Sphingosine 1-Phosphate Restrains Insulin-Mediated Keratinocyte Proliferation via Inhibition of Akt through the $S1P_2$ Receptor Subtype

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The balance between keratinocyte proliferation and differentiation plays a decisive role for skin formation and development. Among the well-characterized biological mediators, insulin and sphingosine 1-phosphate (S1P) have been identified as major regulators of keratinocyte growth and differentiation. Insulin induces proliferation of keratinocytes, whereas S1P inhibits keratinocyte growth and initiates keratinocyte differentiation. However, it is not clear which S1P receptor subtype and downstream signaling pathways are involved in the antiproliferative action of S1P. In this study, we present evidence that S1P inhibits insulin-mediated keratinocyte growth via the activation of protein kinase C (PKC) followed by a subsequent dephosphorylation of Akt. The inhibition of insulin-mediated Akt activity by S1P is completely abolished in the presence of PKCδ siRNA indicating that this isozyme is selectively potent at causing dephosphorylation of Akt and modifying keratinocyte proliferation. Further experiments by downregulation of S1P receptor subtypes and the use of specific receptor agonists/antagonists clearly indicated that the $S1P_2$ receptor is dominantly involved in the S1P-induced dephosphorylation of Akt and keratinocyte growth arrest. This is of great clinical interest, as the immunomodulator FTY720, after being phosphorylated by sphingosine kinase, activates all of the five S1P receptors except $S1P_2$ and therefore fails to inhibit keratinocyte proliferation.

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INTRODUCTION

The equilibrium between cell growth and cell differentiation plays a pivotal role in many physiological processes. In skin, the maintenance of the balance between proliferation of mitotically active epidermal keratinocytes and differentiation of postmitotic skin cells is particularly critical for skin formation and development (Koria and Andreadis, 2006). Indeed, under certain pathological conditions, the interplay between proliferation and differentiation can be disturbed, which may result in an impaired wound healing, hyperproliferative skin diseases, or even tumorigenesis (Fuchs and Raghavan, 2002). Thus, it is not astonishing that keratinocyte proliferation and differentiation are controlled by a complex network of several growth factors and cytokines.

Among the well-characterized growth factors, insulin and IGF-I have been identified as critical regulators of cellular proliferation and differentiation (Wertheimer *et al.*, 2000; Sadagurski *et al.*, 2006). Both mediators bind to specific receptors belonging to the tyrosine kinase family of growth factor receptors (Cheatham and Kahn, 1995; LeRoith *et al.*, 1995; Dupont and LeRoith, 2001). Receptor expression studies have demonstrated the presence of the insulin receptor (IR) and the IGF-I receptor (IGF-IR) in dermal and epidermal cells, suggesting their function in skin development (Verrando and Ortonne, 1985; Misra *et al.*, 1986; Goren *et al.*, 2006). Indeed, increasing IR/IGF-IR signaling is associated with enhanced cell proliferation, skin hyperplasia, and tumorigenesis (Giovannucci, 1999; DiGiovanni *et al.*, 2000; Shen *et al.*, 2001). In conclusion, mice with disrupted IGF-IR are characterized by a thinner and defective epidermis (Martin, 1997). It has been well established that insulin induces proliferation not only via the ligation to IR but also to IGF-IR (Kuhn *et al.*, 1999; Edmondson *et al.*, 2003). In response to IR and IGF-IR stimulation, the signal is propagated via at least two main signaling pathways, the Ras/Raf/mitogen-activated protein kinase pathway and the phosphoinositide-3 kinase/Akt pathway, respectively (Kooijman, 2006).

Especially in keratinocytes, the phosphoinositide-3 kinase/Akt signaling pathway is of great importance in regulation of cell proliferation (Murayama *et al.*, 2007; Ouyang *et al.*, 2007). The serine/threonine kinase Akt has been identified as

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Abbreviations: ASO, antisense phosphorothioate oligonucleotide; FTY720-P, FTY720-phosphate; IGF-IR, IGF-I receptor; IR, insulin receptor; PBS, phosphate-buffered saline; PKC, protein kinase C; siRNA, small interfering RNA; S1P, sphingosine 1-phosphate; TPA, 12-O-tetradecanoylphorbol-13-acetate

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a key downstream target of phosphoinositide-3 kinase in insulin-regulated processes leading to the formation of phosphatidylinositol-3,4,5-trisphosphate and subsequent phosphorylation of Akt by the phosphoinositide-dependent kinase-1 at the two regulatory phosphorylation sites Thr^{308} and Ser^{473} (Kandel and Hay, 1999; Lawlor and Alessi, 2001; Scheid and Woodgett, 2003; Song *et al.*, 2005). In keratinocytes, these phosphorylations result in cell cycle progression via regulation of D-type cyclins and cyclin-dependent kinase inhibitors, $p21^{WAF1/CIP1}$ (p21) and $p27^{KIP1}$ (p27) (Blagosklonny, 2002; Liang *et al.*, 2002; Shin *et al.*, 2002). Thus, it is not astonishing that activation of Akt is enhanced in hyperproliferative states and malignant transformation of keratinocytes (Wilker *et al.*, 2005; Pankow *et al.*, 2006; Segrelles *et al.*, 2006).

In addition, the protein kinase C (PKC) family of serine/threonine kinases was identified as a critical regulator of keratinocyte proliferation and differentiation (Matsui *et al.*, 1992; Gartsbein *et al.*, 2006). Among the 12 members of the PKC family, PKCα, PKCδ, PKCε, PKCη, PKCζ, and PKCμ have been localized in the epidermis (Dlugosz *et al.*, 1992, 1994; Gschwendt *et al.*, 1996). Skin targeting of PKC isozymes in transgenic mice has indicated that overexpression of PKCδ and PKCε is connected with a reduction in the number of benign tumors induced by chemical carcinogenesis (Newton, 2003). Moreover, it has been shown that PKCδ and PKCε are selectively potent at causing dephosphorylation of Akt, which may explain their tumor-inhibiting action (Li *et al.*, 2006).

Sphingolipids have long been recognized as important structural components of the epidermis, securing its permeability barrier. Moreover, recent findings suggest a substantial role of the sphingolipid sphingosine 1-phosphate (S1P) as a bioactive mediator involved in the regulation of keratinocyte growth and differentiation (Vogler *et al.*, 2003; Kim *et al.*, 2004; Sauer *et al.*, 2004). Although S1P is a potent mitogen in a variety of cells, an opposed effect is visible in human keratinocytes, as S1P inhibits cell growth of epidermal cells (Manggau *et al.*, 2001; Vogler *et al.*, 2003; Radeff-Huang *et al.*, 2004).

S1P has been suggested to exert most of its actions as a ligand for G-protein-coupled receptors (Spiegel and Milstien, 2003; Ishii *et al.*, 2004; Chalfant and Spiegel, 2005; Hait *et al.*, 2006). To date, five members of the S1P receptor family have been identified, namely $S1P_{1-5}$, which are all expressed in human keratinocytes (Vogler *et al.*, 2003). The first available drug acting on S1P receptors is FTY720 (2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol), which is presently in clinical development for suppression of transplant rejection and multiple sclerosis. FTY720, after being phosphorylated by sphingosine kinase, activates all of the five S1P receptors except $S1P_2$ (Brinkmann *et al.*, 2004).

In this report, we identified an interplay between insulin and S1P, but not FTY720, on Akt signaling, PKC activity, and keratinocyte cell growth. Most interestingly, these S1P-mediated actions are transduced via the receptor subtype $S1P_2$. Therefore, true agonists on the $S1P_2$ may be of great interest in the treatment of hyperproliferative skin diseases.

RESULTS

S1P inhibits insulin-dependent Akt phosphorylation and keratinocyte growth

Several studies indicate that insulin induces proliferation of human keratinocytes, whereas S1P mediates keratinocyte cell growth arrest (Shen *et al.*, 2001; Vogler *et al.*, 2003). As Akt has been identified as an important regulator of cell proliferation, we have further evaluated the interplay of both mediators on Akt phosphorylation (Ouyang *et al.*, 2007). Therefore, primary human keratinocytes were grown under serum-free conditions and then stimulated with either S1P or insulin, or both stimulants. As indicated in Figure 1a, S1P significantly reduced phosphorylation of constitutively active Akt in cultured basal keratinocytes in a transient manner, whereas the levels of total Akt were not affected. A time-course study indicated that the dephosphorylation of Akt on Ser^{473} was first visible after 10 minutes and persisted over a time period of 1 hour. In contrast to S1P, insulin induced a substantial phosphorylation of Akt on Ser^{473}. A maximal effect was detected after 10 minutes with 1 μM of insulin (Figure 1b). Most interestingly, pretreatment of keratinocytes with S1P reduced the ability of insulin to induce Akt phosphorylation. Figure 1c indicates that S1P-induced inhibition of insulin-dependent Akt activation was dose-dependent. Thus, S1P (10 μM) completely abolished insulin-mediated Akt activation. It should be mentioned that the capability of insulin to mediate Akt phosphorylation was significantly reduced when keratinocytes were pretreated with S1P over a time period of 10 minutes. To investigate the biological consequences of the S1P/insulin interplay on the Akt-mediated cell growth pathway, proliferation experiments were performed. As expected, insulin induced a strong mitogenic effect of quiescent primary human keratinocytes (Figure 2a). But when cells were pretreated with S1P, insulin failed to evoke its mitogenic signaling. As shown in Figure 2b, S1P inhibited insulin-mediated proliferation in a dose-dependent manner. When human keratinocytes were pretreated with 10 μM of S1P, the insulin-dependent increase of proliferation was almost completely annihilated, which is in agreement with the inhibitory effect of S1P at this concentration on Akt phosphorylation.

S1P induces Akt dephosphorylation via activation of PKCδ

It has been indicated that activation of PKC is a negative modulator of Akt phosphorylation in primary mouse keratinocytes (Li *et al.*, 2006). Therefore, it was of interest to prove whether S1P is able to activate PKC in primary human keratinocytes and whether this effect contributes to the S1P-mediated inhibition of Akt activation. To this end, phosphorylation of the C-terminal residue in the hydrophobic motif of PKC isozymes, homologous to Ser^{660} of PKCβII, was measured. S1P at several concentrations was added to quiescent keratinocytes for several incubation periods, and cell lysates were analyzed for PKC activation. Indeed, S1P induced phosphorylation of PKC. As presented in Figure 3a, the time course of activation of PKC was rapid and similar to the time course of the S1P-mediated inhibitory effect on Akt activation. A maximal response on PKC activity occurred

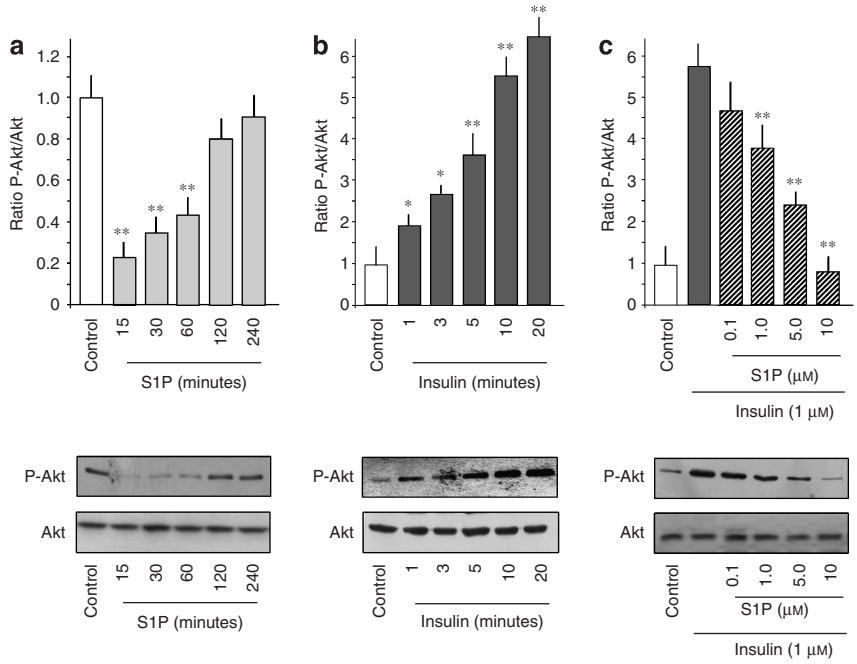

Figure 1. S1P inhibits basal and insulin-induced Akt phosphorylation. Human keratinocytes were treated with (**a**) 10 μM S1P or (**b**) 1 μM insulin for the indicated time periods followed by the detection of Akt activity using western blot analysis. (**c**) Cells were pretreated with the indicated S1P concentrations for 10 minutes followed by stimulation with 1 μM insulin for another 10 minutes. Values of the densitometric analysis are expressed as *x*-fold increase of phospho-Akt formation (**a** and **b**) compared to untreated cells ± SED or (**c**) compared to insulin-treated cells ± SED from three experiments. **P*<0.05 and ***P*<0.001 indicate a statistically significant difference (**a** and **b**) versus unstimulated control cells and (**c**) versus insulin-treated cells.

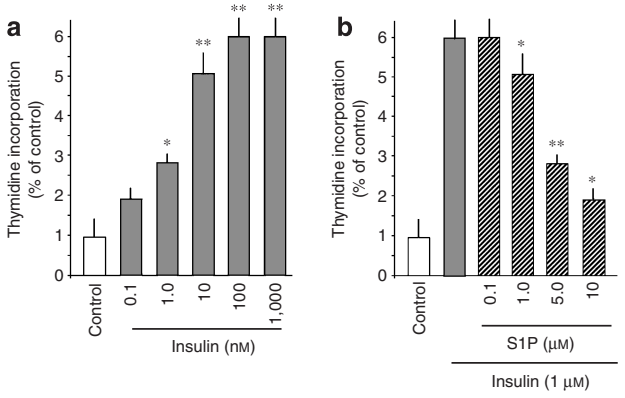

Figure 2. S1P inhibits insulin-induced proliferation. Keratinocytes were (**a**) incubated with the indicated concentrations of insulin or (**b**) pretreated for 10 minutes with the indicated concentrations of S1P followed by a subsequent stimulation with 1 μM insulin. After 24 hours, [^{3}H]thymidine incorporation was measured. Values are expressed as *x*-fold increase compared to control and are the mean ± SED from three experiments. **P*<0.05 and ***P*<0.001 indicate a statistically significant difference (**a**) versus unstimulated control cells and (**b**) versus insulin-treated cells.

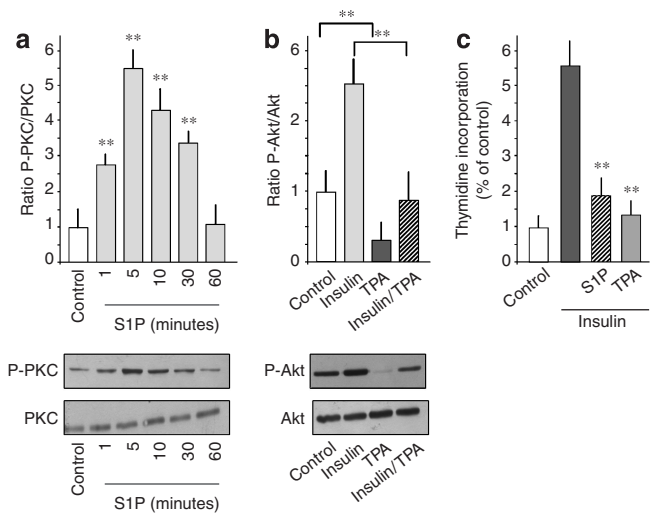

Figure 3. Stimulation of PKC by S1P impairs Akt activity and proliferation. (**a**) Human keratinocytes were treated with 10 μM S1P for various time periods followed by the detection of basal and phosphorylated PKC levels using western blot analysis. (**b**) Cells were preincubated with 250 nM TPA or vehicle for 15 minutes followed by stimulation with 1 μM insulin for 10 minutes, and Akt activity was detected by western blot analysis. (**c**) Keratinocytes were pretreated with either 10 μM S1P for 10 minutes or 250 nM TPA for 15 minutes followed by incubation with 1 μM insulin. After 24 hours, cells were pulsed with [^{3}H]thymidine. Values are expressed as *x*-fold increase compared to control and are the mean ± SED from three experiments. ***P*<0.001 indicates a statistically significant difference (**a**) versus unstimulated control cells and (**b** and **c**) versus insulin-treated cells.

after 5 minutes of S1P treatment and returned to basal levels after 60 minutes (Figure 3a). The most effective dose to stimulate PKC activity was 10 μM of S1P, which is similar to the concentration inhibiting Akt phosphorylation (data not shown). To further support the fact that PKC activation leads to a dephosphorylation of Akt in human keratinocytes, the

direct PKC activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was used. In analogy to S1P, TPA caused dephosphorylation of basal Akt activity and significantly abrogated the insulin-dependent phosphorylation of Akt on Ser^{473} in human keratinocytes (Figure 3b). In accordance, measurement of proliferation indicated that pretreatment of keratinocytes with TPA inhibited the insulin-mediated increase of keratinocyte cell growth (Figure 3c). As previous studies indicated the involvement of especially PKCδ on Akt dephosphorylation, the ability of S1P to stimulate this PKC isozyme was examined (Li *et al.*, 2006). As presented in Figure 4a, S1P strongly activated PKCδ in a time-dependent manner. The most effective concentration of S1P to phosphorylate PKCδ was 10 μM (data not shown). To further substantiate the role of PKCδ in S1P-induced Akt modulation, on the one hand, the selective inhibitor rottlerin was used and, on the other hand, PKCδ was downregulated by small interfering RNA (siRNA). Treatment of siRNA-transfected cells with TPA confirmed the effective downregulation of PKCδ (Figure 4c, upper panel). Indeed, S1P did not reduce insulin-induced Akt phosphorylation when PKCδ was abrogated by siRNA technology (Figure 4b). These findings were confirmed by the use of rottlerin, as it also prevented the effect of S1P on Akt modulation (data not shown). Finally, proliferation was measured in PKCδ-downregulated cells. According to the Akt phosphorylation status, S1P lost its ability to inhibit insulin-induced proliferation (Figure 4c).

S1P mediates its action on Akt, PKCδ, and proliferation via the S1P$_2$ receptor subtype

Next, it was of interest to evaluate whether Akt dephosphorylation induced by S1P is a receptor-mediated process. Recently, we have indicated that human keratinocytes express mRNA transcripts of all five S1P receptors (Vogler *et al.*, 2003). On the basis of these studies, we performed real-time PCR to quantify mRNA of S1P receptors in primary human keratinocytes. The relative amount of S1P receptor mRNA was $S1P_5 > S1P_1 > S1P_4 > S1P_2 > S1P_3$ (Figure 5a). Employing antisense technique, we further sought to elucidate which receptor subtype is responsible for the inhibition of Akt activation. Real-time PCR analysis revealed that treatment of cells with $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$ antisense phosphorothioate oligonucleotides (ASOs) resulted in a serious reduction of mRNA levels (Figure 5b). As presented in Figure 6, abrogation of the receptor subtypes $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$ did not abolish the S1P-induced Akt dephosphorylation. Most interestingly, only abrogation of the $S1P_2$ receptor subtype influenced the ability of S1P to modulate Akt phosphorylation. Thus, downregulation of the $S1P_2$ prevents the inhibitory effect of S1P on insulin-mediated

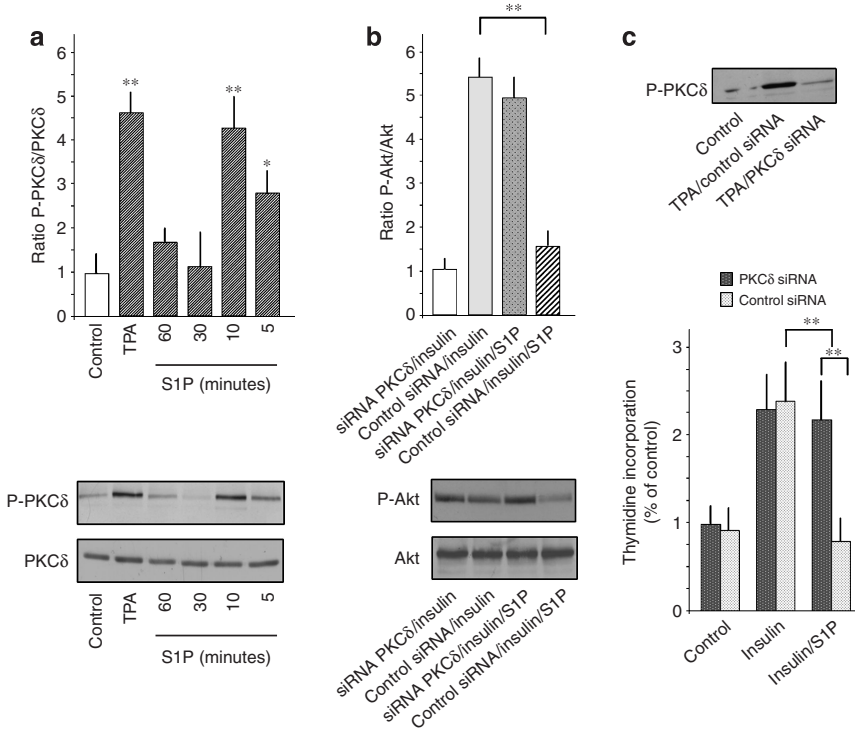

Figure 4. PKCδ activation by S1P inhibits keratinocyte proliferation via Akt dephosphorylation. (**a**) Cells were treated with 10 μM S1P for the indicated time periods or with 250 nM TPA for 15 minutes, and PKCδ activation was measured by western blot analysis. (**b**) Keratinocytes were transfected with siRNA against PKCδ or control siRNA before stimulation with 10 μM S1P for 10 minutes and the subsequent treatment with 1 μM insulin for additional 10 minutes. Then Akt activity was determined by western blot analysis. (**c**) Efficiency of downregulation of PKCδ by siRNA was proved by detection of phospho-PKCδ levels in siRNA-transfected cells toward stimulation with 250 nM TPA for 15 minutes (upper panel). (**c**) PKCδ-downregulated and control cells were pretreated with 10 μM S1P for 10 minutes and then stimulated with 1 μM insulin. After 24 hours, [^{3}H]thymidine incorporation was examined. Data are expressed as *x*-fold increase of control. All results were confirmed in three independent experiments and represented as means ± SED. $^*P<0.05$ and $^{**}P<0.001$ indicate a statistically significant difference.

Akt activation, indicating that this receptor subtype is responsible for the interplay between S1P and insulin signaling (Figure 7a). To further substantiate the involvement of $S1P_2$, actions of S1P were examined in the presence of JTE013, a well-established antagonist of this receptor subtype. In accordance to the findings using ASO technique against $S1P_2$, S1P completely lost its ability to impair insulin-induced Akt phosphorylation in the presence of JTE013 (Figure 7b).

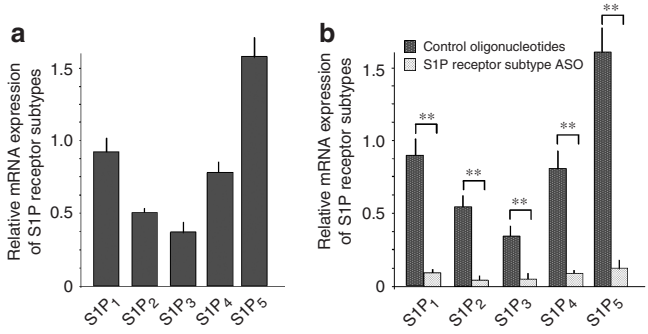

Figure 5. Expression of S1P receptor subtypes and their downregulation in response to $S1P_{1-5}$ ASO in human keratinocytes. (**a**) Quantitative real-time PCR analysis of the human S1P receptor subtypes $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$ in human keratinocytes was performed using cyclophilinA as reference gene. The efficiency of ASO technique to downregulate S1P receptors was demonstrated by real-time PCR. (**b**) Human keratinocytes were pretreated with control oligonucleotides or $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, or $S1P_5$ ASO (each 500 nM) for 2 days. Values are means ± SED from three independent experiments. $^{**}P<0.001$ indicates a statistically significant difference versus control oligonucleotide-treated cells.

Additionally, we proved whether $S1P_2$ receptor subtype is also responsible for the activation of PKCδ. As presented in Figure 7c, abrogation of $S1P_2$ almost completely diminished the stimulatory effect of S1P on PKCδ. To substantiate the role of the $S1P_2$ receptor subtype not only on PKCδ activation and Akt dephosphorylation but also on inhibition of insulin-mediated proliferation, thymidine incorporation was performed in the absence of $S1P_2$. Actually, S1P did not significantly reduce insulin-mediated proliferation in case of $S1P_2$ downregulation (Figure 7d).

The S1P analogue FTY720-phosphate does not inhibit insulin-mediated Akt phosphorylation and proliferation

Next, it was of great interest to investigate the role of FTY720-phosphate (FTY720-P; phosphoric acid mono[(*R*/*S*)-2-amino-2-hydroxymethyl-4-(4-octylphenyl)butyl]ester) on Akt modulation and keratinocyte proliferation, as this immunomodulator acts on all S1P receptor subtypes except $S1P_2$. Consequently, FTY720-P neither influenced the basal activity of Akt nor inhibited insulin-mediated Akt phosphorylation (Figure 8a). As we have identified Akt dephosphorylation as the essential pathway of S1P via $S1P_2$ to inhibit keratinocyte cell growth, we also investigated the influence of FTY720-P on keratinocyte proliferation. As expected, proliferation measurements revealed that FTY720-P, in contrast to S1P, was not able to inhibit the insulin-mediated cell growth (Figure 8b). Taken together, our results indicate that S1P inhibits insulin-dependent cell growth of human keratinocytes via the activation of PKC, followed by a subsequent inhibition of Akt phosphorylation. $S1P_2$ has been identified as the crucial receptor subtype for this action. This

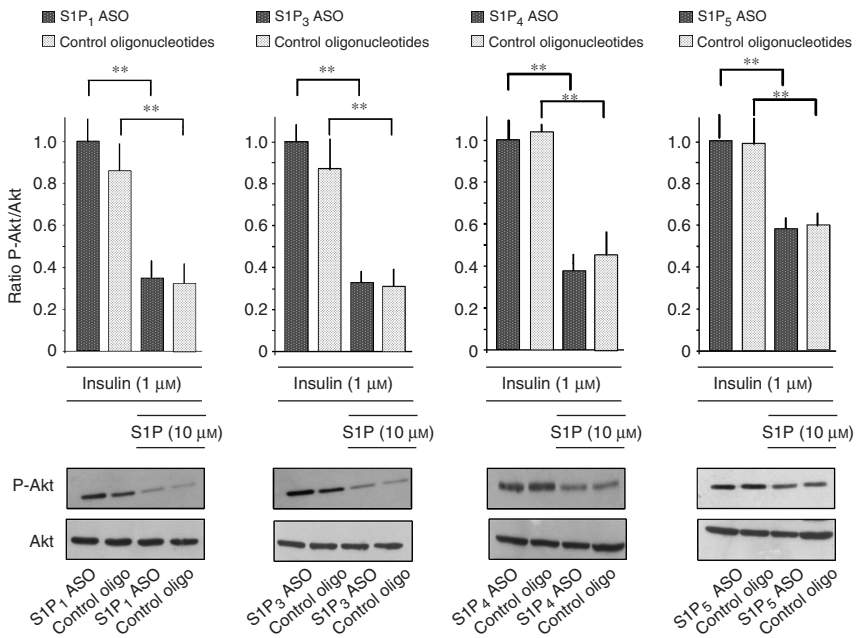

Figure 6. Akt dephosphorylation toward S1P in the presence of $S1P_{1-5}$ ASO or control oligonucleotides. Keratinocytes were transfected with $S1P_1$-, $S1P_3$-, $S1P_4$-, or $S1P_5$-specific ASO or control oligonucleotides for 2 days. Then cells were pretreated with 10 µM S1P for 10 minutes followed by stimulation with 1 µM insulin for another 10 minutes. Akt phosphorylation was detected by western blot analysis. Values are presented as the mean ± SED from three experiments. $^{**}P<0.001$.

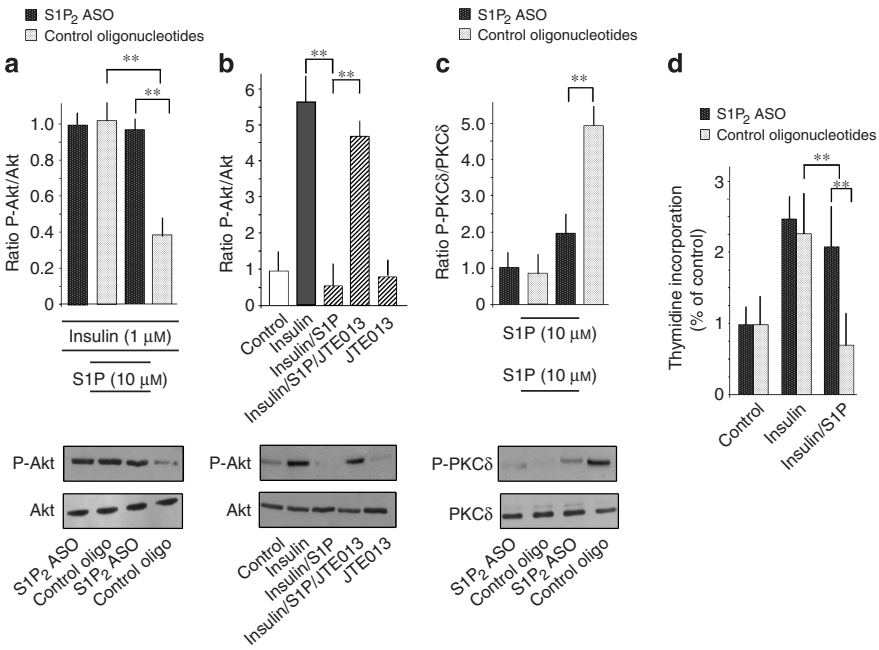

Figure 7. The S1P$_2$ receptor subtype modulates PKCδ- and Akt-activation as well as keratinocyte growth arrest. Human keratinocytes were (**a**) pretreated with control oligonucleotides or S1P$_2$ ASO for 2 days or (**b**) with 10 μM of the S1P$_2$ receptor antagonist JTE013 for 10 minutes. Then cells were stimulated with 10 μM S1P for 10 minutes followed by an incubation with 1 μM insulin for another 10 minutes. (**a** and **b**) Akt phosphorylation was measured by western blot analysis. (**c**) Keratinocytes transfected with S1P$_2$ ASO or control oligonucleotides were stimulated with 10 μM S1P for 10 minutes and phosphorylation of PKCδ was measured by western blot analysis. (**c**) Cells transfected with S1P$_2$ ASO or control oligonucleotides were pretreated with 10 μM S1P for 10 minutes followed by stimulation with 1 μM insulin. After 24 hours, [^{3}H]thymidine incorporation was performed. Values are expressed as *x*-fold increase compared to control and are the mean ± SED from three experiments. $^{**}P<0.001$ indicates a statistically significant difference versus control cells.

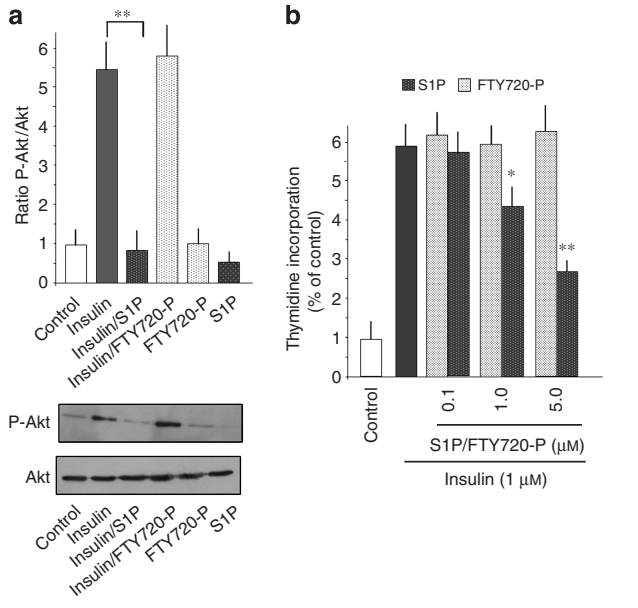

Figure 8. FTY720-P fails to inhibit insulin signaling. Keratinocytes were pretreated with either S1P (10 μM) or FTY720-P (1 μM) for 10 minutes and then stimulated with 1 μM insulin for another 10 minutes. (**a**) Phosphorylated Akt was determined by western blot analysis. (**b**) Cells were pretreated with the indicated concentrations of S1P or FTY720-P for 10 minutes and then stimulated with 1 μM insulin. After 24 hours, cells were pulsed with [^{3}H]thymidine. Data of DNA incorporation are expressed as *x*-fold increase of control and are the mean ± SED from three experiments. $^{*}P<0.05$ and $^{**}P<0.001$ indicate a statistically significant difference versus (**a**) insulin- and (**b**) FTY720-P-treated cells.

is of importance, as the immunomodulator FTY720 is not a true agonist at this receptor subtype and therefore fails to suppress growth factor-mediated proliferation of human keratinocytes.

DISCUSSION

Cross-talk of multiple signaling pathways activated by growth factors and cytokines plays a pivotal role in the regulation of proliferation and differentiation of many cell types. Most interestingly, our study demonstrates a complex interplay of the bioactive molecules insulin and S1P in human keratinocytes. Herein, we present evidence that activation of the S1P$_2$ receptor subtype by its physiological ligand S1P inhibits insulin-mediated cell growth of human keratinocytes via the activation of PKCδ followed by a subsequent dephosphorylation of Akt.

Several studies have pointed out a role for insulin and IGF-I in the pathogenesis of psoriasis. Thus, keratinocytes derived from psoriasis patients are more susceptible to IGF-I-stimulated proliferation *in vitro* than keratinocytes from non-affected individuals (Ristow, 1993). Moreover, the IGF-IR is more abundant in psoriatic lesions (Hodak *et al.*, 1996). Therefore, it has been proposed that inhibition of IR/IGF-IR signaling would be an effective way of treating psoriatic epidermal hyperplasia. Indeed, antisense oligonucleotides against IGF-IR caused a dramatic normalization of the hyperplastic epidermis in mice, indicating that interfering of IGF-I signaling forms the basis of a potential psoriasis therapy (Wraight *et al.*, 2000).

Our data indicate that the sphingolipid metabolite S1P interferes with IR/IGF-IR signaling, resulting in an almost complete inhibition of insulin-mediated keratinocyte proliferation. Although it is well known that S1P acts as a positive regulator of cell growth in many cell types (Hla, 2003; Spiegel and Milstien, 2003), an antiproliferative effect of the sphingolipid has been previously reported in hepatic myofibroblasts, rat hepatocytes, mouse myoblasts, and in human keratinocytes (Davaille *et al.*, 2000; Ikeda *et al.*, 2003; Kim *et al.*, 2004; Donati *et al.*, 2005).

Despite our increasing knowledge of S1P as cell growth inhibitor, the involved signaling pathways in epidermal cells have not been well characterized. It is well established that Akt phosphorylation plays an important role in regulating cell growth of a variety of cells. Overexpression of Akt has been reported in a number of human cancers (Testa and Bellacosa, 2001; Itoh *et al.*, 2002; Bellacosa *et al.*, 2005). In addition, a marked enhancement of Akt phosphorylation can also be detected in psoriatic skin (Rosenberger *et al.*, 2007). S1P possesses a divergent role on Akt phosphorylation depending on the cell type (Igarashi *et al.*, 2001; Baudhuin *et al.*, 2002; Kim *et al.*, 2003, 2004; Balthasar *et al.*, 2006). In a variety of cells, S1P is able to induce Akt phosphorylation, which is in accordance to its positive role on proliferation in these cells (Rosenberger *et al.*, 2007). In keratinocytes, it has been described that S1P inhibits cyclin D2 and induces p21 and p27 (Kim *et al.*, 2004). Thus, the cell cycle arrest correlates with the S1P-induced inhibition of Akt activation.

This study has indicated that PKC activation reduces Akt phosphorylation under both basal and insulin-stimulated conditions. Moreover, S1P induces PKC phosphorylation, and inhibition of PKC completely abolished S1P-induced Akt dephosphorylation in human keratinocytes. Both positive and negative regulation of Akt phosphorylation by PKC has been reported previously, and it has been suggested that individual PKC isozymes have specific functions in the regulation of Akt phosphorylation (Thors *et al.*, 2003; Li *et al.*, 2006). Most interestingly, a decreased PKC activity has been identified in psoriatic epidermis, suggesting the important role of PKC activity in the regulation of proliferation (Horn *et al.*, 1987). Using adenovirus-mediated overexpression of PKC isozymes in keratinocytes, especially PKCδ has been identified as the crucial isozyme that determines the sensitivity of Akt to TPA-induced dephosphorylation (Li *et al.*, 2006). This is consistent to our results, as the use of rottlerin and siRNA against PKCδ confirmed the essential role of this isozyme on the modulation of proliferation and Akt activity. In accordance to the present findings, PKCδ activity induces differentiation and inhibits proliferation in HaCaT cells (Papp *et al.*, 2004). As we clearly indicate that S1P stimulates PKCδ, it is not astonishing that this sphingolipid is also able to induce differentiation of epidermal cells (Balasubramanian *et al.*, 2006). But it should be mentioned that the effect of PKCδ overexpression on keratinocyte cell growth has been controversially discussed (Li *et al.*, 1999; Denning *et al.*, 2002; Gartsbein *et al.*, 2006). Although Shen *et al.* (2001) suggested that PKCδ activation is involved in insulin-mediated proliferation of mouse keratinocytes, our results clearly indicate that in case of abrogation of PKCδ, S1P fails to inhibit insulin-induced Akt phosphorylation and proliferation.

To our knowledge, this study figures out a previously unreported function of the $S1P_2$ receptor subtype in human keratinocytes. Abrogation of $S1P_2$ restored not only the inhibitory effect of S1P on Akt phosphorylation and proliferation but also prevented the S1P-induced PKCδ activation. In congruence to our results, the antiproliferative effect of S1P in rat hepatocytes and mouse myoblasts was mediated by the same receptor subtype (Ikeda *et al.*, 2003; Donati *et al.*, 2005). However, in disagreement, $S1P_2$ was found as the crucial receptor subtype to induce a mitogenic signaling in mouse mesangial cells and in mouse hepatic myofibroblasts (Katsuma *et al.*, 2002; Serriere-Lanneau *et al.*, 2007). This apparent discrepancy can be explained taking into account the high cellular specificity of the signaling pathways initiated by individual S1P receptor subtypes.

In particular, our findings indicate that $S1P_2$ activation is to be considered a therapeutic target for hyperproliferative skin diseases. Therefore, the development of $S1P_2$ receptor subtype-specific ligands might be highly important for such medicinal interventions. It is of interest that the first available drug acting on S1P receptors, the phosphorylated FTY720, activates all of the five S1P receptors except $S1P_2$ and therefore fails to inhibit keratinocyte proliferation.

MATERIALS AND METHODS

Materials

S1P was purchased from Biomol (Hamburg, Germany). S1P was dissolved in methanol and stored at −80 °C. For each experiment, stored S1P was dried and freshly diluted in 0.4% fatty acid-free serum albumin (BSA)/phosphate-buffered saline (PBS). FTY720 was from Biozol Diagnostica (Eching, Germany). JTE013 was obtained from Tocris (Ellisville, MO). TPA was purchased from Calbiochem/Merck Biosciences (Darmstadt, Germany). Insulin, rottlerin, fatty acid-free BSA, trypsin, PBS, Ipegal, sodium desoxycholate, SDS, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, pepstatin, EDTA, sodium fluoride, sodium orthovanadate, DMSO, DMEM, and penicillin/streptomycin were purchased from Sigma-Aldrich (Taufkirchen, Germany). Fetal bovine serum and Optimem were obtained from Biochrom (Berlin, Germany). Keratinocyte basal medium, epidermal growth factor, insulin, hydrocortisone, bovine pituitary extract, gentamycin sulfate, and amphotericin B were purchased from Lonza (Verviers, Belgium). Polyclonal rabbit anti-phospho-Akt (Ser^{473}) antibody, polyclonal rabbit anti-phospho-PKC (pan) (βII Ser^{660}) antibody, polyclonal rabbit anti-phospho-PKCδ (Thr^{505}) antibody, polyclonal rabbit anti-Akt-antibody, anti-rabbit IgG, anti-mouse IgG, SDS sample buffer, dithiothreitol, LumiGlo reagent, and peroxide were obtained from New England Biolabs (Beverly, MA). Monoclonal mouse anti-PKC antibody, PKCδ siRNA, control siRNA, siRNA transfection reagent, and transfection medium were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyvinylidene difluoride membranes were obtained from Millipore (Schwalbach, Germany). TransIT-LT1® transfection reagent was purchased from KMF Laborchemie (Lohmar, Germany). Control oligonucleotides, ASO, and primers were synthesized by TIB Molbiol (Berlin, Germany). [methyl-^{3}H]Thymidine (35 Ci mM^{-1}) was purchased from Amersham Pharmacia Biotech (Freiburg, Germany).

Synthesis of FTY720-P

FTY720-P was synthesized from FTY720, as recently described (Albert *et al.*, 2005). Briefly, FTY720 was protected as an oxazolidinone by the addition of benzyl chloroformate. Then, phosphorylation of the free hydroxyl group was carried out by addition of 3-(diethylamino)-1,5-dihydro-2,4,3-benzodioxaphosphepintriphenyl phosphite and a subsequent oxidation to obtain (*R/S*)-4-[2-(4-octylphenyl)ethyl]-4-(3-oxo-1,5-dihydro-3λ^5-benzo[*e*][1,3,2]dioxaphosphepin-3-yloxymethyl) oxazolidin-2-one. The phosphate-protecting group was removed by hydrogenation, and the oxazolidinone was cleaved with lithium hydroxide yielding to FTY720-P. Identity was checked by electrospray ionization time-of-flight (TOF) mass spectrometry using an Agilent 6210 TOF LC/MS (Agilent, Waldbronn, Germany). Electrospray ionization–mass spectrometry, *m/z*: 386 $(M-H)^-$. Moreover, purity of FTY720-P was measured by high-performance liquid chromatography. Therefore, synthesized FTY720-P or standard FTY720 was dissolved in 275 µl methanol/0.07 M K_2HPO_4 (9:1). A derivatization mixture of 10 mg *o*-phthaldialdehyde, 200 µl ethanol, 10 µl 2-mercaptoethanol, and 10 ml 3% boric acid was prepared and adjusted to pH 10.5 with KOH. A 25 µl portion of the derivatization mixture was added to the resolved FTY720 (respectively, FTY720-P) for 15 minutes at room temperature. The derivatives were analyzed by a Merck Hitachi LaChrom HPLC system (Merck Hitachi, Darmstadt, Germany) using an RP 18 Kromasil column (Chromatographie Service, Langerwehe, Germany). Separation was performed with a gradient of methanol and 0.07 M K_2HPO_4. Resulting profiles were evaluated using the Merck system manager software, indicating no detectable amount of FTY720 in the synthesized FTY720-P. FTY720-P was dissolved in DMSO/HCl and for all cell culture experiments further diluted in keratinocyte growth medium (KGM).

Keratinocyte culture

Human keratinocytes were isolated from juvenile foreskin by surgery as described recently (Vogler *et al.*, 2003). Briefly, skin was incubated at 4 °C in a solution of 0.25% trypsin and 0.2% EDTA for 20 hours. Trypsinization was terminated by addition of ice-cold DMEM containing 10% fetal bovine serum. Keratinocytes were separated from the epidermis by gently pivoting, washed with PBS, and centrifuged at 250 *g* for 5 minutes. After washing the cells with PBS, the pellet was resuspended in KGM, which was prepared from keratinocyte basal medium by the addition of 0.1 ng ml^{-1} epidermal growth factor, 5.0 µg ml^{-1} insulin, 0.5 µg ml^{-1} hydrocortisone, 0.15 mM Ca^{2+}, 30 µg ml^{-1} bovine pituitary extract, 50 µg ml^{-1} gentamycin sulfate, and 50 ng ml^{-1} amphotericin B. Keratinocytes were pooled from several donors and cultured at 37 °C in 5% CO_2. For all cell experiments, keratinocytes were cultured for 24 hours in KGM without insulin. The study was conducted according to the Declaration of Helsinki Principles. An institutional approval for the performed experiments was not necessary.

Quantitative real-time PCR

Keratinocytes were cultured in KGM, and RNA was collected using an RNeasy kit (Qiagen, Foster City, CA). cDNA was generated from total RNA using the FermentasAid™ First strand cDNA synthesis kit (Fermentas GmbH, St Leon-Rot, Germany), according to the instructions of the manufacturer. Quantitative real-time PCR was performed using a LightCycler480 and the SYBR Green PCR master mix (Roche Diagnostics–Applied Science, Mannheim, Germany). CyclophilinA was used as a normalization control for all experiments. For the measurement of the S1P receptor subtypes, the following primers were used: $S1P_1$: 5′-CGTGTTCAGTCTCCTCG-3′ (forward), 5′-CTGATGCAGTTCCAGCC-3′ (reverse); $S1P_2$: 5′-GTTAGCCAGGATGGTCTT-3′ (forward), 5′-CAACAGAGCGAGACTTCA-3′ (reverse); $S1P_3$: 5′-CGCTTCAGTGTAAACAACG-3′ (forward), 5′-GAGGGTCACACAGCATT-3′ (reverse); $S1P_4$: 5′-AAGACCAGCCGCGTCTA-3′ (forward), 5′-CCAGGCAGAAGAGGATGT-3′ (reverse); $S1P_5$: 5′-GGAAATGCAGCCAAAGG-3′ (forward), 5′-CCATTATTTCATCACCGAGTT-3′ (reverse); cyclophilinA: 5′-TTTGCTTAATTCTACACAGTACTTAGAT-3′ (forward), 5′-CTACCCTCAGGTGGTCTT-3′ (reverse). Total RNA (10 ng) of three different sets of keratinocytes was used to analyze gene expression. Relative mRNA expression was quantified using the comparative threshold cycle method. Data were obtained in triplicate, and the specific mRNA levels were expressed as the mean ± SEM of relative mRNA expression relative to control cells.

Western blot analysis

Keratinocytes were seeded in six-well plates and cultured for 24 hours in KGM without insulin. After stimulation, cells were rinsed twice with ice-cold PBS and harvested in lysis buffer (PBS without Ca^{2+}/Mg^{2+}, 1% Ipegal, 0.5% sodium desoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 µg ml^{-1} leupeptin, 1 µg ml^{-1} aprotinin, 1 µg ml^{-1} pepstatin, 1 mM sodium orthovanadate, and 50 mM sodium fluoride). Lysates were centrifuged at 14,000 *g* for 30 minutes. Samples containing 20–40 µg protein were boiled in SDS sample buffer (100 mM Tris/HCl (pH 6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM dithiothreitol) and separated by 10% SDS-PAGE. Gels were blotted overnight onto polyvinylidene difluoride membranes. After blocking with 5% non-fat dry milk for 1 hour at 37 °C, membranes were incubated with the appropriate primary antibodies at a dilution of 1:1,000 for 2 hours at room temperature, and further incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour. Then blots were developed according to the manufacturer's protocol. Densitometry measurements were recorded using a Syngene Gene Genius imaging system (Syngene, Cambridge, UK). Values of the phosphorylated proteins were normalized to the corresponding total protein level.

Deletion of S1P receptors using antisense technique

$S1P_{1–5}$ receptor ASOs were designed to surround the translational initiation site, a place empirically known to be most effective for inhibition of gene expression. S1P receptor-specific ASOs were synthesized based on the sequence of the mammalian S1P receptor cDNAs. Same-length control oligonucleotides (with the same nucleotides but randomly scrambled sequence) to the S1P receptor subtypes were also designed: $S1P_1$ ASO (5′-GACGCTGGTGGGCCCCAT-3′) and $S1P_1$ control oligonucleotides (5′-ATGGGGCCCACCAGCGTC-3′); $S1P_2$ ASO (5′-GTTGAGCAGGGAATTCAGGGTGGAGA-3′) and $S1P_2$ control oligonucleotides (5′-CATCACTAGCCACTTGAAGCAGGCCA-3′); $S1P_3$ ASO (5′-CGGGAGGGCAGTTGCCAT-3′) and $S1P_3$ control oligonucleotides (5′-ATGGCAACTGCCCTCCCG-3′); $S1P_4$ ASO (5′-GAAGGCCAGCAGGATCATCAGCAC-3′) and $S1P_4$ control oligonucleotides (5′-ACCTAGCCAACCCTCCATGAAGGC-3′); $S1P_5$ ASO (5′-CAACATGCCACAAAGGCCAGGAG-3′) and $S1P_5$ control oligonucleotides (5′-GCAACAACATAACGGGCCAGCA-3′).

For all antisense experiments, cells were treated with the indicated ASO or control oligonucleotides for 2 days at a final concentration of 500 nM using TransIT-LT1® in Optimem.

Abrogation of PKCδ by siRNA

Downregulation of PKCδ by siRNA (target sequence: 5′-CCAUGAG UUUAUCGCCACCTT-3′) was performed according to the manufacturer's protocol (Santa Cruz Biotechnology). Briefly, keratinocytes were transfected with 50 nM of PKCδ siRNA or control siRNA using siRNA transfection reagent. Thereafter, siRNA and transfection reagent were diluted in transfection medium and incubated at room temperature for 30 minutes. Cells were then incubated at 37 °C for 5 hours with the siRNA transfection complexes. After the transfection period of 5 hours, the medium was changed to antibiotic-free KGM. After a recovering time for 48 hours, proliferation and western blot experiments were performed. The silencing efficiency was detected by western blot analysis using anti-phospho-PKCδ-antibody in response to stimulation with TPA.

[^{3}H]Thymidine incorporation assay

To determine DNA synthesis rates in cell culture experiments, keratinocytes were grown in 24-well plates for 24 hours. Then medium was replaced by fresh KGM without insulin and cells were incubated with the indicated substances for 24 hours. After 20 hours, cells were pulse labeled with 1 μCi [methyl-^{3}H]thymidine per well for 4 hours. The medium was removed and cells were washed twice with PBS and ice-cold trichloroacetic acid (5%). The precipitated material was dissolved in 0.3 N NaOH solution and incorporated [methyl-^{3}H]thymidine was determined in a scintillation counter (MicroBeta Plus, Wallac Oy, Turku, Finland).

Statistical analysis

Data are expressed as the mean ± SEM of results from at least three experiments, each run in triplicate. Statistics were performed using Student's *t*-test. $*P<0.05$ and $**P<0.01$ indicate a statistically significant difference versus control experiments.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

Albert R, Hinterding K, Brinkmann V, Guerini D, Muller-Hartwieg C, Knecht H *et al.* (2005) Novel immunomodulator FTY720 is phosphorylated in rats and humans to form a single stereoisomer. Identification, chemical proof, and biological characterization of the biologically active species and its enantiomer. *J Med Chem* 48:5373–7

Balasubramanian S, Zhu L, Eckert RL (2006) Apigenin inhibition of involucrin gene expression is associated with a specific reduction in phosphorylation of protein kinase Cdelta Tyr311. *J Biol Chem* 281:36162–72

Balthasar S, Samulin J, Ahlgren H, Bergelin N, Lundqvist M, Toescu EC *et al.* (2006) Sphingosine 1-phosphate receptor expression profile and regulation of migration in human thyroid cancer cells. *Biochem J* 398:547–56

Baudhuin LM, Cristina KL, Lu J, Xu Y (2002) Akt activation induced by lysophosphatidic acid and sphingosine-1-phosphate requires both mitogen-activated protein kinase kinase and p38 mitogen-activated protein kinase and is cell-line specific. *Mol Pharmacol* 62:660–71

Bellacosa A, Kumar CC, Di Cristofano A, Testa JR (2005) Activation of AKT kinases in cancer: implications for therapeutic targeting. *Adv Cancer Res* 94:29–86

Blagosklonny MV (2002) Are p27 and p21 cytoplasmic oncoproteins? *Cell Cycle* 1:391–3

Brinkmann V, Cyster JG, Hla T (2004) FTY720: sphingosine 1-phosphate receptor-1 in the control of lymphocyte egress and endothelial barrier function. *Am J Transplant* 4:1019–25

Chalfant CE, Spiegel S (2005) Sphingosine 1-phosphate and ceramide 1-phosphate: expanding roles in cell signaling. *J Cell Sci* 118:4605–12

Cheatham B, Kahn CR (1995) Insulin action and the insulin signaling network. *Endocr Rev* 16:117–42

Davaille J, Gallois C, Habib A, Li L, Mallat A, Tao J *et al.* (2000) Antiproliferative properties of sphingosine 1-phosphate in human hepatic myofibroblasts. A cyclooxygenase-2 mediated pathway. *J Biol Chem* 275:34628–33

Denning MF, Wang Y, Tibudan S, Alkan S, Nickoloff BJ, Qin JZ (2002) Caspase activation and disruption of mitochondrial membrane potential during UV radiation-induced apoptosis of human keratinocytes requires activation of protein kinase C. *Cell Death Differ* 9:40–52

DiGiovanni J, Bol DK, Wilker E, Beltran L, Carbajal S, Moats S *et al.* (2000) Constitutive expression of insulin-like growth factor-1 in epidermal basal cells of transgenic mice leads to spontaneous tumor promotion. *Cancer Res* 60:1561–70

Dlugosz AA, Cheng C, Williams EK, Dharia AG, Denning MF, Yuspa SH (1994) Alterations in murine keratinocyte differentiation induced by activated rasHa genes are mediated by protein kinase C-alpha. *Cancer Res* 54:6413–20

Dlugosz AA, Mischak H, Mushinski JF, Yuspa SH (1992) Transcripts encoding protein kinase C-alpha, -delta, -epsilon, -zeta, and -eta are expressed in basal and differentiating mouse keratinocytes *in vitro* and exhibit quantitative changes in neoplastic cells. *Mol Carcinog* 5: 286–92

Donati C, Meacci E, Nuti F, Becciolini L, Farnararo M, Bruni P (2005) Sphingosine 1-phosphate regulates myogenic differentiation: a major role for S1P2 receptor. *FASEB J* 19:449–51

Dupont J, LeRoith D (2001) Insulin and insulin-like growth factor I receptors: similarities and differences in signal transduction. *Horm Res* 55(Suppl 2): 22–6

Edmondson SR, Thumiger SP, Werther GA, Wraight CJ (2003) Epidermal homeostasis: the role of the growth hormone and insulin-like growth factor systems. *Endocr Rev* 24:737–64

Fuchs E, Raghavan S (2002) Getting under the skin of epidermal morphogenesis. *Nat Rev Genet* 3:199–209

Gartsbein M, Alt A, Hashimoto K, Nakajima K, Kuroki T, Tennenbaum T (2006) The role of protein kinase C delta activation and STAT3 Ser727 phosphorylation in insulin-induced keratinocyte proliferation. *J Cell Sci* 119:470–81

Giovannucci E (1999) Insulin-like growth factor-I and binding protein-3 and risk of cancer. *Horm Res* 51(Suppl 3):34–41

Goren I, Muller E, Pfeilschifter J, Frank S (2006) Severely impaired insulin signaling in chronic wounds of diabetic ob/ob mice: a potential role of tumor necrosis factor-alpha. *Am J Pathol* 168:765–77

Gschwendt M, Dieterich S, Rennecke J, Kittstein W, Mueller HJ, Johannes FJ (1996) Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase c isoenzymes. *FEBS Lett* 392: 77–80

Hait NC, Oskeritzian CA, Paugh SW, Milstien S, Spiegel S (2006) Sphingosine kinases, sphingosine 1-phosphate, apoptosis and diseases. *Biochim Biophys Acta* 1758:2016–26

Hla T (2003) Signaling and biological actions of sphingosine 1-phosphate. *Pharmacol Res* 47:401–7

Hodak E, Gottlieb AB, Anzilotti M, Krueger JG (1996) The insulin-like growth factor 1 receptor is expressed by epithelial cells with proliferative potential in human epidermis and skin appendages: correlation of increased expression with epidermal hyperplasia. *J Invest Dermatol* 106: 564–70

Horn F, Marks F, Fisher GJ, Marcelo CL, Voorhees JJ (1987) Decreased protein kinase C activity in psoriatic versus normal epidermis. *J Invest Dermatol* 88:220–2

Igarashi J, Bernier SG, Michel T (2001) Sphingosine 1-phosphate and activation of endothelial nitric-oxide synthase: differential regulation of Akt and MAP kinase pathways by EDG and bradykinin receptors in vascular endothelial cells. *J Biol Chem* 276:12420–6

Ikeda H, Satoh H, Yanase M, Inoue Y, Tomiya T, Arai M *et al.* (2003) Antiproliferative property of sphingosine 1-phosphate in rat hepatocytes involves activation of Rho via Edg-5. *Gastroenterology* 124:459–69

Ishii I, Fukushima N, Ye X, Chun J (2004) Lysophospholipid receptors: signaling and biology. *Annu Rev Biochem* 73:321–54

Itoh N, Semba S, Ito M, Takeda H, Kawata S, Yamakawa M (2002) Phosphorylation of Akt/PKB is required for suppression of cancer cell apoptosis and tumor progression in human colorectal carcinoma. *Cancer* 94:3127–34

Kandel ES, Hay N (1999) The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Exp Cell Res* 253:210–29

Katsuma S, Hada Y, Ueda T, Shiojima S, Hirasawa A, Tanoue A *et al.* (2002) Signalling mechanisms in sphingosine 1-phosphate-promoted mesangial cell proliferation. *Genes Cells* 7:1217–30

Kim DS, Hwang ES, Lee JE, Kim SY, Park KC (2003) Sphingosine-1-phosphate promotes mouse melanocyte survival via ERK and Akt activation. *Cell Signal* 15:919–26

Kim DS, Kim SY, Kleuser B, Schafer-Korting M, Kim KH, Park KC (2004) Sphingosine-1-phosphate inhibits human keratinocyte proliferation via Akt/protein kinase B inactivation. *Cell Signal* 16:89–95

Kooijman R (2006) Regulation of apoptosis by insulin-like growth factor (IGF)-I. *Cytokine Growth Factor Rev* 17:305–23

Koria P, Andreadis ST (2006) Epidermal morphogenesis: the transcriptional program of human keratinocytes during stratification. *J Invest Dermatol* 126:1834–41

Kuhn C, Hurwitz SA, Kumar MG, Cotton J, Spandau DF (1999) Activation of the insulin-like growth factor-1 receptor promotes the survival of human keratinocytes following ultraviolet B irradiation. *Int J Cancer* 80:431–8

Lawlor MA, Alessi DR (2001) PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J Cell Sci* 114:2903–10

LeRoith D, Werner H, Beitner-Johnson D, Roberts CT Jr (1995) Molecular and cellular aspects of the insulin-like growth factor I receptor. *Endocr Rev* 16:143–63

Li L, Lorenzo PS, Bogi K, Blumberg PM, Yuspa SH (1999) Protein kinase Cdelta targets mitochondria, alters mitochondrial membrane potential, and induces apoptosis in normal and neoplastic keratinocytes when overexpressed by an adenoviral vector. *Mol Cell Biol* 19:8547–58

Li L, Sampat K, Hu N, Zakari J, Yuspa SH (2006) Protein kinase C negatively regulates Akt activity and modifies UVC-induced apoptosis in mouse keratinocytes. *J Biol Chem* 281:3237–43

Liang J, Zubovitz J, Petrocelli T, Kotchetkov R, Connor MK, Han K *et al.* (2002) PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. *Nat Med* 8:1153–60

Manggau M, Kim DS, Ruwisch L, Vogler R, Korting HC, Schafer-Korting M *et al.* (2001) 1Alpha,25-dihydroxyvitamin D_3 protects human keratinocytes from apoptosis by the formation of sphingosine-1-phosphate. *J Invest Dermatol* 117:1241–9

Martin P (1997) Wound healing—aiming for perfect skin regeneration. *Science* 276:75–81

Matsui MS, Chew SL, DeLeo VA (1992) Protein kinase C in normal human epidermal keratinocytes during proliferation and calcium-induced differentiation. *J Invest Dermatol* 99:565–71

Misra P, Nickoloff BJ, Morhenn VB, Hintz RL, Rosenfeld RG (1986) Characterization of insulin-like growth factor-I/somatomedin-C receptors on human keratinocyte monolayers. *J Invest Dermatol* 87:264–7

Murayama K, Kimura T, Tarutani M, Tomooka M, Hayashi R, Okabe M *et al.* (2007) Akt activation induces epidermal hyperplasia and proliferation of epidermal progenitors. *Oncogene* 26:4882–8

Newton AC (2003) Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. *Biochem J* 370:361–71

Ouyang W, Li J, Zhang D, Jiang BH, Huang DC (2007) PI-3K/Akt signal pathway plays a crucial role in arsenite-induced cell proliferation of human keratinocytes through induction of cyclin D1. *J Cell Biochem* 101:969–78

Pankow S, Bamberger C, Klippel A, Werner S (2006) Regulation of epidermal homeostasis and repair by phosphoinositide 3-kinase. *J Cell Sci* 119:4033–46

Papp H, Czifra G, Bodo E, Lazar J, Kovacs I, Aleksza M *et al.* (2004) Opposite roles of protein kinase C isoforms in proliferation, differentiation, apoptosis, and tumorigenicity of human HaCaT keratinocytes. *Cell Mol Life Sci* 61:1095–105

Radeff-Huang J, Seasholtz TM, Matteo RG, Brown JH (2004) G protein mediated signaling pathways in lysophospholipid induced cell proliferation and survival. *J Cell Biochem* 92:949–66

Ristow HJ (1993) Effect of insulin-like growth factor-I/somatomedin C on thymidine incorporation in cultured psoriatic keratinocytes after growth arrest in growth factor-free medium. *Growth Regul* 3:129–37

Rosenberger C, Solovan C, Rosenberger AD, Jinping L, Treudler R, Frei U *et al.* (2007) Upregulation of hypoxia-inducible factors in normal and psoriatic skin. *J Invest Dermatol* 127:2445–52

Sadagurski M, Yakar S, Weingarten G, Holzenberger M, Rhodes CJ, Breitkreutz D *et al.* (2006) Insulin-like growth factor 1 receptor signaling regulates skin development and inhibits skin keratinocyte differentiation. *Mol Cell Biol* 26:2675–87

Sauer B, Vogler R, von Wenckstern H, Fujii M, Anzano MB, Glick AB *et al.* (2004) Involvement of Smad signaling in sphingosine 1-phosphate-mediated biological responses of keratinocytes. *J Biol Chem* 279:38471–9

Scheid MP, Woodgett JR (2003) Unravelling the activation mechanisms of protein kinase B/Akt. *FEBS Lett* 546:108–12

Segrelles C, Moral M, Lara MF, Ruiz S, Santos M, Leis H *et al.* (2006) Molecular determinants of Akt-induced keratinocyte transformation. *Oncogene* 25:1174–85

Serriere-Lanneau V, Teixeira-Clerc F, Li L, Schippers M, de Wries W, Julien B *et al.* (2007) The sphingosine 1-phosphate receptor S1P2 triggers hepatic wound healing. *FASEB J* 21:2005–13

Shen S, Alt A, Wertheimer E, Gartsbein M, Kuroki T, Ohba M *et al.* (2001) PKCdelta activation: a divergence point in the signaling of insulin and IGF-1-induced proliferation of skin keratinocytes. *Diabetes* 50:255–64

Shin I, Yakes FM, Rojo F, Shin NY, Bakin AV, Baselga J *et al.* (2002) PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. *Nat Med* 8:1145–52

Song G, Ouyang G, Bao S (2005) The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med* 9:59–71

Spiegel S, Milstien S (2003) Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol* 4:397–407

Testa JR, Bellacosa A (2001) AKT plays a central role in tumorigenesis. *Proc Natl Acad Sci USA* 98:10983–5

Thors B, Halldorsson H, Clarke GD, Thorgeirsson G (2003) Inhibition of Akt phosphorylation by thrombin, histamine and lysophosphatidylcholine in endothelial cells. Differential role of protein kinase C. *Atherosclerosis* 168:245–53

Verrando P, Ortonne JP (1985) Insulin binding properties of normal and transformed human epidermal cultured keratinocytes. *J Invest Dermatol* 85:328–32

Vogler R, Sauer B, Kim DS, Schafer-Korting M, Kleuser B (2003) Sphingosine-1-phosphate and its potentially paradoxical effects on critical parameters of cutaneous wound healing. *J Invest Dermatol* 120:693–700

Wertheimer E, Trebicz M, Eldar T, Gartsbein M, Nofeh-Moses S, Tennenbaum T (2000) Differential roles of insulin receptor and insulin-like growth factor-1 receptor in differentiation of murine skin keratinocytes. *J Invest Dermatol* 115:24–9

Wilker E, Lu J, Rho O, Carbajal S, Beltran L, DiGiovanni J (2005) Role of PI3K/Akt signaling in insulin-like growth factor-1 (IGF-1) skin tumor promotion. *Mol Carcinog* 44:137–45

Wraight CJ, White PJ, McKean SC, Fogarty RD, Venables DJ, Liepe IJ *et al.* (2000) Reversal of epidermal hyperproliferation in psoriasis by insulin-like growth factor I receptor antisense oligonucleotides. *Nat Biotechnol* 18:521–6