Activation of Epidermal Akt by Diverse Mouse Skin Tumor Promoters

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Abstract

Akt is a serine/threonine kinase involved in a variety of cellular responses, including cell proliferation and cell survival. Recent studies from our laboratory suggest that Akt signaling may play an important role in skin tumor promotion. To explore this premise, we examined epidermal Akt activation and signaling in response to chemically diverse skin tumor promoters. Mice received single or multiple applications of 12-O-tetradecanoylphorbol-13-acetate (TPA), okadaic acid, or chrysin. All three tumor promoters were able to activate epidermal Akt as early as 1 h after treatment. Activation of Akt following tumor promoter treatment led to enhanced downstream signaling, including hyperphosphorylation of glycogen synthase kinase-3β and Bad. Structure activity studies with phorbol ester analogues revealed that the magnitude of activation paralleled tumor-promoting activity. In cultured primary keratinocytes, TPA treatment also led to activation of Akt. Activation of the epidermal growth factor receptor (EGFR) seemed to underlie the ability of TPA to activate Akt as both PD153035, an inhibitor of EGFR, and GW2974, a dual-specific inhibitor of both EGFR and erbB2, were able to effectively reduce TPA-induced Akt phosphorylation as well as TPA-stimulated EGFR and erbB2 tyrosine phosphorylation in a dose-dependent manner. Furthermore, inhibition of protein kinase C (PKC) activity blocked TPA-stimulated heparin-binding EGF production and EGFR transactivation. Inhibition of PKC also led to a decreased association of Akt with the PP2A catalytic subunit, leading to increased Akt phosphorylation. However, combination of EGFR inhibitor and PKC inhibitor completely abrogated TPA-induced activation of Akt. Collectively, the current results support the hypothesis that elevated Akt activity and subsequent activation of downstream signaling pathways contribute significantly to skin tumor promotion. In addition, signaling through the EGFR via EGFR homodimers or EGFR/erbB2 heterodimers may be the primary event leading to Akt activation during tumor promotion in mouse skin.

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Introduction

Akt is a serine/threonine kinase involved in a variety of cellular responses, including cell proliferation and cell survival (1, 2). In response to phosphatidylinositol 3-kinase (PI3K) activation, the 3'-phosphorylated phosphoinositides PI(3,4,5)P3 and PI(3,4)P2 are synthesized at the plasma membrane. These phospholipids are then capable of recruiting pleckstrin homology domain-containing proteins, such as Akt and PI(3,4,5)P3-dependent protein kinases (PDK-1 and PDK-2), to the inner leaf of the plasma membrane and facilitating phosphorylation of Akt at Thr308 and Ser473 (1, 2). On activation, Akt modulates numerous signaling pathways affecting such proteins as Bad, glycogen synthase kinase-3β (GSK-3β), mammalian target of rapamycin (mTOR), endothelial nitric oxide synthase, caspase-9, and forkhead transcription factors (3-8).

Previously, we reported an up-regulation of PI3K and Akt activity and cell cycle regulatory proteins in the epidermis of transgenic mice that overexpress human insulin-like growth factor-I (IGF-I) under control of a bovine keratin 5 (BK5) promoter (9, 10). These biochemical changes were inhibited by the PI3K inhibitor LY294002 (9), and furthermore, this compound inhibited skin tumor promotion mediated by IGF-I in a dose-dependent manner (10). Collectively, these data support the hypothesis that the PI3K/Akt signaling pathway may play a critical role in IGF-I–mediated mouse skin tumor promotion through the modulation of signaling pathways that affect cell cycle progression and possibly cell survival. In addition, Segrelles et al. (11) also reported sustained activation of epidermal Akt throughout tumorigenesis using a standard two-stage regimen. More recently, this group used Akt-transformed keratinocytes to examine the signaling pathways involved in Akt-mediated cellular proliferation. Analysis of tumors generated by s.c. injection of the transformed keratinocytes revealed an increase in Foxo3a phosphorylation and an increase in the expression and nuclear localization of ΔNp63, β-catenin, and Lef1. Northern blot analysis revealed an increase in expression of cyclin D1 and c-myc (12).

The process of skin tumor promotion is complex and the exact cellular and biochemical mechanisms that underlie this
process remain to be determined (13-15). This is underscored by the fact that many different types of chemicals may act as tumor promoters in the mouse skin model of tumorigenesis (14, 15). Unlike the phorbol esters, many of these compounds do not interact with the protein kinase C (PKC) family, thus presenting an initial mechanism(s) different from that of the phorbol esters (15-17). In addition, multiple cellular signaling pathways seem to be involved. It is assumed that, within the diversity of tumor-promoting stimuli, some common mechanisms exist. There is considerable evidence that signaling through the epidermal growth factor receptor (EGFR) plays an important role during multistage skin carcinogenesis in mice. We have previously shown that the expression (mRNA and protein) of both the EGFR and EGFR ligands was constitutively elevated in primary papillomas and squamous cell carcinomas generated by an initiation-promotion regimen (18-20). In addition, treatment of SENCAR mice with 12-O-tetradecanoylphorbol-13-acetate (TPA), okadaic acid, or chrysurarin led to increased tyrosine phosphorylation of the EGFR in the epidermis (21-23). Furthermore, levels of phosphorylated erbB2, a heterodimer partner of EGFR, were elevated in a dose-dependent manner in response to TPA as well as an increase in erbB2:EGFR heterodimer formation (22). In cultured human keratinocytes, treatment with TPA leads to a significant elevation in mRNA expression and secretion of transforming growth factor-α protein (24). In JB6 (murine epidermal cell line) cells, it was also reported that exposure to TPA leads to activation of the EGFR through a mechanism that involves release of EGFR ligands through ectodomain shedding (25). Finally, studies with both EGFR knockout keratinocytes transduced with v-Ha-ras and mice overexpressing a dominant-negative EGFR support a role for EGFR signaling in skin carcinogenesis and tumor promotion (26, 27).

In the present study, we show that activation of epidermal Akt is a common underlying event for three different classes of tumor promoters in mouse skin. In addition, activation of epidermal Akt leads to changes in phosphorylation of downstream signaling substrates of this pathway. Furthermore, we show that the mechanism whereby TPA activates epidermal Akt involves signaling through the EGFR. Thus, the current results provide support for the hypothesis that elevated Akt activity and subsequent activation of downstream signaling pathways contribute significantly to the tumor-promoting action of diverse skin tumor promoters and that signaling through the EGFR may be the primary event leading to epidermal Akt activation during this process.

Results
Diverse Tumor Promoters Activate Epidermal Akt following Topical Application

Initial experiments were designed to examine whether topical treatment with TPA, okadaic acid, or chrysurarin would modulate epidermal Akt activity. Therefore, groups of mice were treated with single topical applications of each tumor promoter and epidermal Akt kinase activity was assessed at various time points following treatment (data not shown). Akt activity was increased as early as 1 or 2 h after treatment with all three tumor promoters. The maximum increase in Akt activity was found at 4 h after treatment for TPA, 2 h after treatment with okadaic acid, and 6 h after treatment with chrysurarin. This early activation of Akt was confirmed in multiple experiments in which epidermal Akt kinase activity was assessed at a single time point at which either maximum or near maximum activation occurred for each tumor promoter. These results are shown in Fig. 1A. Using the same treatment regimen, epidermal lysates were prepared at 4 h after treatment with either TPA or okadaic acid and at 6 h after treatment with chrysurarin. All three tumor promoters caused a significant increase in epidermal Akt activity (P < 0.05, ANOVA). UVB exposure (125 mJ/cm²) was also able to induce Akt activity 8 h after exposure (data not shown).

It is widely accepted that maximal activation of Akt involves phosphorylation at Thr308 followed by phosphorylation at Ser473 (28). These phosphorylation events are believed to be mediated by PDK-1 and PDK-2, respectively (29, 30). Thus, as shown in Fig. 1B, we assessed the status of epidermal Akt following treatment with TPA, okadaic acid, and chrysurarin by Western blotting using phospho-specific antibodies. By 6 h after treatment, all three tumor promoters had caused an increase in phosphorylation of Akt at both Ser473 (Fig. 1B) and Thr308 (data not shown). There were no remarkable alterations in the total level of Akt. As part of this experiment, we examined whether representative Akt downstream signaling molecules were also modulated in epidermis of tumor promoter–treated mice. As also shown in Fig. 1B, phosphorylation of mTOR (Ser428), GSK-3β (Ser9), and Bad (Ser136) was increased in response to treatment with the panel of tumor promoters used. Thus, Akt activation by diverse tumor promoters leads to increased phosphorylation of at least three known Akt substrates. Note that in this experiment different doses of the promoting agents were used compared with Fig. 1A. The higher activation level of Akt after okadaic acid treatment (as assessed by phosphorylation status) compared with the activity data shown in Fig. 1A is likely due to the fact that a 2-fold higher dose of okadaic acid (10 nmol) was used in Western blot analysis (Fig. 1B). This dose of okadaic acid still falls within the range of tumor-promoting doses for this compound.

Structure Activity Study of Epidermal Akt Activation by Phorbol Esters

As an approach to further establish a link between phorbol ester skin tumor promotion and activation of epidermal Akt, we did a structure activity experiment in which the ability of two phorbol ester analogues, phorbol 12,13-didecanoate (PDD) and 4α-PDD, to activate epidermal Akt was examined. PDD has less tumor-promoting activity than TPA and 4α-PDD has no tumor-promoting activity. As shown in Fig. 1C, treatment with PDD did result in an increase in epidermal Akt activity but to a lesser extent than treatment with TPA even at twice the dose. There was no significant change in epidermal Akt kinase activity in response to 4α-PDD. Thus, the data support the hypothesis that Akt activation is related to tumor-promoting ability and provide further evidence that activation of epidermal Akt plays a role in skin tumor promotion by phorbol esters and possibly other classes of tumor promoters.
Cellular Localization of Activated Akt by Tumor Promoters in Mouse Epidermis

To identify the cells in the epidermis displaying activated Akt following tumor promoter treatment, we used immunofluorescence to examine the cellular localization of phosphorylated Akt. Phospho-Akt (Ser473) staining was primarily localized to the epidermal basal layer following treatment with TPA (Fig. 2) and okadaic acid and chrysarobin (data not shown). These results indicate that the early activation of Akt in epidermis following treatment with diverse types of tumor promoters occurs primarily in the basal keratinocytes.

TPAInducesAktPhosphorylationinCulturedMousePrimaryKeratinocytes

To further investigate the mechanisms of Akt phosphorylation by TPA, primary mouse keratinocytes were isolated, grown to confluency, and then switched to medium lacking growth factors and serum for 24 h. Following starvation, cells were exposed to TPA (0.68 nmol/mL) in DMSO or EGF (40 ng/mL) for the indicated time points. Elevated phosphorylation of Akt at Ser473 was observed in response to TPA as early as 30 min after treatment and maximal phosphorylation occurred 2 to 4 h after the addition of TPA (Fig. 3A). EGF treatment of keratinocytes under these culture conditions also significantly induced Akt phosphorylation (Fig. 3A). The membranes were stripped and probed for \( \text{h-tubulin} \) as the loading control.

TPALeadstoActivationofAktviatheEGFRinCulturedKeratinocytes

As noted in the Introduction, previous studies from our laboratory have shown that activation of the EGFR is a common response in mouse epidermis following treatment with both phorbol ester and non–phorbol ester tumor promoters (21-23). Treatment with TPA leads to an elevation in tyrosine phosphorylation of both the EGFR and erbB2 and an increase in the formation of EGFR:erbB2 heterodimers (21, 22). Phosphorylation at Tyr1086, Tyr992 (Fig. 3B), and Tyr845 (data not shown) of the EGFR were seen as early as 15 min after TPA treatment. Furthermore, phosphorylation of erbB2 at Tyr877 was also increased by TPA treatment (Fig. 3C). Interestingly, phosphorylation of other EGFR tyrosine residues, notably Tyr1045, Tyr1068, and Tyr1173 of the EGFR, was not affected by TPA treatment at the observed time points under the experimental conditions used (data not shown).

Given the fact that TPA treatment of cultured primary mouse keratinocytes leads to activation of both Akt and EGFR, we examined the role of the EGFR in TPA-induced Akt activation (phosphorylation) using an EGFR/erbB2 inhibitor, GW2974, and an inhibitor specific for the EGFR, PD153035. Mouse primary keratinocytes were prepared as described above and incubated for 1 h with either 1 \( \mu \)mol/L GW2974 and an inhibitor specific for the EGFR, PD153035. Mouse primary keratinocytes were prepared as described above and incubated for 1 h with either 1 \( \mu \)mol/L GW2974 or a range of concentrations of GW2974 (0.1, 0.5, 1, or 5 \( \mu \)mol/L) or PD153035 (0.05, 0.1, 0.5, or 1 \( \mu \)mol/L). Treatment with inhibitors was followed by exposure to TPA for the indicated time points. As shown in Fig. 4A, TPA-induced Akt phosphorylation was significantly blocked by 1 \( \mu \)mol/L GW2974. Furthermore, both GW2974 and PD153035 inhibited TPA-induced Akt activation in a dose-dependent manner as shown in
Phosphorylation of tyrosine residues on erbB2/EGFR in response to TPA treatment was blocked by the erbB2/EGFR inhibitor GW2974 and also by the EGFR specific inhibitor PD153035 (again see Figs. 4B and 5C, respectively). TPA-induced tyrosine phosphorylation of erbB2 was also inhibited by GW2974 (data not shown). Collectively, inhibition of EGFR signaling whether by targeting the EGFR alone or targeting both the EGFR and erbB2 with a dual-specific inhibitor effectively reduced TPA-induced Akt phosphorylation as well as TPA-stimulated EGFR tyrosine phosphorylation.

**Inhibition of PKC Activity by Bisindolylmaleimide I Abrogates TPA-Induced Heparin-Binding EGF Production and EGFR Phosphorylation in Cultured Keratinocytes**

All of the EGFR ligands are synthesized as membrane-anchored precursors that can be proteolytically cleaved and released from the plasma membrane to generate bioactive molecules that bind EGFR (reviewed in ref. 31). Previous reports have shown that the processing of pro-EGF ligands is induced by a variety of stimuli, including TPA (32-34). As shown in Fig. 5A, TPA induced heparin-binding EGF (HB-EGF) production and increased tyrosine phosphorylation of the EGFR in cultured mouse keratinocytes. Figure 5A shows, consistent with previous reports (32, 34-36), that HB-EGF is synthesized as a 20- to 30-kDa heterogenous product of transmembrane precursor and stimulation with TPA leads to a cleavage processing as well as synthesis of the pro-HB-EGF.

Previous studies indicate that PKC mediates the effect of TPA on pro-EGFR ligand release (36, 37) and selective PKC inhibitors abrogate TPA-induced EGFR transactivation in PC-3 human prostate cancer cells (38). To examine whether inhibition of PKC affected TPA-induced HB-EGF expression and EGFR transactivation in mouse epidermal cells, primary keratinocytes were treated with TPA for the indicated time points in the presence or absence of 1 μmol/L bisindolylmaleimide I (BIM), which was added 1 h before or after TPA treatment. As shown in Fig. 5B, the pan PKC inhibitor BIM given before TPA blocked HB-EGF production as well as EGFR phosphorylation in mouse keratinocytes. Surprisingly, PKC inhibition by BIM before or after TPA treatment increased Akt phosphorylation as early as 15 min and prolonged Akt phosphorylation for at least 18 h (Fig. 5C), whereas EGFR activation was reduced by BIM (Fig. 5B). Several phosphatases have been shown to regulate Akt activity, including PP2A, which is a phosphoserine/phosphothreonine phosphatase that renders Akt inactive (39) and is induced by TPA in HeLa cells (40). Thus, PKC inhibition following TPA treatment may lead to inhibition of PP2A activity and increased phosphorylation of Akt even in the presence of reduced signaling through the EGFR. To verify the contribution of PP2A to Akt phosphorylation in keratinocytes treated with TPA and BIM, we did coimmunoprecipitation studies with Akt antibody using lysates from TPA-treated cells with or without BIM pretreatment. As shown in Fig. 5D, the catalytic subunit of PP2A coimmunoprecipitated with Akt and the physical interaction between Akt and PP2A catalytic subunit was induced by TPA. Indeed, inhibition of PKC by BIM led to a decreased association of Akt with PP2A catalytic subunit (Fig. 5D). These results suggest that PP2A regulation of Akt is PKC dependent in TPA-stimulated mouse keratinocyte cells. To further investigate this phenomenon and the role of the EGFR in Akt phosphorylation following TPA treatment, keratinocytes were pretreated with GW2974 or PD153035 and BIM for 1 h and incubated with TPA for either 15 min or 2 h. As clearly shown in Fig. 5E, Akt phosphorylation at both time points was completely blocked in cell lysates from both GW2974- and PD153035-treated cells, although PP2A was inactivated by BIM treatment. Thus, this result strongly suggests that signaling through the EGFR is the major pathway leading to increased Akt phosphorylation during tumor promotion with TPA in mouse skin.

**Effects of Downstream Kinase Inhibitors on Akt Activation by Diverse Tumor Promoters**

Using a panel of downstream kinase inhibitors, experiments were done to further establish possible downstream signaling pathways responsible for Akt activation by TPA. The three compounds selected for this study were LY294002 (a PI3K inhibitor), PD98059 (a mitogen-activated protein kinase inhibitor), and 7-hydroxystaurosporine (UCN-01), a derivative...
of the serine/threonine inhibitor staurosporine. Maximal activation of Akt requires phosphorylation at Thr308 and Ser473 (1, 30, 41). Phosphorylation of Akt at Thr 308 is catalyzed by PDK-1 (30, 42). UCN-01 was initially thought to be highly specific for the Ca2+- and phospholipid-dependent PKC isoforms (43). However, recent articles have reported that the PKC inhibitory actions of UCN-01 did not correlate with enhanced lethality and apoptosis (44), and instead, UCN-01 is also a potent inhibitor of PDK-1, which is directly upstream of Akt kinase (45, 46). As shown in Fig. 6A, the mitogen-activated protein kinase inhibitor PD98059 did not inhibit Akt activation by TPA at doses of either 1 or 5 nmol (i.e., no statistically significant decreases were observed). In contrast, by targeting either PI3K (LY294002) or PDK-1 (UCN-01), we were able to significantly modulate the activation of Akt by TPA at both doses used in vivo. Collectively, these results from experiments in vivo strengthen the argument for a PI3K-dependent and PDK-1–dependent mechanism of Akt activation by TPA, which is downstream of the EGFR.

In a second set of experiments, we examined the effect of these distinct signaling pathways on TPA-induced phosphorylation of Akt in cultured keratinocytes. For these experiments, primary keratinocytes were again starved for 24 h before treatment with TPA with or without pretreatment with LY294002, UCN-01, or PD98059 (Fig. 6B). Preincubation with either LY294002 (50 μmol/L) or UCN-01 (100 nmol/L) completely inhibited TPA-induced Akt phosphorylation. Pretreatment with UCN-01 at a concentration of 100 nmol/L before TPA had no effect on pan PKC phosphorylation at a COOH-terminal residue and PKC protein expression (data not shown). It has previously been shown that TPA leads to the activation of mitogen-activated protein kinase (47, 48); however, in our studies, PD98059 did not affect TPA-induced phosphorylation of Akt at Ser473 (Fig. 6B). These data further suggest that TPA-induced activation of Akt primarily is mediated via EGFR activation and subsequent PI3K-dependent and PDK-1–dependent pathways but not through mitogen-activated protein kinase pathways. These results were consistent with the experiments done with mouse epidermis in vivo (Fig. 6A).

Discussion

In this study, we provide the first evidence that diverse chemical skin tumor promoters rapidly activate epidermal Akt following a single topical application to mouse skin. In addition, at least three downstream effectors of Akt (mTOR,
GSK-3β, and Bad) were shown to be hyperphosphorylated in tumor promoter–treated epidermis. These data indicate that activation of Akt during tumor promoter treatment leads to modulation of downstream signaling components that may contribute to the tumor promotion process. Recent studies from our laboratory showed that activation of PI3K/Akt signaling, including downstream cell cycle targets of this signaling pathway, contributed to IGF-1–mediated skin tumor promotion in BK5.IGF-1 transgenic mice (9). Inhibition of PI3K/Akt signaling by LY294002 inhibited IGF-I–mediated skin tumor promotion and reduced levels of specific cell cycle proteins found to be elevated in epidermis of BK5.IGF-1 transgenic mice. Given the similarity between TPA-induced cell cycle changes and those observed in epidermis of BK5.IGF-1 transgenic mice, we hypothesized that activation of Akt may be a common event underlying, at least in part, the ability of diverse tumor-promoting agents to stimulate epidermal cell proliferation and skin tumor formation.

Segrelles et al. (11) reported that Akt activity increased during the process of two-stage carcinogenesis. In this regard, activity of Akt was examined in hyperplastic mouse skin resulting from treatment with TPA (multiple treatments) as well as early-stage, mid-stage, and late-stage papillomas and squamous cell carcinomas. This study reported slight activation of Akt in hyperplastic skin following multiple applications of TPA, whereas we observed significant elevations of epidermal Akt activity after only a single application of several types of tumor promoters. Notably, Segrelles et al. used whole skin in their analyses of Akt activity, which may not have accurately reflected Akt activation in the epidermal compartment. Our current data clearly show that Akt activation is an early and reproducible event in mouse epidermis following treatment with diverse skin tumor-promoting agents. The localization of this increased activity in the basal cell compartment suggests that it may play a role either in the proliferation or survival of keratinocytes during the early stages of skin tumor promotion.
Akt is activated downstream of PI3K via signaling through a variety of cell surface receptors, including the EGFR (49, 50). Previously, we reported that treatment with TPA as well as okadaic acid and chrysarobin up-regulated ligands for the EGFR and induced phosphorylation of the EGFR, suggesting that EGFR activation is a common event following tumor promoter treatment (18-23). In addition, we showed that TPA activated both the EGFR and erbB2 and that downstream signaling pathways (i.e., signal transducers and activators of transcription 3 and src) are activated and contribute to skin tumor promotion (22, 23, 51). Therefore, we hypothesized that Akt activation following tumor promoter treatment also could result from activation of the EGFR. In the current study, treatment of cultured keratinocytes with TPA led to phosphorylation of the EGFR, erbB2, and Akt. TPA-induced phosphorylation of Akt was effectively blocked by a potent inhibitor of the EGFR (PD153035) and also by bisindolylmaleimide, a pan PKC inhibitor. PD153035 inhibits the EGFR with an IC50 of 29.5 pmol/L and also inhibits erbB2 with an IC50 of 2.3 pmol/L (52). As shown in Fig. 4C, the highest concentration of PD153035 used in mouse primary keratinocytes was 1 µmol/L, which significantly inhibited TPA-induced Akt activation as well as TPA-stimulated EGFR tyrosine phosphorylation. These data suggest that EGFR/EGFR homodimers and/or EGFR/erbB2 heterodimers may be the primary mediator of TPA-induced Akt activation in mouse keratinocytes. Previously, our laboratory reported that EGFR, erbB2, and erbB3, but not erbB4, were expressed in cultured keratinocytes and in mouse epidermis (22). In addition, exposure of primary mouse keratinocytes to EGF led to phosphorylation of both the EGFR and erbB2 but not erbB3 (22). These data support the above hypothesis. Interestingly, erbB2 does not contain a PI3K-binding site nor a docking protein Gab1 site, whereas EGFR has a docking site for Gab1 and a PI3K-binding motif (53). Furthermore, erbB3 contains six binding sites for PI3K (53). Because erbB3 lacks intrinsic tyrosine kinase activity, signal transduction through erbB3 depends on heterodimer formation with other erbB family members (54). Emkey and Kahn (55) reported that phorbol 12-myristate 13-acetate induced tyrosine phosphorylation (~4-fold) of both erbB2 and erbB3 (without increase in erbB2/erbB3 heterodimer formation), but not EGFR, in a PKC-dependent manner in a Fao rat hepatoma cell line. Although, given our current and previous data, it seems less likely that erbB3 plays a role in Akt phosphorylation by TPA in mouse keratinocytes, this cannot be ruled out completely without further experimentation. The fact that both okadaic acid and chrysarobin treatment lead to up-regulation of EGFR ligands and activation of the EGFR in epidermis (18-21), as noted above, suggests that this mechanism may also apply to activation of Akt by these other tumor promoters. Preliminary experiments indicate that treatment of cultured mouse keratinocytes with okadaic acid and chrysarobin leads to activation of Akt, which is blocked by GW2974.

As shown in Fig. 5A, TPA stimulated production of HB-EGF in primary mouse keratinocytes with increases seen as early as 30 min. Multiple EGFR ligands, including HB-EGF, are coordinately up-regulated following TPA treatment of mouse epidermis (18). Activation of Akt occurred after activation of the EGFR and erbB2 as shown in Fig. 3. Akt activation could be seen as early as 30 min after TPA treatment. Furthermore, TPA-induced HB-EGF production and EGFR transactivation were blocked by the pan PKC inhibitor BIM. PKC activation by TPA is known to lead to up-regulation of...
transforming growth factor-α and other EGFR ligands through increased mRNA production and subsequent new protein synthesis (18-20, 24). However, the very early activation of the EGFR may occur through a process of proteolytic cleavage of existing membrane-bound forms of transforming growth factor-α and HB-EGF (31, 56). This process referred to as “ectodomain shedding” is stimulated by TPA and also involves activation of PKC (33). For example, the cleavage of pro–transforming growth factor-α in cells stimulated by TPA is a very rapid process with a half-life of ~5 min (37) and the released transforming growth factor-α is rapidly consumed by EGFR (31). In addition, HB-EGF shedding induced by TPA occurs with a very rapid time course (32). Because PKC activation is involved in both increased synthesis and ectodomain shedding of EGFR ligands, the inhibition of EGFR activation by TPA at all time points by BIM is consistent with both processes being involved.

Notably, inhibition of PKC by BIM did not block TPA activation of Akt as shown in Fig. 5C. Recently, it has been shown that TPA treatment of different cell types activates the serine/threonine phosphatase PP2A, leading to dephosphorylation of Akt (40, 57). This activation of PP2A also occurs through activation of PKC. In addition, a recent report showed that treatment of primary keratinocytes from newborn mice with TPA also led to activation of a phosphatase, most likely PP2A (58). In this latter study, TPA did not induce phosphorylation of Akt but only early time points were examined. As shown in Figs. 4 and 5C of our study, we observed increased Akt phosphorylation following TPA treatment at times ≥30 min. Thus, differences in time points examined, use of keratinocytes from adult mice, as well as other differences in experimental conditions may have influenced results obtained in our current study. Nevertheless, our results in cultured keratinocytes match the results obtained from mouse epidermis following TPA treatment in vivo. As shown in Fig. 5D, we found that TPA treatment of cultured keratinocytes led to increased association of PP2A with Akt and that BIM pretreatment significantly inhibited this association. Thus, inhibition of TPA-induced PKC activation by BIM seems to reduce activation of PP2A and thus lead to increased phosphorylation of Akt even in the presence of reduced signaling through the EGFR and erbB2 (Fig. 5B). To explore this further, we examined Akt phosphorylation in the presence of both BIM and PD153035 or GW2974. The lack of Akt phosphorylation following TPA treatment when both the EGFR and PKC were blocked (Fig. 5E) strongly suggests that activation of PKC plays a dual role in regulating Akt phosphorylation.

Another interesting observation from the current study is that TPA increased tyrosine phosphorylation of EGFR at residues 845, 992, and 1086 and of erbB2 at residue 877 in cultured mouse keratinocytes (Fig. 4B and C, respectively), whereas phosphorylation of other tyrosine residues (1045, 1173, and 1068 of EGFR) was relatively unaffected by TPA treatment during the observed time period. There are emerging data indicating that differential phosphorylation of EGFR may be functionally related and cell specific (59, 60). For example, Li et al. (60) showed differential requirements of EGFR phosphorylation and tyrosine kinase activity with respect to activator protein-1 transactivation when induced by EGF versus TPA. These investigators showed that EGFR autophosphorylation at Tyr1173 is not required for TPA-induced activation of extracellular signal-regulated kinase and activator protein-1. Furthermore, Amos et al. (59) showed that treatment of glioblastoma cell lines with TPA induced EGFR phosphorylation only at Tyr1068. However, we found in the current study that TPA elevated the phosphorylation of EGFR at tyrosine residues 845, 992, and 1086, but not 1068, in primary mouse keratinocytes. These differences may be due to the different cell types in which the studies were done (malignant glioblastoma cell lines versus normal primary keratinocytes) and/or other experimental differences. Interestingly, in other cell types, EGF-induced activation of the PI3K/Akt pathway has been associated with phosphorylation of tyrosine sites 1086 and 1068 of the EGFR mediated by the docking protein Gab1 (61, 62). The current data suggest a specific pattern of tyrosine phosphorylation of the EGFR in mouse keratinocytes following TPA treatment. Further work will be necessary to determine the significance of these observations and its effect on specific downstream signaling pathways.

In conclusion, our current data suggest that activation of epidermal Akt is a common early event following topical application of tumor promoters to mouse skin. The earlier finding that inhibition of PI3K in BK5.IGF-I transgenic mice inhibited IGF-I–mediated skin tumor promotion as shown in our previous work (9), together with the data presented in the current study, suggests that activation of Akt plays an important role in skin tumor promotion by diverse tumor promoters. Furthermore, we found that TPA treatment leads to activation of Akt, at least in part, via activation of the EGFR (and erbB2) in cultured keratinocytes. The activation of the EGFR (and erbB2) by TPA in cultured keratinocytes occurs via activation of PKC through up-regulation of EGFR ligands (likely by both ectodomain shedding as well as increased synthesis). TPA treatment of mouse primary keratinocytes led to activation of PP2A through activation of PKC. However, this activation of PP2A did not prevent subsequent activation of Akt through the EGFR/erbB2 axis. Collectively, these results support the hypothesis that elevated Akt activity and subsequent activation of downstream signaling pathways contribute significantly to the tumor-promoting action of diverse tumor-promoting agents. Furthermore, signaling through the EGFR (through EGFR/EGFR and/or EGFR/erbB2 dimers) may be the primary event leading to Akt activation during tumor promotion in mouse skin. Targeting this pathway may be an effective strategy for cancer prevention.

Materials and Methods

Chemicals and Reagents

TPA, PD98059, okadaic acid, and LY294002 were obtained from Alexis Corp. Chrysarobin was purchased from ICN Pharmaceuticals, Inc. GW2974, PDD, 4e-PDD, proteinase inhibitor cocktails, and phosphatase inhibitor cocktails 1 and 2 were purchased from Sigma Chemical Co. PD153035 and BIM were purchased from Calbiochem. UCN-01 was generously provided by D. Johnson (University of Texas M. D. Anderson Cancer Center, Smithville, TX). The following were purchased from Cell Signaling Technology, Inc.: phospho-specific antibody directed against the EGFR at Tyr992, anti-EGFR antibody,
anti-phospho-erbB2 (Tyr877), phospho-specific antibody against dual-phosphorylated extracellular signal-regulated kinase 1/2, anti-phospho-Akt (Ser473) and anti-Akt antibodies, anti-PP2A catalytic subunit, anti-phospho-Bad (Ser155) and anti-Bad antibodies, anti-phospho-mTOR (Ser2448) and anti-mTOR antibodies, and anti-phospho-GSK-3β (Ser9). The phospho-specific antibody against the EGFR at Tyr1066 was purchased from Abcam, Inc. Anti-HB-EGF (H-88) and protein A/G PLUS-agarose beads were purchased from Santa Cruz Biotechnology. Anti-β-tubulin and anti-GSK-3β antibodies were obtained from Sigma Chemical and BD Transduction Laboratories, respectively. The anti-rabbit IgG horseradish peroxidase–conjugated secondary antibody was purchased from Amersham Biosciences. Enhanced chemiluminescence detection kits were purchased from Amersham Biosciences.

**Animals and Treatments**

The dorsal skin of female ICR mice in the resting stage of the hair cycle (7–9 weeks of age) was shaved 48 h before treatment. From three to five animals were used per group for each experiment. The mice then received a single topical application of TPA (5 or 6.8 nmol), okadaic acid (5 or 10 nmol), chrysarobin (220 or 440 nmol), or acetone (0.2 mL) depending on the experiment. To determine possible mechanisms involved in Akt activation by these diverse tumor promoters, dorsal skin was treated with either 1 or 5 nmol of inhibitors (LY294002, PD98059, or UCN-01) in 0.2 mL acetone, where indicated, to inhibit Akt and dual-phosphorylated extracellular signal-regulated kinase 1/2, respectively. The anti-rabbit IgG horseradish peroxidase–conjugated secondary antibody was purchased from Amersham Biosciences. Enhanced chemiluminescence detection kits were purchased from Amersham Biosciences.

**Preparation of Epidermal Lysates**

For whole-cell extracts, the dorsal skin was excised and the epidermis was scraped directly into radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 1% NP40, 0.25% sodium deoxycholate, proteinase inhibitor cocktail, phosphatase inhibitor cocktails 1 and 2]. After centrifugation at 10,000 rpm for 30 min at 4°C, the supernatants were either used immediately for Western analysis or snap frozen in liquid nitrogen and stored at −80°C until analysis.

**Cell Culture**

Epidermal basal cells were isolated from female ICR mice, 6 to 8 weeks of age, and cultured in MEM-2 medium containing 1% fetal bovine serum and a Cu2+ concentration of 0.04 mmol/L as described previously (63). Cells were plated at a density of 1 × 10⁷ per 100-mm tissue culture dish. Cells were grown to confluence and then starved for 24 h in 1% bovine serum albumin/MEM-2 in the absence of all growth factors. After two washes with the starvation medium, cells were stimulated with 40 ng/mL EGF or 0.68 mmol/mL TPA for 15 min to 4 h and lysed with ice-cold lysis buffer [1% Triton X-100, 10% glycerol, 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 2.5 mmol/L EGTA, proteinase inhibitor cocktail, phosphatase inhibitor cocktails 1 and 2]. In studies involving the pharmacologic inhibitors, the inhibitors were added to the cells for 1 h before TPA treatment.

**Immunoprecipitation and Western Blot Analysis**

To immunoprecipitate Akt, 200 μg of whole-cell lysate were incubated with 4 μL of rabbit anti-Akt polyclonal antibody at 4°C overnight with gentle agitation. Protein A/G-agarose (20 μL) was then added, and the sample was kept at 4°C for 4 h with gentle agitation. The pellet was collected by centrifugation and washed five times with cold cell lysis buffer at 4°C, solubilized in 20 μL of SDS sample loading buffer, and boiled for 5 min. The immunoprecipitates were resolved by SDS-PAGE (10%) and analyzed by Western blotting. For Western blot analysis, whole-cell lysates were resolved on 4% to 20% SDS-PAGE gels and then transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked for 1 h in 5% bovine serum albumin in TTBS [20 mmol/L Tris-HCl, 136 mmol/L NaCl, 0.1% Tween 20 (pH 7.6)] and incubated overnight at 4°C in appropriate primary antibodies. After incubation, the membranes were subjected to three washes, 10 min each, in TTBS before incubation with secondary antibody for 1 h at room temperature. Unbound secondary antibody was removed by three successive washes of 10 min each. Protein bands were visualized using chemiluminescence detection (Amersham Biosciences).

**Akt Kinase Assay**

Kinase assays for Akt were done according to the manufacturer’s instructions (Upstate Biotechnology). Briefly, 750 μg of epidermal protein were added to an Akt immunocomplex and rotated for 90 min at 4°C. After washing and centrifugation, assay buffer, protein kinase A inhibitor, Akt substrate, and [γ-32P]ATP were added to the enzyme immunocomplex and allowed to incubate for 10 min at 30°C with continuous shaking. The reaction was stopped with 40% trichloroacetic acid. After centrifugation, the supernatant was spotted onto P81 phosphocellulose paper and washed in 0.75% phosphoric acid. Assay squares were then placed into a vial containing scintillation fluid and assayed for radioactivity in a scintillation counter.

**Immunohistochemistry**

Tissues were fixed in 10% buffered formalin and embedded in paraffin. After deparaffinization, the slides were microwaved twice for 5 min each time in 0.1% NP40 in 0.1 mol/L sodium citrate buffer (pH 7.2). After blocking the nonspecific Fc receptor in tissue, the slides were incubated with a 1:50 dilution of the primary antibody, phospho-Akt (Ser473), overnight at 4°C. After three washes with PBS containing 1% bovine serum albumin, the sections were incubated with secondary FITC-conjugated F(ab')2 fragments of donkey anti-rabbit IgG antibody for 40 min.

**Statistical Analyses**

Experiments were conducted in triplicate. Means ± SE were calculated where applicable. To compare differences among treatment groups of mice, ANOVA was used. A P value of ≤0.05 was considered statistically significant.
References


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Jerry Lu, Okkyung Rho, Erik Wilker, et al.


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