases lipogenesis in SEB-1 cells through both SREBP-dependent and SREBP-independent pathways.

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INTRODUCTION

Sebum production is pivotal in the pathogenesis of acne. An understanding of the factors that regulate this process is important in designing strategies to improve acne. The peak incidence of acne occurs during adolescence when serum IGF-1 levels reach their peak during the growth spurt (Deplewski and Rosenfield, 1999), and IGF-1 levels correlate positively with severity of acne in women with clinical acne (Cappel *et al.*, 2005). The IGF-1 receptor is expressed on sebocytes and can be activated by either IGF-1 or high doses of insulin (Rudman *et al.*, 1997).

Insulin, in combination with the hormones methylisobutylxanthine and dexamethasone, is commonly used to induce the differentiation of 3T3-L1 preadipocytes into adipocytes, a process which is characterized by a marked increase in lipogenesis (Student *et al.*, 1980). A role for IGF-1 and insulin in stimulating sebaceous gland lipogenesis was first demonstrated in rat preputial sebocytes (Deplewski and Rosenfield, 1999).

The sterol response element-binding proteins (SREBPs) are nuclear transcription factors that regulate the synthesis of cholesterol and fatty acids. As suggested by their name, SREBPs bind sterol response elements in the promoters of genes involved in lipogenesis, including fatty acid synthase, long-chain fatty acyl elongase, stearoyl CoA desaturase, HMG CoA synthase, HMG CoA reductase, and squalene synthase (Horton, 2002). SREBPs are produced as precursors embedded in the endoplasmic reticulum. In sterol-depleted cells, two sequential cleavages release the amino terminal fragment of the protein, which in turn enters the nucleus and binds to promoters of genes containing sterol response elements, activating transcription (Hua *et al.*, 1996; Sakai *et al.*, 1996). There are three known SREBPs that regulate synthesis of cholesterol and fatty acids. SREBP-1a and 1c are derived from a single gene with alternate start sites, yielding a different first exon (Shimomura *et al.*, 1997). SREBP-2 is the third member of the family and is believed to be more active in cholesterol homeostasis, though there is some functional overlap between the two forms (Sakai *et al.*, 1996).

The goal of this study was to examine the mechanism(s) by which adipogenic hormones increase lipogenesis in human SEB-1 sebocytes and to test the hypothesis that SREBPs are key regulators of this process. Here we report that: (1) the increased lipogenesis observed in SEB-1 cells treated with

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Abbreviations: MDI, methylisobutylxanthine, dexamethasone, and insulin; QPCR, quantitative reverse transcription PCR; SREBP, sterol response element-binding protein

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methylisobutylxanthine, dexamethasone, and a high dose of insulin (MDI) is attributed to insulin; (2) physiologically relevant doses of IGF-1 increase lipogenesis in SEB-1 sebocytes; and (3) increased expression of SREBP-1 mRNA and protein mediates the increase in lipid production by IGF-1 and augments the increase by 1 μM insulin. Additionally, 100 nM insulin increases lipogenesis to a statistically lesser extent than 1 μM insulin or 20 ng/ml IGF-1 in SEB-1 sebocytes. From this we conclude that insulin and IGF-1 can potentially activate lipogenesis by different mechanisms. These data also suggest that the IGF-1 receptor is instrumental in activating the SREBP pathway and the downstream increases in lipogenesis in SEB-1 cells.

RESULTS

Insulin is the component in MDI media that increases lipogenesis in SEB-1 cells

Statistically significant increases in the incorporation of ^{14}C acetate into sebaceous lipids were noted when SEB-1 cells were treated with methylisobutylxanthine (0.5 mM), dexamethasone (0.3 μM), and insulin (1.74 μM) together with 10% fetal bovine serum (Figure 1a). Specifically, cholesterol was increased 4.4-fold, fatty alcohol was increased 3.6-fold, triglycerides were increased 4.8-fold, cholesterol oleate was increased 9.5-fold, and squalene was increased 14.2-fold. However, when SEB-1 cells were treated with each of the three hormones individually in 10% fetal bovine serum, only insulin significantly increased lipogenesis (Figure 1b). There were no significant changes in lipogenesis when cells were treated with either methylisobutylxanthine or dexamethasone alone (data not shown).

Lipogenesis is increased by 100 nM insulin, 1 μM insulin, and IGF-1 in SEB-1 sebocytes

Because insulin at high concentrations is capable of acting through the IGF-1 receptor (Prisco *et al.*, 1999), we tested whether a physiologically relevant dose of IGF-1 (20 ng/ml) could mimic the lipogenic effect observed with 1.74 μM insulin. We also wanted to determine if a lower dose of insulin would cause the same increase in lipogenesis as the high dose of insulin which was likely acting through both the IGF-1 and insulin receptor.

Statistically significant increases in the production of cholesterol, cholesterol oleate, fatty alcohol, and triglycerides were noted when SEB-1 cells were treated with 100 nM insulin (Figure 2). Treatment with either IGF-1 or 1 μM insulin significantly increased all eight lipid species when compared to the vehicle control (Figure 2). Further, the increases induced by 1 μM insulin were significantly greater than those induced by 100 nM insulin in all eight lipids; and significantly greater incorporation of acetate into cholesterol, oleic acid, triglycerides, and wax esters was achieved with IGF-1 compared to 100 nM insulin (Figure 2).

SREBP-1 protein is induced in SEB-1 cells by IGF-1 and 1 μM insulin but not by 100 nM insulin

Since SREBPs regulate numerous genes involved in lipid metabolism, we tested whether the effects of IGF-1 on lipogenesis were mediated by SREBPs. Western blot demonstrated that both the precursor and mature forms of the SREBP-1 protein are increased by 6- and 3-fold, respectively, in response to IGF-1 (Figure 3a and b). Additionally, the precursor is increased by 2.7-fold and the mature protein is increased by 1.7-fold in response to 1 μM insulin, a dose known to activate the IGF receptor. Interestingly, although the lower dose of insulin (100 nM) increased lipogenesis, it did not increase SREBP protein expression (Figure 3c and d). These results suggest that the moderate increase in lipogenesis in SEB-1 cells induced by 100 nM insulin does not involve upregulation of SREBP-1, while the greater increase seen with 1 μM insulin or 20 ng/ml IGF-1 are at least, in part, mediated by the SREBP-1 pathway.

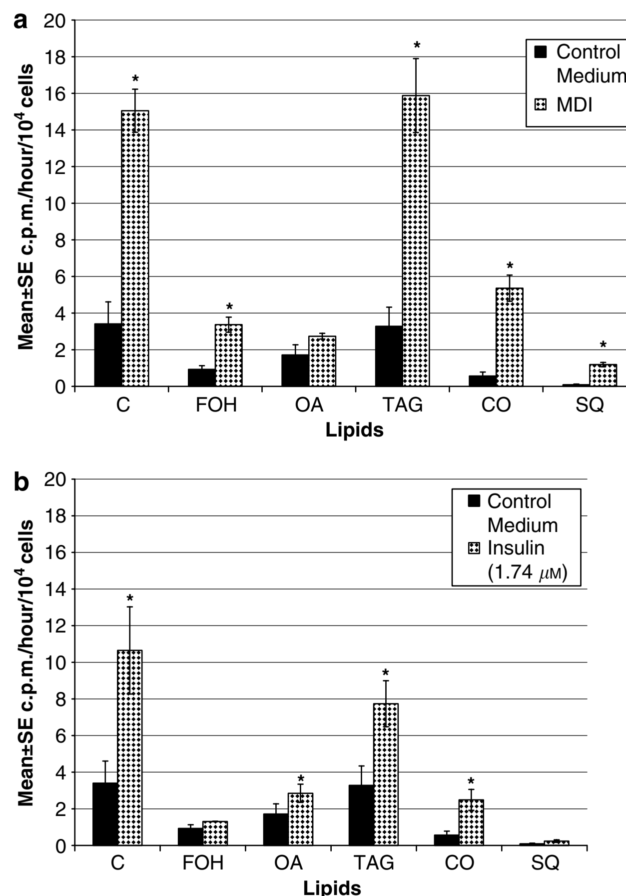

Figure 1. Treatment of SEB-1 sebocytes with adipogenic hormones increases ^{14}C acetate incorporation into non-polar lipids. This increase can be attributed to insulin. (**a**) SEB-1 sebocytes were treated with methylisobutylxanthine (0.5 mM), dexamethasone (0.3 μM), and insulin (1.74 μM) for 72 hours. Cells were then incubated with ^{14}C acetate for 2 hours. Lipids were extracted and separated by thin layer chromatography. (**b**) Insulin alone increases lipogenesis in SEB-1 sebocytes. Treatment with methylisobutylxanthine or dexamethasone alone did not change lipogenesis (not shown). Units are expressed as mean ± SE c.p.m./hour/10^4 cells. Significance was determined by *t*-test and a *P*-value of <0.05 in comparison to the vehicle was determined to be significant and denoted with an *. The following abbreviations are used: C = cholesterol; FOH = fatty alcohol; OA = oleic acid; TAG = triglycerides; CO = cholesterol oleate; SQ = squalene.

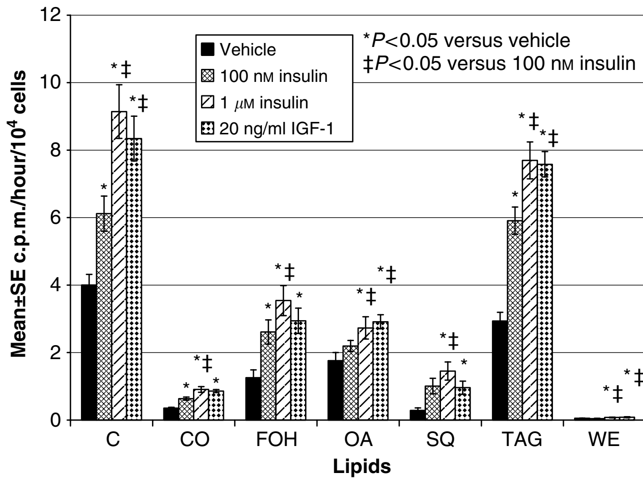

Figure 2. Treatment of SEB-1 sebocytes with 100 nM insulin increases lipogenesis, though to a lesser extent than 1 μM insulin or 20 ng/ml IGF-1. SEB-1 sebocytes were treated for 24 hours with 100 nM insulin, 1 μM insulin, or 20 ng/ml IGF-1. Cells were then incubated with ^{14}C acetate for 2 hours. Lipids were extracted and separated by thin layer chromatography. Units are expressed as mean ± SE c.p.m./hour/10^4 cells. Analysis of variance was used to determine statistical significance. Date by treatment interaction was observed for some data points. A *P*-value of <0.05 in comparison to the vehicle was determined to be significant and denoted with an *, while a *P*-value of <0.05 compared to 100 nM insulin is denoted by an ‡. The following abbreviations are used: C = cholesterol; FOH = fatty alcohol; OA = oleic acid; TAG = triglycerides; CO = cholesterol oleate; SQ = squalene; WE=wax esters.

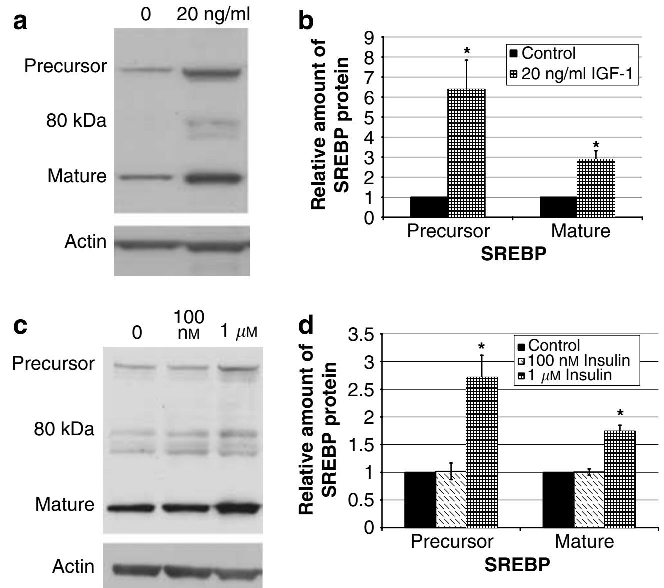

Figure 3. Both forms of the SREBP-1 protein are increased in SEB-1 sebocytes treated with 20 ng/ml IGF-1 or 1 μM insulin, but not with 100 nM insulin. SEB-1 sebocytes were treated 24 hours with insulin or IGF-1. Protein was isolated and a Western blot was performed, probing with antibodies to SREBP-1 and actin. (**a**) is a representative blot probed for SREBP-1 following 20 ng/ml IGF-1 treatment. (**b**) These data were normalized to actin and quantified. (**c**) Representative blot probed for SREBP-1 and normalized to actin following insulin treatment: 100 nM (lane 2) and 1 μM (lane 3). (**d**) These data are quantified. (**b** and **d**) All blots were repeated a minimum of five times, analyzed by densitometry and normalized to actin. Densitometer data were compared to vehicle-treated blots and Student's *t*-test was performed. A *P*-value <0.05 was determined to be statistically significant.

Both the SREBP-1a and 1c mRNA transcripts are increased in SEB-1 cells treated with IGF-1 and insulin

Quantitative reverse-transcription PCR (QPCR) was performed to determine if the SREBP-1a or 1c transcripts were increased in response to IGF-1 or insulin treatment. Treatment of SEB-1 sebocytes with 20 ng/ml IGF-1 produced a statistically significant increase in mRNA for both SREBP-1a (3.3-fold increase) and SREBP-1c (4.7-fold increase) (Figure 4a). Treatment with either 100 nM insulin or 1 μM insulin also increased SREBP-1a and 1c mRNA (Figure 4b). The increase in response to 100 nM insulin was surprising, as no changes in SREBP-1 protein were noted at this dose (Figure 3d). The increase in both SREBP-1a and 1c transcripts in response to 100 nM insulin, combined with the lack of increase in SREBP-1 protein at this dose, implies another level of regulation, perhaps post-transcriptional, that has not been observed for SREBP-1.

The ratios of SREBP-1c:SREBP-1a differ between human sebaceous glands and SEB-1 sebocytes

Several reports have indicated that the SREBP-1c:1a ratio is markedly different in cell lines compared to tissue (Shimomura *et al.*, 1997). It is typical for the SREBP-1c form to predominate in tissue, while immortalized cell lines have much more SREBP-1a than 1c. We performed QPCR to compare the respective SREBP-1c:1a ratios and found this to be true for SEB-1 cells as well. Sebaceous glands had a 1c:1a ratio of 9.8:1, whereas, the SEB-1 cell line had a SREBP-1c:1a ratio of 0.59:1 (Figure 5).

The ratio of insulin receptor isoform A:isoform B is 5.1:1 in SEB-1 sebocytes

The isoform of insulin receptor present in cells can have a profound impact on the insulin signaling. There have been two isoforms of the insulin receptor described, isoform A lacks exon 11, while isoform B retains exon 11. We performed QPCR to determine which isoform was prevalent in the SEB-1 sebocytes. We found that the isoform A (exon 11−) was more prevalent than isoform B (exon 11+) by a ratio of 5.1:1 (Figure 6).

DISCUSSION

Acne is the most common skin condition observed by clinicians, and at times it has been considered ''physiologic'' in the adolescent population (Kligman, 1974). Sebum production by the sebaceous gland is fundamental in the pathophysiology of acne and, apart from isotretinoin or hormonal therapy, there are no effective means to reduce sebum production. An understanding of the factors that regulate sebum production is crucial to making advances in acne therapy (Harper and Thiboutot, 2003).

While sebocytes are epithelial cells and adipocytes are mesenchymal cells, similarities exist in that each of these cell types make lipid as part of their process of differentiation, as first noted by Rosenfield *et al.* (1999). The adipogenic hormones methylisobutylxanthine, dexamethasone, and

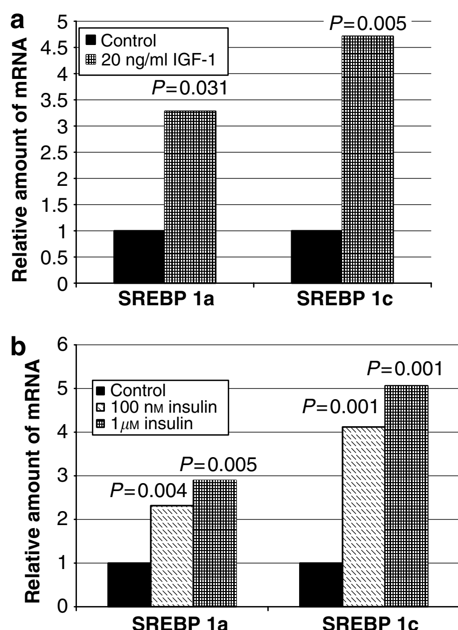

Figure 4. Both SREBP-1a and SREBP-1c mRNA transcripts are increased in SEB-1 sebocytes treated with 20 ng/ml IGF-1, 100 nM insulin, or 1 μM insulin. SEB-1 sebocytes were treated with (**a**) IGF-1 or (**b**) insulin for 14 hours. Following treatment, RNA was isolated and subjected to QPCR to determine abundance of the SREBP-1a and SREBP-1c transcript in comparison to vehicle-treated cells. Data were analyzed using the relative expression software tool program and Student's *t*-test was performed. A *P*-value <0.05 was considered statistically significant. Using the relative expression software tool program, it is not possible to generate SD or SE unless the same RT reaction is run repeatedly, thus *P*-values are used to gauge variation.

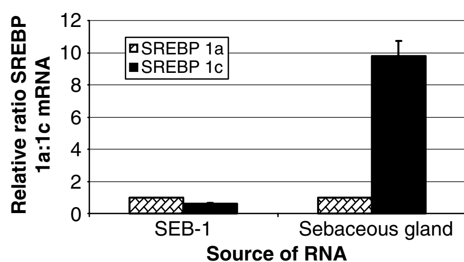

Figure 5. The ratio of SREBP-1c:1a mRNA in SEB-1 sebocytes is 0.59:1, while the ratio is 9.8:1 in sebaceous gland. Human sebaceous glands were microdissected. RNA was extracted from the glands and was subjected to QPCR to determine the relative ratio of SREBP-1c:1a. RNA was also extracted from SEB-1 cells to determine the SREBP-1c:1a ratio by QPCR. Data represent the mean ratios of five determinations ± SE.

insulin induce the differentiation of preadipocytes into adipocytes which is accompanied by a change in morphology, increased expression of the peroxisome proliferator-activated receptor gamma, and the accumulation of intracellular lipid (Rosen *et al.*, 2000). In this paper, we show that adipogenic hormones increase lipid production in SEB-1 sebocytes, similar to their actions in adipocytes, and that this effect can be induced by insulin. Additionally, this effect can be reproduced by IGF-1.

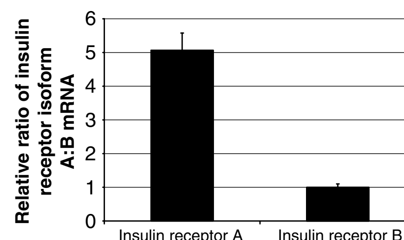

Figure 6. The ratio of insulin receptor isoform A:isoform B mRNA is 5.1:1 in SEB-1 sebocytes. RNA was extracted from SEB-1 sebocytes and QPCR was performed to determine the ratio of insulin receptor isoform A to insulin receptor isoform B. Data represent the mean ratios of five determinations ± SE.

Both insulin and IGF-1 are members of the receptor tyrosine kinase subfamily. It is crucial to note that although each molecule has its own receptor, insulin can bind to the IGF-1 receptor with an affinity 100-fold less than to its native receptor. Likewise, IGF-1 will bind to the insulin receptor with 100–1,000 times less affinity than it has for its own receptor (Jones and Clemmons, 1995). This matter is further complicated by alternative splicing that has been observed for the insulin receptor, which alters both the signaling and affinity for insulin, IGF-1, and IGF-II (Denley *et al.*, 2003). As 1 μM insulin can activate the IGF-1 receptor in addition to the insulin receptor (Prisco *et al.*, 1999), we also treated the SEB-1 cells with a lower dose of insulin (100 nM) or with IGF-1 (20 ng/ml). The lower dose of insulin was able to induce lipogenesis to a moderate extent, but the IGF-induced lipogenesis to a significantly greater extent than 100 nM insulin, and to approximately the same levels as 1 μM insulin (Figure 2). From this we conclude that IGF-1 receptor activation is a key driver of lipogenesis in SEB-1 sebocytes. This hypothesis is supported by the fact that 3T3-L1 preadipocytes can be differentiated by IGF-1 or 2 μM insulin, from which it was deduced that IGF-1 is the essential regulator of differentiation in the 3T3-L1 line (Smith *et al.*, 1988).

Our data support those of Deplewski and Rosenfield (1999) who found increased lipid content in rat preputial cells following treatment with insulin and IGF-1. IGF-1 at 1 nM (7.7 ng/ml) stimulates differentiation to the same degree as 1 μM insulin in the rat preputial sebocyte model. In another study, isolated human sebaceous glands from chest skin that were grown in the presence of 50 ng/ml IGF-1 for 3 days showed an increase in ^{14}C acetate incorporation into non-polar lipids that was comparable to that achieved by 1.8 μM insulin (Downie *et al.*, 2002, 2004). Our data demonstrate that IGF-1 at a dose of 20 ng/ml increases lipogenesis to the same extent as insulin at 1 μM. Taken together, these data indicate that the lipogenic enzymes in sebaceous glands and sebocytes are maximally stimulated by physiologically relevant doses of IGF-1 as evidenced by the increase in acetate incorporation into lipids. Under the experimental conditions used in this study (serum-free medium), an increase in the mass of lipids was not noted (data not shown).

A possible mechanism for this increase in rate of lipogenesis in response to insulin and IGF-1 treatment involves the SREBP pathway. The SREBPs are key transcriptional regulators of lipogenic enzymes (Horton, 2002). Their expression is upregulated in response to insulin in tissues such as liver, fat, and skeletal muscle (Kim *et al.*, 1998; Foretz *et al.*, 1999; Guillet-Deniau *et al.*, 2002). We hypothesized that the effects of insulin and IGF-1 on lipogenesis in SEB-1 sebocytes may be mediated via SREBPs.

When SEB-1 cells were treated with 20 ng/ml IGF-1 or 1 μM insulin, we found that both the precursor and cleaved SREBP-1 protein levels were increased, while 100 nM insulin had no effect on SREBP-1 protein. The modest increase in lipogenesis in cells treated with 100 nM insulin combined with the lack of induction of SREBP-1 protein in this group provides evidence for an SREBP-1-independent mechanism by which sebaceous gland lipogenesis can be increased. However, activation of the IGF-1 receptor, and ultimately, SREBP-1 is required for maximal stimulation.

The two transcripts of the SREBP-1 protein are formed from a single gene with alternate transcription start sites which yields two mRNA species that have different first exons, but are the identical from the second exon onward (Shimomura *et al.*, 1997). In this first exon, the 1a variant has a much longer activation domain, making it a much more potent activator of sterol response elements than the 1c variant. As such, SREBP-1c has been reported to be regulated more at the transcriptional level, particularly by insulin, while SREBP-1a appears to be regulated at the protein processing stage (Eberle *et al.*, 2004). Here, we show that in SEB-1 sebocytes, both the SREBP-1a and SREBP-1c mRNA transcripts are increased in response to IGF-1 treatment and two doses of insulin. The increase in SREBP-1c mRNA was expected as this response to insulin has been well characterized. However, the increase in SREBP-1a transcript was not as robust as the increase in SREBP-1c. This is attributed to the fact that the 1a promoter contains only two Sp1 elements which are relatively weak activators of transcription (Zhang *et al.*, 2005). Sp1 elements have been shown to activate transcription in response to insulin (Samson and Wong, 2002), which is most likely responsible for the increase in response to IGF-1 as well. In agreement with our findings, increases in SREBP-1a mRNA have been reported in 3T3-L1 adipocytes when differentiated by adipogenic hormones (Shimomura *et al.*, 1997). SEB-1 cells, like HepG2 cells, or 3T3-L1 adipocytes have more SREBP-1a compared to SREBP-1c, which is reversed compared to the tissues from which these cell lines were derived (Shimomura *et al.*, 1997).

Whereas, numerous studies link the action of insulin to SREBPs, there is a very limited body of work connecting the IGF-1 and SREBP pathways, none of which has been investigated in the sebaceous gland. IGF-1 increases low-density lipoprotein receptor expression in HepG2 cells. Significantly less activation of an low-density lipoprotein receptor reporter construct is noted, however, in a HepG2-derived cell line that exhibits decreased expression of SREBP-1 (Streicher *et al.*, 1996). Although artificial and indirect, these data indicate that the effect of IGF-1 on low-density lipoprotein receptor expression may be mediated by SREBP-1 which would support the relationship between IGF-1 and SREBP-1 we report here. In addition, a functional IGF receptor is necessary for insulin to induce SREBP protein and its downstream effects in an immortalized brown fat cell line (Mur *et al.*, 2003). In this model IGF-1 can stimulate fatty acid synthase RNA (Arribas *et al.*, 2003). Fatty acid synthase RNA may correlate directly with SREBP activity (Kim *et al.*, 1998), though there are exceptions (Palmer *et al.*, 2002; Louveau and Gondret, 2004). Taken together, these studies support our finding that IGF-1 activates SREBP-1, which in turn increases *de novo* lipid production. To our knowledge, we are the first to directly show an increase in SREBP-1 protein in response to IGF-1.

Our data suggest that different molecular mechanisms might underlie the increase in lipogenesis induced by low doses of insulin (acting via the insulin receptor) compared to IGF-1 and 1 μM insulin (acting via the IGF-1 receptor). Each of these treatments increased mRNA for SREBP-1a and SREBP-1c, yet 100 nM insulin failed to increase SREBP-1 protein. The reasons for this discrepancy are not apparent but could involve differences in transcript or protein stability. Additional studies are needed to test these hypotheses.

Androgens are major regulators of sebaceous gland function. Interestingly, the SREBP pathway has been implicated in the actions of androgens in stimulating lipogenesis in sebaceous glands. Rosignoli *et al.* (2003) showed that the increased lipogenesis observed in the hamster ear model in response to androgen treatment is mediated by the SREBP pathway. In agreement with our findings with insulin and IGF-1, both the SREBP-1a and 1c transcripts were increased in response to this treatment.

Our work confirms that SREBP proteins are indeed important mediators of sebaceous lipid metabolism. An understanding of the mechanisms by which insulin and IGF-1 increase SREBP-1 expression will move us one step closer to understanding the underlying molecular mechanisms that govern skin lipid production. Finally, this work demonstrates that SREBP proteins, considered to be, ''Master regulators of lipid homeostasis'' (Eberle *et al.*, 2004) are important in the sebaceous gland and are regulated by IGF-1 and high-dose insulin. The SEB-1 sebocyte model could be a useful tool for dissecting the differences between insulin and IGF-1 signaling which may provide further insight into the factors regulating human sebum production and the development of acne.

MATERIALS AND METHODS

Cell culture

SEB-1 (passage 22–24) SV40 immortalized human sebocytes were grown to confluence in all experiments unless stated otherwise, and were cultured in standard medium consisting of DMEM (Invitrogen, Carlsbad, CA), 5.5 mM glucose/Ham's F-12 3:1 (Invitrogen), fetal bovine serum 2.5% (HyClone, Logan, UT), adenine 1.8×10^{-4} M (Sigma, St Louis, MO), hydrocortisone 0.4 μg/ml (Sigma), insulin 10 ng/ml (Sigma), epidermal growth factor 3 ng/ml (Austral Biologicals, San Ramon, CA), and cholera toxin 1.2×10^{-10} M (Sigma) (Thiboutot *et al.*, 2003).

Cell treatments

To determine if adipogenic hormones induce differentiation of SEB-1 cells, cells were grown to 80% confluence in standard medium and treated with a combination of methylisobutylxanthine (0.5 mM) (Sigma), dexamethasone (0.3 μM) (Sigma), and insulin (1.74 μM) from Sigma in medium containing DMEM 5.5 mM glucose, 10% fetal bovine serum, and antibiotics for 72 hours (Student *et al.*, 1980). Additional plates were assayed using the appropriate drug vehicle controls in the same medium.

For all treatments with insulin or IGF-1, confluent cells were washed twice with phosphate-buffered saline and treated with the appropriate hormone or vehicle in DMEM with no additives or serum for 24 hours. Thus, the control media in experiments with insulin or IGF-1 consisted of DMEM without additives plus the appropriate vehicle. IGF-1 (Invitrogen) was diluted 1:1,000 in 0.1 M acetic acid with 0.1% BSA. The final concentration was 20 ng/ml. Insulin was also added 1:1,000 in 0.005 N HCl.

Lipogenesis assay

The incorporation of ^{14}C-acetate into lipids was used as a measure of lipogenesis. SEB-1 cells were cultured in standard medium until confluent and treated in different experiments with a combination of adipogenic hormones or each hormone individually as above for 72 hours. In experiments examining the effects of IGF-1 on lipogenesis, cells were treated with IGF-1 or the vehicle (0.1% BSA in 0.1 M acetic acid) in DMEM without additives or serum for 24 hours. In all experiments, cell counts were used to normalize the data. The remaining cells were suspended in a DMEM solution containing 1 μCi ^{14}C-acetate (New England Nuclear, Boston, MA), incubated for 2 hours at 37°C with agitation, and extracted twice with ethyl ether and non-radioactive carrier lipids. Samples were dissolved in a small volume of ethyl acetate and spotted on 20 cm silica gel thin layer chromatography plates (Macherey-Nagel, Easton, PA) which were run until the solvent front reached 19.5 cm in hexane, followed by 19.5 cm in benzene, and finally to 11 cm in hexane:ethyl ether:glacial acetic acid (69.5:30:1.5). Lipids were visualized and all lipid-containing zones were counted in a liquid scintillation counter. Negative controls for each experiment consisted of plates of untreated cells where radioactivity was added and cells were incubated on ice for 2 hours; lipids were extracted and analyzed in parallel. All experiments were repeated a minimum of three times with each sample in triplicate within each experiment. Data analysis is described in the individual figure legends.

Western blot

Cytoplasmic and nuclear lysates were obtained using the Ne-Per kit (Pierce, Rockford, IL) following manufacturer's instructions. A protease inhibitor cocktail (Sigma) containing 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatinA, E-64, bestatin, leupeptin, and aprotinin was added. Both the mature and precursor forms of the SREBP-1 proteins were found in the nuclear fraction in SEB-1 cells. This appears to be unique to the SEB-1 cells, since 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 Western blots shown using SEB-1 extracts are the nuclear fractions. Total protein for each sample was determined using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Twenty-five micrograms of protein, was run on a 4–12% Bis-Tris NuPage polyacrylamide gel (Invitrogen). Protein was then transferred to a polyvinylidene fluoride membrane and probed using standard methods. The SREBP-1 (K-10) antibody was obtained from Santa Cruz and used at a dilution of 1:2,000. Preliminary experiments showed that this antibody reacts non-specifically with a protein at 80 kDa. This was confirmed to be nonspecific using the SREBP-1 (K-10) blocking peptide (Santa Cruz) (data not shown). The actin antibody and goat anti-rabbit secondary antibody (7074) were obtained from Cell Signaling Technologies (Beverly, MA) and were used at 1:5,000 and 1:2,000 dilutions, respectively. Blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to film. Films of blots were analyzed and quantified by densitometry with QuantityOne Software (Bio-Rad) after background subtraction. Western blots were repeated a minimum of three times. Data were analyzed using Student's *t*-test and results were considered significant if $P<0.05$.

QPCR

QPCR was performed to compare the ratios of SREBP-1a and 1c mRNA transcripts in SEB-1 sebocytes and human sebaceous glands. For QPCR with SEB-1 cells, cells were grown in standard medium for 6 days. On day 7, cells were washed twice with phosphate-buffered saline and treated with insulin, IGF-1 or the appropriate vehicle in serum-free DMEM for 14 hours. RNA was isolated and complimentary DNA was generated from 4.2 μg RNA/reaction primed with oligo-dT using the SuperScript First-Strand Synthesis System for RT-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, a housekeeping gene, was used as a reference gene. The following primer sequences were used: TATA-binding protein upstream (GenBank number NM_003194) 5′c acg gca ctg att ttc agt tct, TATA-binding protein downstream 5′ttc ttg ctg cca gtc tgg act, SREBP-1a upstream (GenBank number NM_004176) 5′gct gct gac cga cat cga a, SREBP-1c upstream (GenBank number NM_001005291) 5′gga gcc atg gat tgc act tt, and SREBP-1a,c downstream 5′tca aat agg cca ggg aag tca. Data were analyzed using the REST-XL© program (Pfaffl *et al.*, 2002). A *P*-value <0.05 was considered significant.

Facial skin samples were obtained under a protocol approved by the Institutional Review Board of the Pennsylvania State University College of Medicine; informed consent was obtained from the subjects and the study was conducted according to the principles outlined in the Declaration of Helsinki Principles. Sebaceous glands were microdissected as previously described (Thiboutot *et al.*, 1995). Owing to a limited number of sebaceous gland samples, threshold values (C_t) were transformed using the formula $(2^{-C_t})\times 10^7$ as described (Spangler *et al.*, 2003) and normalized to TATA-binding protein. The relative difference between SREBP-1c and 1a is a ratio of normalized 1c to 1a.

The ratio of insulin receptor A-type (exon 11−) to insulin receptor B-type (exon-11+) was determined by relative QPCR as described previously (Trivedi *et al.*, 2006). SEB-1 sebocytes were grown to 50% confluence and the RNA was extracted. Both primer/probe sets were purchased from ABI. For the insulin receptor A-type (GenBank number AB208861) the primer/probe set Hs00965956_m1 was used. For the B-type (GenBank number NM_000208) the primer/probe set Hs00169631_m1 was used.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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