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Epiregulin is More Potent Than EGF or TGFα in Promoting In Vitro Wound Closure due to Enhanced ERK/MAPK Activation

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Abstract Epiregulin (EPR) is a broad specificity EGF family member that activates ErbB1 and ErbB4 homodimers and all possible heterodimeric ErbB complexes. We have previously shown that topical EPR enhances the repair of murine excisional wounds. The purpose of this study was to determine whether EPR was more effective than EGF or TGFα in promoting in vitro wound closure and to compare the EPR induced signal transduction pathways with those activated by EGF and TGFα. Normal human epidermal keratinocytes or A431 cells were scratch wounded and treated for 24 h with varying doses of EPR, EGF or TGFα. Five-fold lower doses of EPR were significantly better than EGF or TGFα in stimulating in vitro wound closure. Mitomycin-c reduced EPR induced wound closure by 59%, versus a 9% and 25% decrease in EGF and TGFα induced closure. The ERK/MAPK inhibitor PD-98059 decreased EPR induced wound closure by 88%. By contrast, the PLC inhibitor U-73122, only reduced the EPR induced response by 21%. Immunoblot analysis revealed that 2 nM EPR stimulated a six-fold increase in p-ERK1/2, whereas 10 nM EGF or TGFα stimulated only a 3- and 2.5-fold increase in p-ERK1/2. When compared with EGF or TGFα, EPR is a more potent and more effective inducer of in vitro wound closure due to its ability to promote significantly greater ERK/MAPK activation. J. Cell. Biochem. 89: 1126–1137, 2003.

Key words: signal transduction; phosphorylation; cell proliferation; migration; wound healing

The ever expanding epidermal growth factor family of growth factors currently consists of at least seven different hormones: Epidermal growth factor (EGF) [Cohen and Elliot, 1963], transforming growth factor alpha (TGFα) [Derynck et al., 1984], amphiregulin (AR) [Shoyab et al., 1989], heparin-binding epidermal growth factor-like factor (HB-EGF) [Higashiyama et al., 1991], betacellulin (BTC) [Shing et al., 1993], epiregulin (EPR) [Toyoda et al., 1995], and the recently identified epigen [Strachan et al., 2001]. In addition to these growth factors, a more distantly related family of EGF-like peptides known as the neuregulins

Abbreviations used: EPR, epiregulin; EGF, epidermal growth factor; TGFα, transforming growth factor-alpha; AR, amphiregulin; HB-EGF, heparin-binding epidermal growth factor-like factor; BTC, betacellulin; EGFR, epidermal growth factor receptor; MAPK, mitogen activated protein kinase; MEK, mitogen activated protein kinase kinase; PLC, Phospholipase C; PI3K, phosphatidyl-inositol 3-kinase; PKC, protein kinase C; JAK/STAT, Janus kinase/signal transducer and activator of transcription; DPBS, Dulbecco's phosphate buffered saline; FBS, fetal bovine serum; DMEM/F12, Dulbecco's modified Eagle medium: Ham's F-12 nutrient mixture; TBST, tris buffered saline 0.05% Tween-20; p-ERK1/2, phosphorylated ERK1/2; p-PLCγ1, phosphorylated PLCγ1, Cyp-40, cyclophilin 40.

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can interact with certain members of the EGF receptor family [Burden and Yarden, 1997]. All members of the EGF family are synthesized as membrane-anchored precursors, which are proteolytically cleaved into the bioactive mature soluble forms that act as paracrine growth factors. Transmembrane forms of these growth factors are also capable of stimulating the growth of adjacent cells in a juxtacrine manner [Brachmann et al., 1989; Wong et al., 1989; Higashiyama et al., 1991; Goishi et al., 1995; Inui et al., 1997]. In addition to a transmembrane domain, all members of the EGF family share a common domain known as the EGF motif. This motif encompasses three of the six conserved cysteine residues and contains additional residues important for tertiary structure stabilization and receptor binding [Groenen et al., 1994].

The mammalian EGF receptor family of receptor tyrosine kinases is composed of four structurally similar members [Wells, 1999]: ErbB1, also referred to as EGFR or Her1 [Ullrich et al., 1984], ErbB2 (Neu/Her2) [Semba et al., 1985], ErbB3 (Her3) [Kraus et al., 1989] and ErbB4 (Her4) [Plowman et al., 1993]. The ligands of the EGF family may be divided into groups based on their receptor affinities. EGF, TGFα, AR, HB-EGF, BTC, EPR and epigen all bind ErbB1. HB-EGF, BTC and EPR also bind ErbB4 [Riese et al., 1996; Elenius et al., 1997; Komurasaki et al., 1997; Strachan et al., 2001]. EPR is unique among the EGF-like peptides because it not only stimulates both ErbB1 and ErbB4 homodimers, but activates all possible ErbB complexes as well [Shelly et al., 1998].

As with many other receptor tyrosine kinases, EGFR dependent signal transduction is initiated by ligand binding, which alters the cell surface to produce receptor dimers. Dimerization activates intrinsic receptor tyrosine kinase activity resulting in transphosphorylation of multiple tyrosine residues in the cytoplasmic domain [Yarden and Schlessinger, 1987]. Subsequent phosphorylation events lead to recruitment of multiple proteins bearing phosphotyrosine binding (PTB) domains. The concerted action of these downstream effector proteins serves to regulate many cellular functions, including cell migration, proliferation, differentiation and apoptosis [Wells, 1999; Carpenter, 2000]. In the case of EGFR however, multiple ligands and the capacity of family members to homo- and hetero-dimerize make receptor binding and dimerization a complex regulatory step in the signaling pathway. Although many ErbB receptors activate similar signaling pathways, signal diversification results from differential utilization of downstream pathways by variable ErbB receptor combinations [Olayioye et al., 1999] as well as different ErbB ligands acting through a given receptor [Sweeney et al., 2000].

Cutaneous wound healing is a complex, dynamic process involving a number of precisely coordinated events including inflammation, cell migration, cell proliferation, angiogenesis, matrix deposition and remodeling [Martin, 1997; Singer and Clark, 1999]. Particularly critical to reepithelialization of wounds is the induction of cell proliferation and migration by the coordinate release of growth factors at the site of injury. EGF and TGFα have been shown to play important roles in promoting reepithelialization [Brown et al., 1986; Barrandon and Green, 1987; Schultz et al., 1987; Brown et al., 1989]. We recently demonstrated that daily topical application of EPR significantly accelerates the repair of full thickness murine excisional wounds as compared to EGF or vehicle [Draper et al., 2003]. Similar to in vivo wound repair, in vitro wound closure results from both migratory and proliferative activities. Since EPR is a potent pan-ErbB ligand, we hypothesized that EPR would promote greater in vitro wound closure than its well characterized relatives EGF and TGFα. We also investigated the EGFR signal transduction pathways utilized in the EPR induced response. Here we report that EPR is both more potent and more effective than EGF or TFGα in promoting in vitro wound closure. Furthermore, we provide evidence that increased mitogenesis secondary to enhanced ERK/MAPK signaling is responsible for the superior EPR induced wound closure response.

MATERIALS AND METHODS

Cell Culture

A431 Cells [Kawamoto et al., 1983] or normal human epidermal keratinocytes (NHEK) were maintained at 37°C, 5% CO_2. A431 cells were cultured in 1:1 Dulbecco's modified Eagle medium: Ham's F-12 nutrient mixture (DMEM/F12; Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), 10,000 U/ml penicillin and 10 mg/ml streptomycin (P/S, Sigma,

St. Louis, MO). NHEK were cultured in keratinocyte growth medium (KGM; BioWhittaker, Walkersville, MD) containing 0.1 μg/ml hEGF, 5.0 mg/ml insulin, 0.5 mg/ml hydrocortisone, 50 mg/ml gentamicin, 50 μg/ml amphotericin-b and 7.5 mg/ml bovine pituitary extract (BPE). Human recombinant EGF was purchased from Upstate Biotechnology (Lake Placid, NY). Human recombinant TGFα was purchased from PeproTech, Inc. (Rocky Hill, NJ). Human recombinant EPR [Riese et al., 1998] was a very generous gift of Dr. Toshi Komurasaki (Taisho Pharmaceutical Co. Ltd., Saitama, Japan). Mitomycin-c was purchased from Sigma. In all experiments involving the addition of growth factors, A431 cells were washed free of serum containing media and placed in serum free DMEM/F12 for 24 h before growth factor addition. Likewise, NHEK were washed free of KGM and placed in growth factor and serum free keratinocyte basal medium (KBM, BioWhittaker) containing only 50 mg/ml gentamicin and 50 μg/ml amphotericin-b.

Determination of In Vitro Wound Closure

A431 cells or NHEK were subcultured and maintained in their respective growth media in Nunc Lab-Tek II, single well chamber slides coated with 10 μg/ml fibronectin (Invitrogen). Following 24 h of serum deprivation, confluent monolayers were scratched with a p1000 blue pipette tip in a standardized manner to create uniform cell free wounds in the culture monolayers as described by Cha et al. [1996]. The monolayers were washed vigorously 3× with Dulbecco's phosphate buffered saline (DPBS) to remove cellular debris. Wounded monolayers were then incubated for either 24 h in serum free media alone as a control, or with the addition of varying doses of EPR, EGF or TGFα. To conclude the experiment, monolayers were washed 2× in DPBS, fixed for 5 min in 4% paraformaldehyde and stained with hematoxylin and eosin. Wounded monolayers were photographed at standard magnification and the degree of in vitro wound closure measured using Zeiss Image Scientific Image Analysis Software (Carl Zeiss, Inc., Thornwood, NY). In vitro wound closure was expressed in square millimeter and represents the inverse of the total area under magnification remaining uncovered by cells. Wound closure results from proliferative as well as migratory activity. To inhibit cell proliferation, parallel experiments were conducted with the addition of 10 μg/ml of mitomycin-c throughout the duration of the experiment. Cell migration was assessed by direct measurement, while proliferative activity was determined indirectly by subtractive analysis.

Pharmacologic Inhibitor Studies

Detection of EPR induced, ErbB signal cascade activation was performed by adding cell permeable, biochemical inhibitors of specific signal transduction molecules to our in vitro wounding assays. The following inhibitors were purchased from Biomol Research Laboratories (Plymouth Meeting, PA): genistein, an inhibitor of protein tyrosine kinases including EGFR autophosphorylation; PD-98059, an inhibitor of MEK (MAP kinase kinase); wortmannin, a potent and selective inhibitor of phosphatidylinositol 3-kinase (PI3K); U-73122, an inhibitor of agonist induced phospholipase C (PLC) activation; and hypericin, a selective inhibitor of protein kinase C (PKC).

Immunoblot Analysis

Sub-confluent NHEK or A431 cells grown in 100 mm culture dishes were serum starved for 24 h, rinsed 2× with DPBS and incubated at 37°C for 0 to 120 min in their respective serum free media containing either 2 nM EPR, 10 nM EGF or 10 nM TGFα. The growth factor concentrations used were the same as those shown to be optimal in our in vitro wound closure assays. At selected time points, the cells were rinsed 2× with ice cold Ca^{++} and Mg^{++} free DPBS and lysed with ice cold lysate buffer containing 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA pH 8.0, 0.2 mM $NaVO_3$ and 0.2 mM PMSF. The cells were incubated in lysate buffer at 4°C for 30 min with constant agitation, scraped from the culture dish with a sterile rubber policeman and passed several times through a 26 gauge needle to disperse any large aggregates. The lysates were subjected to centrifugation for 15 min at 16,000*g*, 4°C. The collected supernatants represented the native total cell lysates. The lysate total protein concentration was determined using the Micro BCA Protein Assay (Pierce, Rockford, IL). Samples containing equal protein amounts were mixed with Laemmli electrophoresis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol), boiled for 5 min

and electrophoresed through an 8% sodium dodecyl sulfate-polyacrylamide gel. Protein was transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ) and blocked with buffer containing 1% bovine serum albumin, 10 mM Tris-HCl pH 7.5, 100 mM NaCl and 0.1% Tween-20 for 1 h at room temperature (RT). Membranes were incubated at RT for 1 h with 0.5 μg/ml of mouse monoclonal antibody to phosphorylated ERK1/2 (p-ERK1/2) or goat polyclonal antibody to phosphorylated PLCγ1 (p-PLCγ1; Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer then washed 4× for 5 min each in tris-buffered saline, 0.05% Tween-20 (TBST). Thereafter, the membranes were incubated for 1 h at RT with a 1:1000 dilution of anti-mouse IgG-horseradish peroxidase conjugate (IgG-HRP) or anti-goat IgG-HRP (Promega, Madison WI) in blocking buffer and washed 4× for 5 min each in TBST. Detection of the reactive bands was performed using SuperSignal West Pico chemiluminescent substrate (Pierce) and the membranes were exposed to Biomax film (Eastman Kodak, Rochester, NY). In order to normalize the data, as well as document equal protein loading and transfer, membranes were stripped and reprobed in a similar fashion with a polyclonal antibody against cyclophilin-40 (Cyp-40; Affinity Bioreagents, Inc., Golden, CO). The developed autoradiograms were analyzed with Kodak Digital Science 1D Image Analysis software (Eastman Kodak). The signal intensity of the immunoblot band in question was normalized for each sample with respect to the intensity of the corresponding Cyp-40 signal.

Statistical Analysis

The results were expressed as mean ± SEM. The significance of the differences between groups was determined by ANOVA or Student's *t*-test, as appropriate.

RESULTS

EPR is a More Potent Inducer of In Vitro Wound Closure Than EGF or TGFα

To establish an EPR dose response curve for induction of in vitro wound closure in NHEK, we treated growth factor starved, scratch wounded monolayers with EPR in concentrations ranging from 0 to 40 nM. EPR induced maximum wound closure at a concentration of 0.2 nM ($P < 0.001$). We observed inhibition of wound closure at doses of 2 to 40 nm. When compared to EGF, 0.2 nM EPR was as efficient as 10 nM EGF in promoting in vitro wound closure in NHEK (Fig. 1).

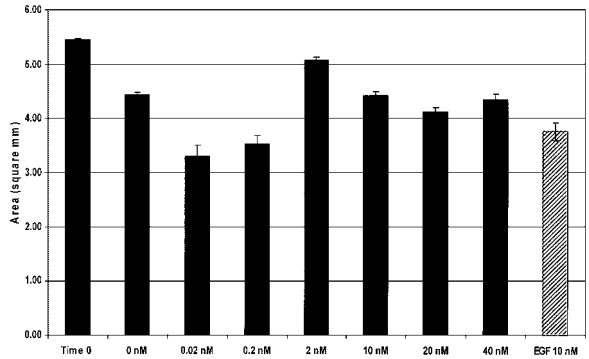

Fig. 1. Dose response of EPR induced in vitro wound closure in NHEK. Serum and growth factor starved, confluent NHEK were scratched wounded, washed and incubated for 72 h in serum and growth factor free media alone, or with the addition of varying doses of EPR, or 10 nM EGF. Following incubation, cell monolayers were fixed, stained and photographed. Measured in vitro wound closure was expressed in square millimeter and represents the inverse of the total area under magnification remaining uncovered by cells. The time 0 control represents the size of the wounded area prior to incubation. The data presented are the mean ± SEM, n = 6. All dose response data were significant ($P < 0.0005$) as determined by ANOVA.

In an attempt to observe and quantify greater differences of in vitro wound closure between EPR and other EGF family members, we turned to the A431 cell line as a model of EGFR overexpression. The A431 epidermoid carcinoma line [Kawamoto et al., 1983] expresses approximately 100-fold more EGFR on its surface than NHEK. As a result of EGFR overexpression, growth factor treated A431 cells proliferate and migrate in a more dynamic fashion than NHEK, thus rendering them considerably more useful in the in vitro wound closure model.

Comparative dose response curves for A431 cells were determined using EPR, EGF or TGFα in concentrations ranging from 0 to 40 nM. In EPR treated cells, a dose dependant increase in in vitro wound closure was observed over a concentration from 0 to 4 nM, with optimal closure at 2–4 nM (Fig. 2). Addition of EPR in concentrations of 10 nM or greater resulted in dose dependent inhibition of wound closure. At 40 nM, the degree of inhibition approximated the lack of closure observed in untreated controls. EGF induced progressive dose related increases in A431 in vitro wound closure with concentrations ranging from 0 to 10 nM, with

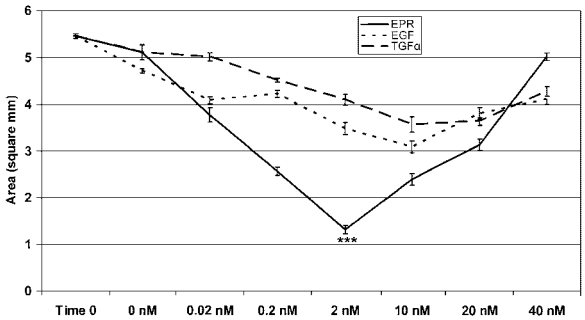

Fig. 2. Effects of EPR, EGF, and TGFα in A431 cell in vitro wound closure. Serum starved, confluent A431 cells were scratched wounded, washed and incubated for 24 h in serum free media alone, or with the addition of varying doses of EPR, EGF or TGFα Following incubation, cell monolayers were fixed, stained and photographed. Measured in vitro wound closure was expressed in square millimeter and represents the inverse of the total area under magnification remaining uncovered by cells. The time 0 control represents the size of the wounded area prior to incubation. The data presented are the mean ± SEM, n = 6. Significant differences (***$P < 0.0005$) from the corresponding untreated control (0 nM, no addition of growth factor), were determined using ANOVA.

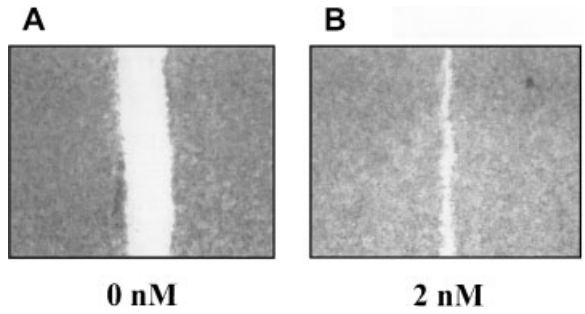

Fig. 3. In vitro wounding assay at 24 h. Serum starved, scratch wounded, A431 monolayers were treated over 24 h with either (**A**) serum free media or (**B**) 2 nM EPR as described in ''Materials and Methods.'' B: EPR treated monolayer shows considerable in vitro wound closure as compared to control monolayer A.

the maximal response seen at 10 nM. EFG dose dependent inhibition of wound closure was noted at concentrations of 20 nM and higher (Fig. 2). TGFα dose effects were very similar to those of EGF. TGFα induced progressive dose related wound closure from 0 to 10 nM. Similar to EGF, the optimal dose of TGFα was 10 nM, with dose dependent inhibition of closure was seen at concentrations of 20 nM or higher (Fig. 2).

Comparative analysis of the three closely related ligands revealed that EPR was both more potent and more effective at inducing in vitro wound closure than EGF or TGFα. The optimal dose of EPR (2 nM) was five-fold less than the ideal 10 nM doses of both EGF and TGFα. In addition, 2 nM EPR was 25% ($P < 0.0005$) more effective than 10 nM EGF, and 35% ($P < 0.0005$) more effective than 10 nM TGFα in promoting in vitro wound closure. There is minimal and non-significant wound closure activity in the untreated controls (Fig. 2). A representative comparison of A431 cells treated with 2 nM EPR versus untreated controls is shown in Figure 3.

Increased Mitogenesis is Responsible for the Enhanced In Vitro Wound Closure Caused by EPR

In vitro wound closure results from both proliferative and migratory activities. To investigate the relative contributions of proliferation and cell migration to in vitro wound closure, experiments were performed using the established optimal doses of EPR, EGF, and TGFα in the presence or absence of mitomycin-c, an inhibitor of cell proliferation. Addition of mitomycin-c reduced the amount of EPR induced in vitro wound closure by 59%, suggesting that proliferation contributes slightly more to the EPR induced response than cell migration. The extent of EPR induced migration is nevertheless noteworthy, accounting for approximately one third of the total EPR induced wound closure and nearly approximating the combined proliferative and motogenic responses engendered by EGF and TGFα. By contrast, addition of mitomycin-c to wounded A431 monolayers treated with EGF or TGFα resulted in a non-significant 9% and 25% decrease in the total in vitro wound closure respectively. These data suggest that cell proliferation plays a lesser role in the promotion of in vitro wound closure by these ligands (Fig. 4).

EPR Induced In Vitro Wound Closure is Mediated Primarily by Activation of ERK/MAPK Signaling

Activation of ErbB receptors has been shown to trigger numerous downstream signaling pathways including ras/MAPK, JAK/STAT, PLCγ , and others [Wells, 1999]. In addition to multiple ErbB signaling cascades, cross talk between signal transduction pathways has made it difficult to ascribe biological responses to specific signaling pathways and events. In an effort to elucidate the downstream signaling pathways associated with EPR induced wound closure, we performed in vitro wounding assays

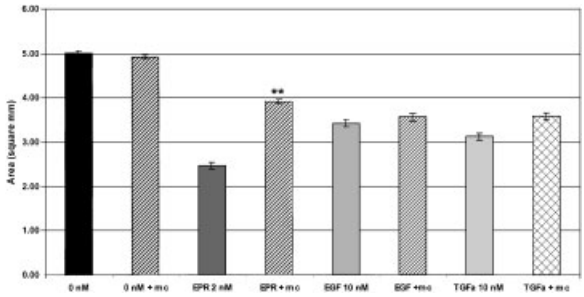

Fig. 4. Effects of mitomycin-c on EPR, EGF or TGFα induced A431 cell in vitro wound closure. Scratch wounded A431 cell monolayers were treated for 24 h with either serum free media alone, 2 nM EPR, 10 nM EGF or 10 nM TGFα with or without the addition of 10 μg/ml of mitomycin-c (mc) to inhibit cell proliferation. Following incubation, cell monolayers were fixed, stained and photographed. Measured in vitro wound closure was expressed in square millimeter and represents the inverse of the total area under magnification remaining uncovered by cells. The data presented are the mean ± SEM, n = 6. The Student's *t*-test was used to determine significant difference between wounded monolayers treated with the same growth factor, with or without the addition of mitomycin-c. $^{**}P < 0.005$.

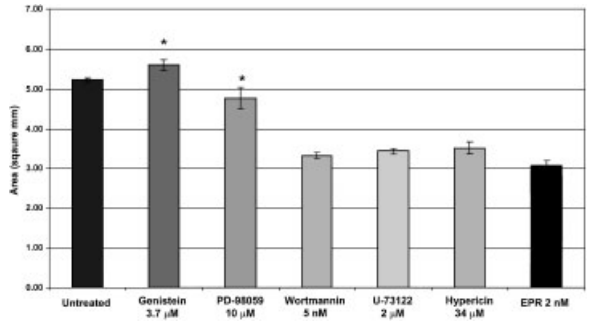

Fig. 5. Pharmacologic inhibition of EPR induced A431 cell in vitro wound closure. Serum starved, confluent A431 cells were scratched wounded, washed and incubated for 24 h with 2 nM EPR and one of the following cell permeable inhibitors: genistein, an inhibitor of protein tyrosine kinases including EGFR autophosphorylation; PD-98059, an inhibitor of MEK (MAP kinase kinase); wortmannin, a potent and selective inhibitor of phosphatidyl-inositol 3-kinase (PI3K); U-73122, an inhibitor of agonist induced phospholipase C (PLC) activation; and hypericin, a selective inhibitor of protein kinase C (PKC). The data presented are the mean ± SEM, n = 6. Significant differences ($^{*}P < 0.05$) from the corresponding untreated control (no addition of growth factor or inhibitor), were determined using Student's *t*-test.

with the addition of cell permeable, pharmacologic inhibitors specific for key intermediates of known signal cascades.

While most of the inhibitors employed in these studies are strictly specific over a wide dose range, some of the selective pharmacologic tyrosine kinase inhibitors are known to inhibit additional kinases at concentrations 100 to 1,000-fold higher than that needed for selective inhibition of the kinase in question. To ensure that our inhibitor doses were selective for the desired signal transduction intermediate, multiple dosing assays were performed using inhibitor concentrations ranging from 100-fold less to 1,000-fold greater than the established IC-50 (data not shown) and the optimal, selective concentrations were used in the following studies.

Treatment of cells with 10 μM PD-98059, an inhibitor of MEK (MAP kinase kinase) [Alessi et al., 1995] reduced EPR induced in vitro wound closure by 88% ($P < 0.05$), suggesting that the ERK/MAPK pathway plays a significant role in promotion of wound closure by EPR (Fig. 5). Treatment with 5 nM wortmannin, a potent and selective inhibitor of PI3K [Nakanishi et al., 1992], resulted in only a 12%, non-significant reduction in total wound closure, indicating that the PI3K pathway is less active in the wound closure response to EPR. The addition of 2 μM U-73122, an inhibitor of agonist induced PLC activation [Smith et al., 1990], reduced wound closure by 18%. PKC acts downstream of PLCγ. Not surprisingly, treatment with 34 μM hypericin, a selective inhibitor of PKC [Takahashi et al., 1989], resulted in a 21% reduction in wound closure, a degree of inhibition almost equivalent to that seen with inhibition of upstream PLC. Addition of 3.7 μM genistein, an inhibitor of protein tyrosine kinases including EGFR autophosphorylation [Akiyama et al., 1987], completely abrogated all EPR induced in vitro wound closure ($P < 0.05$). To serve as negative controls, scratch wounded A431 cells were treated with each of the aforementioned inhibitors in the absence of EPR. The inhibitors alone had no effect on the degree of in vitro wound closure (data not shown).

EPR Induced Phosphorylation of ERK1/2 and PLCγ1 Differs From That of EGF or TGFα

Immunoblot analysis was used to confirm the results of our biochemical inhibition studies, as well as to compare the kinetics of ERK/MAPK and PLCγ activation by EPR as compared to EGF and TGFα. We treated A431 cells with the doses of growth factor that promoted optimal closure in our in vitro wounding assays, specifically, 2 nM EPR and 10 nM of both EGF and TGFα. Even at this five-fold lower concentration, EPR was far more effective than either EGF or TGFα in stimulating ERK1/2 activation.

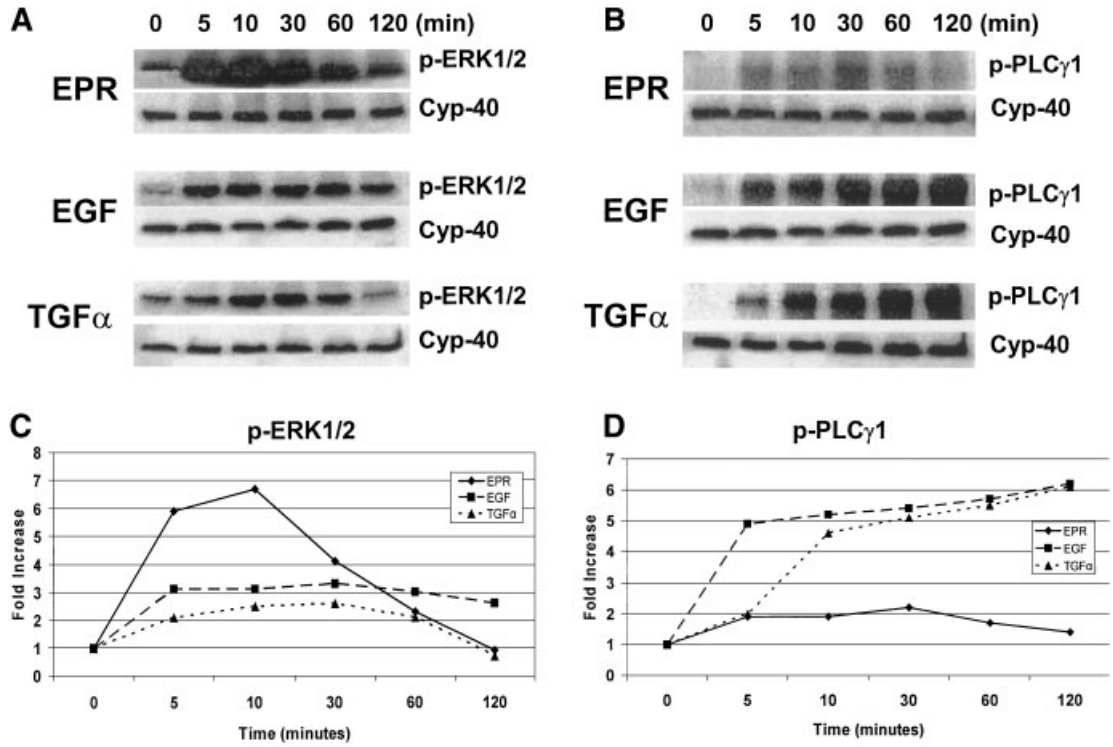

Fig. 6. ERK1/2 and PLCγ1 phosphorylation in EPR, EGF and TGFα treated A431 cells. Sub-confluent, serum starved A431 cells were stimulated for 0 to 120 min with either 2 nM EPR, 10 nM EGF or 10 nM TGFα. At selected time points, native total cell lysates were prepared as outlined under ''Materials and Methods,'' resolved by 7.5% SDS–PAGE and transferred to a nitrocellulose membrane. **A**, **B**: phosphorylated ERK1/2 or PLCγ1 was detected and visualized by immunoblotting with phosphospecific antibody to either ERK1/2 (p-ERK1/2) or PLCγ1 (p-PLCγ1). **C**, **D**: To normalize the data and document equal protein loading and transfer, membranes were stripped and reprobed in a similar fashion with antibody to cyclophilin-40 (Cyp-40). Antiphosphotyrosine immunoblot images were analyzed with Kodak Digital Science 1D Image Analysis software. The signal intensity of the immunoblot band in question was normalized for each sample with respect to the intensity of the corresponding Cyp-40 signal. The data are fold increase over untreated (time 0) cells. Results were confirmed in three independent sets of experiments.

At 5 min after addition of this ligand, EPR promoted an approximate six-fold increase in phosphorylated ERK1/2 (p-ERK1/2; Fig. 6A,C). A maximal increase of 6.7-fold was seen at 10 min. The level of p-ERK1/2 began to decrease by 30 min and by 120 min had fallen to a level less than the basal rate observed in untreated cells. In contrast, 10 nM EGF treatment resulted in an overall weaker, yet more sustained degree of ERK1/2 activation than that of 2 nM EPR. In EGF treated cells, p-ERK1/2 increased approximately three-fold by 5 min and maintained approximately the same level of activation through 60 min before tapering slightly at 120 min (Fig. 6A,C). TGFα induced a two-fold increase in p-ERK1/2 at 5 min, with a slightly higher 2.5-fold maximal increase at 10 and 30 min. Levels of p-ERK1/2 declined by 60 min to the same level as noted at 5 min. By 120 min, p-ERK1/2 had dropped to sub-basal levels. The overall level of ERK1/2 activation in TGFα treated cells was somewhat less than that observed with EGF and markedly less than that induced by EPR. There is a slight amount of constitutive ERK1/2 phosphorylation in serum starved A431 cells.

In contrast to the impressive degree of ERK1/2 phosphorylation, EPR promoted only moderate PLCγ stimulation. In EPR treated A431 cells, p-PLCγ1 levels began to rise approximately two-fold at 5 min and remained unchanged at 10 min. A maximal 2.2-fold increase was noted at 30 min. By 60 min, the amount of p-PLCγ1 decreased to levels comparable to those seen at 5 and 10 min, prior to a continued decline at 120 min that remained only slightly above the near undetectable basal activity noted in untreated cells (Fig. 6B,D). Treatment with EGF resulted in a five-fold increase in p-PLCγ1 by 5 min that continued to rise at all time

intervals to a maximal 6.2-fold increase at 120 min. The kinetics of TGFα induced phosphorylation of p-PLCγ1 mirrored that of EGF except at the early 5 min time point where only a minimal two-fold increase in p-PLCγ1 was observed, versus the near five-fold increase seen with EGF treatment. By 10 min though, TGFα activation promoted a more impressive 4.6-fold increase in p-PLCγ1 that continued to rise parallel to that seen with EGF, reaching a maximum 6.1-fold increase essentially equivalent to that observed with EGF. Both EGF and TGFα promoted markedly higher PLCγ1 phosphorylation than EPR. The maximum EPR induced p-PLCγ1 level was approximately equal to or substantially less than the weakest levels seen with both TGFα and EGF respectively (Fig. 6B,D).

To establish that the differences in ERK1/2 activation induced by EPR, EGF or TGFα were not related to EGFR overexpression in A431 cells, we also performed immunoblot analysis of ERK1/2 phosphorylation in NHEK. In EPR treated NHEK, we observed a near five-fold rise in pERK1/2 by 5 min. A maximum greater than seven-fold increase was seen at 10 min followed by a decline to near five-fold by 30 min that remained relatively constant through 120 min (Fig. 7A,B). The ERK1/2 phosphorylation curves in EGF and TGFα treated NHEK were nearly identical with a 3 to 3.5-fold increase at 5 min, a maximal 4.5-fold increase at 10 min, followed by a gradual taper thereafter (Fig. 7A,B). For any given growth factor, the phosphorylation kinetics seen in A431 cells were almost identical to those observed in NHEK, thus validating the model of EGFR overexpression in A431 cells.

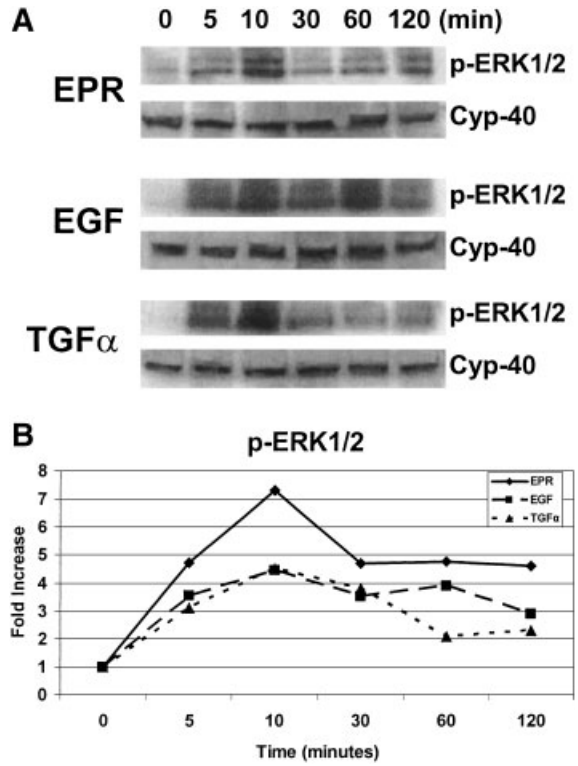

Fig. 7. ERK1/2 phosphorylation in EPR, EGF and TGFα treated NHEK. Sub-confluent, serum starved NHEK were stimulated for 0 to 120 min with either 0.2 nM EPR, 10 nM EGF or 10 nM TGFα. At selected time points, native total cell lysates were prepared as outlined under ''Materials and Methods,'' resolved by 7.5% SDS–PAGE and transferred to a nitrocellulose membrane. **A**: phosphorylated ERK1/2 was detected and visualized by immunoblotting with phosphospecific antibody to ERK1/2 (p-ERK1/2). **B**: to normalize the data and document equal protein loading and transfer, membranes were stripped and reprobed in a similar fashion with antibody to cyclophilin-40 (Cyp-40). Antiphosphotyrosine immunoblot images were analyzed with Kodak Digital Science 1D Image Analysis software. The signal intensity of the immunoblot band in question was normalized for each sample with respect to the intensity of the corresponding Cyp-40 signal. The data are fold increase over untreated (time 0) cells. Results were confirmed in three independent sets of experiments.

DISCUSSION

In this report, we provide evidence that EPR is both more potent and more effective than EGF and TGFα in stimulating in vitro wound closure. Comparative dose analysis studies show that EPR induces significantly greater wound closure than EGF or TGFα, even at a concentration that is five-fold less than the optimal 10 nM dose of either EGF or TGFα. These findings are in agreement with our in vivo murine wound healing studies which demonstrated that topically applied EPR was both more potent and more effective that than topical EGF or vehicle in promoting wound repair [Draper et al., 2003].

Similar to in vivo reepithelialization, in vitro wound closure results from both proliferative and migratory activities. In our experiments, the addition of mitomycin-c significantly reduced the amount of EPR induced in vitro wound closure as compared with EGF and TGFα. Thus, the enhanced in vitro wound closure observed with EPR results primarily from the increased mitogenic capability of this ligand. This is not to suggest that EPR does not promote cell migratory activity. Our data demonstrate that the amount of EPR induced wound closure due to migration alone is not

significantly less than that due to the combined proliferative and migratory responses engendered by EGF and TGFα.

Three lines of evidence suggest that only EGFR is subject to autocrine activation in keratinocytes. First, highly specific anti-EGFR mAbs abrogate clonal keratinocyte proliferation in the absence of fibroblast feeder layers [Elder et al., 1992; Klein et al., 1992]. Second, keratinocyte proliferation was not significantly increased in NDF treated wounds, suggesting that ErbB3 does not play a role in keratinocyte proliferation [Danilenko et al., 1995]. Third, ligand activation of HER2/HER3 was not mitogenic for keratinocytes cultured in serum-free medium [Poumay and Pittelkow, 1995]. It remains unanswered whether the ability of EPR to promote greater wound closure is due to utilization of additional ErbB receptors aside from EGFR. If the dramatically superior wound repair effects of EPR are not due to utilization of ErbB dimer combinations unavailable to other EGF-related ligands such as TGF−α and EGF, then what else may account for the impressive differences in cell response? Perhaps the answer lies in the ability of EPR to potently stimulate EGFR (and perhaps other ErbB receptor subtypes), yet not induce receptor down-regulation.

In their original analysis, Toyoda et al. [1995] found that EPR was more mitogenic than EGF for several types of normal cells despite a 10-fold lower affinity than EGF for the A431 epidermoid carcinoma line. Using engineered myeloid cells expressing only ErbB1, Shelly et al. [1998] demonstrated that EPR was more potent than EGF in activating mitogenesis, although the affinity of EGF to ErbB1 was approximately 100-fold greater. They also noted a similar discrepancy between binding affinity and signaling bioactivity through ErbB4. Strength of ligand binding is critical for receptor routing [Davis et al., 1987; Kadowaki et al., 1990]. Low affinity ErbB-binding peptides have been shown to hinder normal receptor down-regulation and degradation [Tzahar et al., 1998]. This implies that low affinity ligands may be even more potent signal inducers than their high affinity counterparts. Unlike EGF, EPR mediates minimal, if any, down-regulation of ErbB1 [Shelly et al., 1998]. The very low affinity of EPR to ErbB receptors is probably inadequate to direct these receptors to lysosomal degradation, because either the ligand dissociates very quickly, or the tyrosine phosphorylation necessary for rapid internalization [Sorkin et al., 1992] is relatively inefficient. When compared to EGF and TGFα, the ability of EPR to promote greater in vitro wound closure is likely a result of the very low affinity of EPR to ErbB receptors.

The ERK/MAP kinases have been postulated to be involved in mitogenic signaling [Cobb et al., 1994; Bornfeldt et al., 1995; Waskiewicz and Cooper, 1995], whereas the PLCγ pathway is commonly associated with induction of cell motility by numerous growth factors including EGF [Chen et al., 1994]. PD-98059 is a highly specific inhibitor of the activation of MEK1/2 [Alessi et al., 1995]. Since no other major up-stream activators of ERK1/2 other than MEK1/2 have been described [Guan, 1994], PD-98059 may be regarded as a specific inhibitor of ERK/MAPK signaling. In our pharmacologic inhibition studies of A431 cell in vitro wound closure, addition of PD-98059 resulted in a significant 88% decrease in EPR induced wound closure. Treatment with U-73122, an inhibitor of agonist induced PLC activation, caused only a non-significant 18% reduction in wound closure. Taken together, these data suggest that the ERK/MAPK pathway is most important in promotion of in vitro wound closure by EPR.

Inhibition of the ERK/MAPK pathway resulted in a greater decrease in EPR induced wound closure than would be predicted from our mitomycin-c experiments alone. This implies that ERK/MAPK activation is not strictly limited to mitogenic activities, but may also play a limited role in the motility response. Indeed, Xie et al. [1998] found that PD-98059 not only inhibited mitogenesis, but also prevented approximately two-thirds of the focal adhesion disassembly in wild-type NR6 cells, suggesting that activation of the ERK/MAP kinases is required for cell motility.

Treatment with mitomycin-c resulted in significant inhibition of EPR induced in vitro wound closure of A431 cells. In contrast, this inhibitor of cell proliferation had a non-significant impact on the promotion of wound closure by both EGF and TGFα, implying that migration is of greater import than proliferation in the wound closure response engendered by EGF and TGFα. Immunoblot analysis of the kinetics of growth factor dependent activation of ERK1/2 or PLCγ, corroborate our mitomycin-c and pharmacologic inhibitor data. As predicted by our pharmacologic inhibition analysis, EPR is a very potent activator of the ERK/MAPK pathway in

both A431 cells and NHEK. Even at a five-fold lower concentration, EPR was far superior to EGF and TGFα in phosphorylation of ERK1/2. Contrariwise, EPR activation of the motility associated PLCγ pathway was less impressive than that induced by both EGF and TGFα. The relatively weaker PLCγ1 phosphorylation by EPR corroborated our pharmacologic inhibition study wherein only a nominal decrease in wound closure was noted with the addition of the PLC inhibitor U-73122. More investigation will be required to determine exactly why EPR is such a potent activator of ERK/MAPK signaling, yet only a weak activator of the PLCγ pathway.

In conclusion, EPR is both more potent and more effective than EGF or TFGα in promoting in vitro wound closure. The augmented EPR induced wound closure response is primarily due to an increase in cell proliferation. In addition, we have provided evidence that EPR is a more potent activator of ERK/MAPK signaling than EGF or TGFα. Taken together, these findings suggest that enhanced ERK/MAPK activation is likely responsible for the superior EPR induced wound closure response.

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