Irritant Activation of Epithelial Cells Is Mediated via Protease-Dependent EGFR Activation

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Although numerous studies have examined *in vivo* and *in vitro* effects of irritants, most focused on events developing hours to days after exposure. Molecular events occurring immediately after skin contact remain incompletely defined. Characterization of early events could lead to the identification of key molecular signals necessary for the production of inflammatory mediators responsible for the signs and symptoms of irritant contact dermatitis (ICD). HaCaT cells treated with sodium lauryl sulfate (SLS), a model irritant, were used to examine early molecular events of ICD. Western analysis showed SLS-mediated induction of early growth response-1 (egr-1), a transcription factor capable of regulating hallmarks of ICD such as angiogenesis, hyperproliferation, and inflammation. Additionally, *de novo* egr-1 expression was commensurate with transcriptional activation of egr-1 mRNA and heteronuclear RNA. Use of pharmacological inhibitors demonstrated that SLS-induced egr-1 was dependent on MEK1/p44/42 ERK, but not on p38 or JNK signaling. The EGFR inhibitor PD168393 and the metalloprotease inhibitor TAPI-2 both inhibited SLS-induced egr-1. Finally, small interfering RNA silencing of the EGFR diminished SLS-induced egr-1 mRNA. These studies suggest a role of the EGFR in SLS signaling as well as a, to our knowledge, previously unreported association between ICD and EGFR induction of egr-1.

Journal of Investigative Dermatology (2011) **131,** 435–442; doi:10.1038/jid.2010.308; published online 28 October 2010

INTRODUCTION

Irritant contact dermatitis (ICD), a pathological condition induced by exposure of skin to irritant compounds, is characterized clinically by inflammation and, in severe cases, ulcerations (Nethercott, 1996). ICD is particularly prevalent in occupational settings; in industrialized countries, occupational dermatitis may account for ~30% of work-related illness (English, 2004). Thus, identifying components involved in irritant signaling holds therapeutic potential for treating and preventing ICD while decreasing incidences of occupational dermatitis.

The number of known irritants is expansive and includes solvents, surfactants, and metals, (English, 2004), yet traditional *in vitro* and *in vivo* ICD studies utilize the model irritant sodium lauryl sulfate (SLS) (Lee and Maibach, 1995). These studies typically focus on molecular events occurring hours or days after irritant exposure (Grangsjo *et al.*, 1996; Palacio *et al.*, 1997; Newby *et al.*, 2000; Moon *et al.*, 2001; Loffler and Happle, 2003; Torma *et al.*, 2006; de Jongh *et al.*, 2006b). Consequently, early irritant-induced responses are incompletely defined.

In this study, we utilized cultured primary human keratinocytes (PHKs) and immortalized HaCaT cells to investigate a role of SLS mediation of ICD signaling events. Our initial studies demonstrated upregulation of early growth response gene 1 (egr-1) protein with concomitant increased *egr-1* transcription in SLS-treated HaCaT cells. This *in vitro* HaCaT model served to define a, to our knowledge, previously unreported molecular mechanism by which irritants, such as SLS, may trigger inflammatory reactions. We hypothesized that irritants induce analogous signaling cascades as those in wound repair, and thus focused on signaling mechanisms involved in epidermal wounding. Our data strongly support a role of p44/42 ERK activation in SLS-mediated induction of egr-1 in part via protease-dependent engagement of the EGFR. These data provide the first evidence that pro-inflammatory factors such as IL-8, egr-1, and vascular endothelial growth factor (VEGF) are induced via protease-dependent EGFR activation in response to irritant exposure in epithelial cells.

RESULTS

SLS induces egr-1 in HaCaT and PHK cells

Multiple studies have identified the induction of a variety of pro-inflammatory cytokines associated with irritant skin injury, evident hours to days after irritant exposure. Our goal was to characterize early signaling events that

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Abbreviations: egr-1, early growth response-1; hnRNA, heteronuclear RNA; ICD, irritant contact dermatitis; PHK, primary human keratinocyte; SC, stratum corneum; siRNA, small interfering RNA; SLS, sodium lauryl sulfate; TGF-α, transforming growth factor alpha; VEGF, vascular endothelial growth factor

Received 16 March 2010; revised 27 August 2010; accepted 9 September 2010; published online 28 October 2010

occur following cellular stimulation with the model irritant SLS.

Detergents, the most common irritants responsible for ICD (Dickel *et al.*, 2002), are frequently used as standards for *in vivo* models of human ICD (Lee and Maibach, 2006). Before identifying activation pathways relevant to irritant molecular signaling, treatment conditions were established. We examined a range of concentrations (0.001–0.008% v/v) and exposure times (30 minutes to 4 hours) to SLS and determined that >85% of HaCaT cells were viable after 4 hours of exposure to 0.004% SLS using the trypan blue exclusion assay (data not shown).

We postulated that treatment of cultured cells with irritants such as SLS would initiate pro-inflammatory cascades via activation and/or *de novo* synthesis of relevant transcription factors. Many instances of inflammation exhibit activation of NF-κB p50/p65 proteins; thus, this transcription factor was examined first only to find that no evidence of NF-κB DNA binding, let alone nuclear translocation, was present following SLS treatment (data not shown).

Given its responsiveness to injurious stimuli and wound healing (Thiel and Cibelli, 2002), we identified egr-1 as a transcription factor of interest. Induction of nuclear egr-1 protein was both dose- and time-dependent (Figure 1a and Supplementary Figure S1a online, respectively) following SLS treatment in HaCaT cells. Maximal egr-1 protein expression was induced by 0.004% SLS (Figure 1a); moreover, in PHK

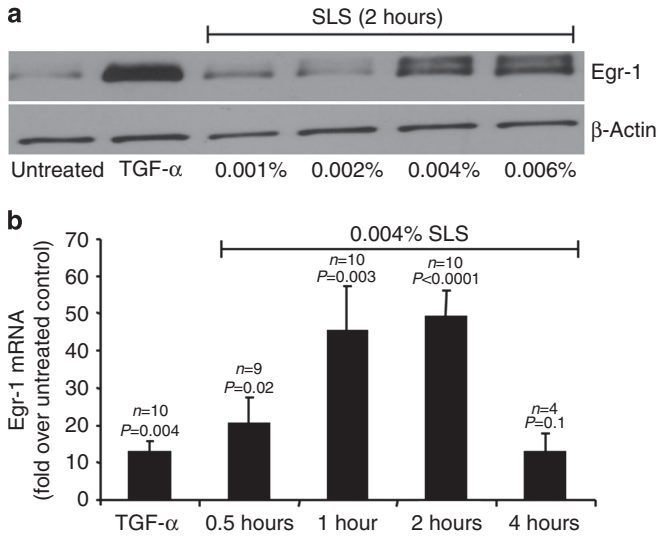

Figure 1. Sodium lauryl sulfate (SLS) induces early growth response-1 (egr-1) biosynthesis. HaCaT cells were switched to low-serum DMEM 18–24 hours before experimentation (serum starved); SLS stimulation was performed in this medium. (**a**) Nuclear egr-1 levels from cells treated for 2 hours with increasing SLS concentrations were determined via western analysis; β-actin serves as a loading control. (**b**) Total RNA from cells stimulated with SLS (0.004%) at increasing times was isolated, DNase treated, reverse transcribed, and subjected to quantitative PCR using primers specific for human egr-1 cDNA. Transforming growth factor alpha (TGF-α) (100 ng ml^{-1}, 2 hours) acts as a positive control. Average data from independent experiments are expressed as fold increase of input RNA from treated samples to untreated control ± SD. Input was normalized to 18S. Statistical significance was determined using a paired Student's *t*-test (two tailed).

0.004% SLS induced a time-dependent increase in egr-1 (Supplementary Figure S1b online). Therefore, in combination with permissible cell viability, 0.004% SLS was selected as a standard for subsequent studies.

Egr-1 mRNA production also showed a similar time-dependency peaking after 1 hour of SLS treatment and diminishing by 4 hours (Figure 1b). Real-time PCR cDNA primers spanning an intron–exon interface measured heteronuclear RNA (hnRNA) before splicing into mRNA, which was used to distinguish increased transcriptional activity from increased mRNA stability (Elferink and Reiners, 1996; O'Reilly *et al.*, 2003). SLS increased egr-1 hnRNA in a time-dependent manner as maximal induction occurred one-half hour after treatment and decreased by 2 hours (Supplementary Figure S1c online). In addition, pretreatment of HaCaT cells with cycloheximide before SLS failed to inhibit egr-1 mRNA production, suggesting that new protein synthesis was unnecessary for *egr-1* gene transcription (data not shown). Therefore, SLS appears to rapidly increase *egr-1* gene transcription as well as *de novo* egr-1 protein synthesis.

The role of MAP kinases in SLS-induced egr-1 production

Treatment of HaCaT cells with SLS activates signaling pathways that result in gene activation and new protein synthesis. However, the signaling cascade responsible for SLS-induced egr-1 in keratinocytes remained to be defined. p38 and JNK are both induced by stress and cytokines, whereas p44/42 ERK is activated by growth factors and the skin irritant phorbol 12-myristate 13-acetate (Dong *et al.*, 2002). Additional studies link activation of MAP kinases to egr-1 biosynthesis (Thiel and Cibelli, 2002); therefore, we employed specific pharmacological MAP kinase inhibitors to probe for MAP kinase-driven egr-1 production. SLS induction of egr-1 protein (Figure 2a), hnRNA (Supplementary Figure S2 online), and mRNA (Figure 2b and c) were significantly inhibited by the MEK1 inhibitor, PD98059. In contrast, neither the JNK inhibitor SP600125 nor the p38 inhibitor SB203580 had an effect on SLS-induced egr-1 expression (data not shown); the same concentration of SB203580 was effective at inhibiting tumor necrosis factor-α-induced tissue factor (data not shown) in human dermal microvascular endothelial cells as previously reported (O'Reilly *et al.*, 2003). Thus, SLS activation of MEK1/p44/42 ERK is consistent with previous studies that demonstrate egr-1 biosynthesis under stress conditions such as heat shock, UV radiation, and sodium arsenite (Lim *et al.*, 1998; Al-Sarraj and Thiel, 2004).

EGFR blockade inhibits SLS induction of egr-1

Following identification of MEK1/p44/42 ERK signaling in SLS-induced egr-1 biosynthesis, we sought to identify the mechanism behind SLS-induced p44/42 ERK activation. EGFR activation might be responsible for SLS-induced egr-1 expression as many EGF-like ligands activate MAP kinases (Yarden and Sliwkowski, 2001). Additionally, egr-1 is induced by EGFR ligands such as transforming growth factor alpha (TGF-α), and MEK1/p44/42 ERK signaling is closely linked to activated EGFR. Finally, we hypothesized that the effect of irritants on the epithelium may have comparable

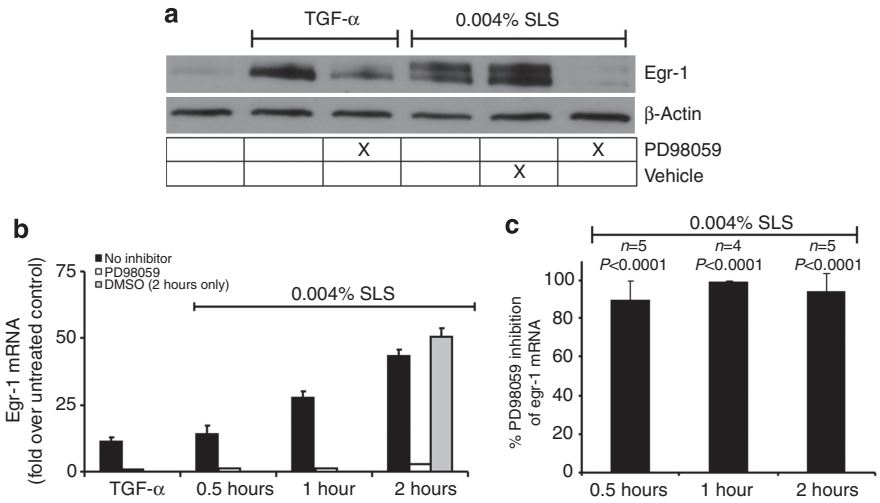

Figure 2. The MAP kinases MEK1/p44/42 ERK are required for early growth response-1 (egr-1) biosynthesis following sodium lauryl sulfate (SLS) stimulation. Serum-starved HaCaT cells were pretreated for 30 minutes with 25 μM PD98059 or DMSO vehicle control before adding transforming growth factor alpha (TGF-α) (100 ng ml^{-1}, 2 hours) or 0.004% SLS as indicated. (**a**) Nuclear protein was isolated 2 hours post-treatment and subjected to western analysis to determine the impact of PD98059 on SLS-induced egr-1; β-actin serves as a loading control. (**b**) Quantitative PCR was performed with primers for human cDNA to determine changes to egr-1 mRNA. Representative results are expressed as fold increase of input RNA in treated samples over untreated control ± SD. Input was normalized using 18S. (**c**) Average inhibition of egr-1 mRNA by PD98059 ± SD; statistical significance was determined using a paired Student's *t*-test (two tailed).

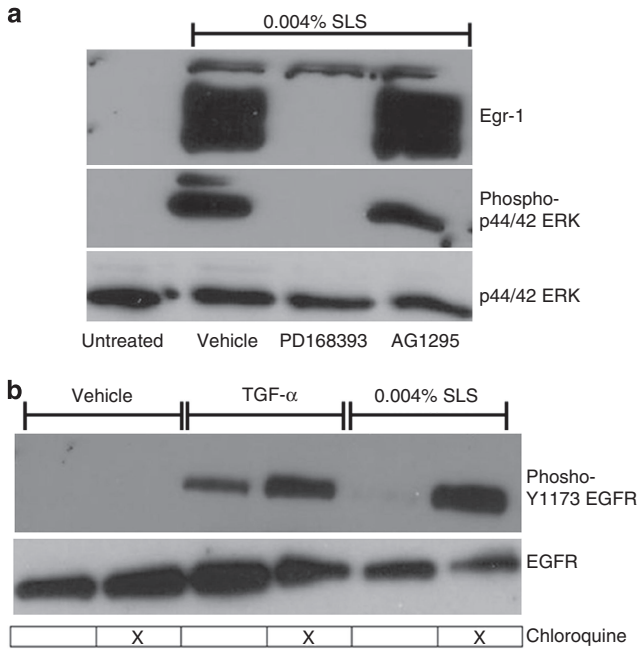

Figure 3. Inhibition of the EGFR inhibits sodium lauryl sulfate (SLS)-mediated p44/42 ERK activation and induction of early growth response-1 (egr-1); SLS induces EGFR activation. (**a**) Nuclear proteins from serum-starved HaCaT cells pretreated for 30 minutes with 62.5 nM of the EGFR inhibitor PD168393 or the platelet-derived growth factor receptor inhibitor AG1295 before addition of SLS (0.004%, 2 hours) were subjected to western analysis using anti-egr-1 and anti-phospho-p44/42 ERK antibodies; p44/42 ERK acts as a loading control. (**b**) Cytoplasmic proteins from serum-starved HaCaT cells pretreated 1 hour with chloroquine (100 μM) before SLS (0.004%, 2 hours) or transforming growth factor alpha (TGF-α) (5 ng ml^{-1}, 2 hours) were subjected to western analysis. The blots were probed with anti-phospho-Y1173 EGFR and anti-EGFR. Data represent at least three independent experiments.

features to programs initiated by injury as EGFR activation is crucial for promoting wound repair (Pastore *et al.*, 2008).

Pretreatment of HaCaT cells with the specific EGFR inhibitor PD168393 completely ablated SLS-induced egr-1 protein (Figure 3a), hnRNA (Supplementary Figure S3a and c online), and mRNA (Supplementary Figure S3b and d online); TGF-α-induced egr-1 protein, hnRNA, and mRNA were significantly impaired (data not shown, Supplementary Figure S3 online). These data were supported by inhibition of SLS-induced egr-1 biosynthesis by an additional and distinct EGFR inhibitor AG1478 (data not shown). PD168393 also significantly inhibited egr-1 mRNA in PHK, although due to high basal levels of egr-1 mRNA, fold induction by SLS was considerably lower than HaCaT cells (Supplementary Figure S4 online). Finally, PD168393 disrupted MEK1/p44/42 ERK signaling in HaCaT cells as determined by inhibition of phospho-p44/42 ERK (Figure 3a). Furthermore, the platelet-derived growth factor receptor inhibitor AG1295 failed to inhibit SLS-induced egr-1 biosynthesis or p44/42 ERK activation supporting specificity for signaling through EGFR (Figure 3; Supplementary Figure S3a online).

The pharmacological inhibitor data strongly support a role of EGFR as an upstream regulator of egr-1 induction following SLS stimulation. To strengthen this claim, small interfering RNA (siRNA) was used to selectively knockdown EGFR expression. No reduction in EGFR mRNA occurred in nonsense siRNA-treated cells (Supplementary Figure S5a online). Compared with both no siRNA and nonsense siRNA controls, EGFR mRNA was reduced 85–90% in EGFR siRNA-treated cells (Supplementary Figure S5a and b online, respectively). As expected, TGF-α-induced egr-1 mRNA was reduced in EGFR siRNA-treated cells compared with the no siRNA control and nonsense siRNA cells (Supplementary

Figure S5c and d online). Furthermore, treatment with specific EGFR siRNA prevented egr-1 mRNA induction that was significant over nonsense siRNA, eliminating any nonspecific effects (Supplementary Figure S5c and d online).

SLS induces EGFR activation

As a receptor tyrosine kinase, EGFR becomes autophosphorylated following activation. We examined SLS-induced EGFR activation by determining the presence of phospho-Y1173, a major autophosphorylation site, via western analysis. Cytoplasmic fractions from both HaCaT cells and primary keratinocytes were resolved by SDS-PAGE and probed with anti-phospho-Y1173 EGFR. Interestingly, SLS induced a time- and dose-dependent increase in phospho-Y1173 EGFR in PHK (Supplementary Figure S6 online, data not shown), but no phosphorylation was evident in HaCaT cells aside from the TGF-α control (data not shown).

Despite the ability of EGFR siRNA and pharmacological inhibitors to inhibit SLS-induced egr-1, phospho-Y1173 EGFR in HaCaT cells could not be readily detected. The rate of EGFR internalization and degradation can vary depending on the stimulating ligand and cell type (Wiley, 2003). Chloroquine has been previously reported to prevent internalization and degradation of the IFN-γ receptor INFγR1 through an unknown mechanism (Gira *et al.*, 2009). HaCaT cells were pretreated with chloroquine before SLS to allow visualization of activated EGFR. Whereas no phospho-Y1173 EGFR was evident in HaCaT cells treated with SLS alone, cells treated with chloroquine before SLS exhibited robust phospho-Y1173 EGFR (Figure 3b); this phosphorylation was not evident until at least 30 minutes of SLS treatment (data not shown), despite activation of p44/42 ERK as soon as 5 minutes after SLS treatment (data not shown). These data confirm that SLS induced EGFR activation in both cell types.

Metalloprotease activity mediates transactivation of EGFR following SLS stimulation

EGFR may become activated via an extracellular, ligand-dependent pathway following either the addition of exogenous ligand, e.g., TGF-α, or release of membrane-bound EGFR ligand(s). This latter mechanism can occur in a variety of contexts via activation of membrane-associated metalloproteases (Sanderson *et al.*, 2006). The data above support a role of EGFR in SLS-induced egr-1 production suggesting the involvement of the EGFR/TGF-α activation pathway in early events in ICD. However, the mode of SLS activation of EGFR required further study.

Many of the ligands that activate the EGFR require protease cleavage to become biologically active (Blobel, 2005). SLS could activate an EGFR ligand-cleaving metalloprotease resulting in EGFR transactivation. To determine whether SLS signaling requires ectodomain shedding, HaCaT cells were pretreated with the metalloprotease inhibitor, TAPI-2, before SLS. TAPI-2 inhibited SLS-induced egr-1 protein (Figure 4a), hnRNA (Supplementary Figure S7 online), and mRNA (Figure 4b and c); moreover, TAPI-2 had no effect on TGF-α-induced egr-1 (Figure 4 and Supplementary Figure S7 online). These data support the hypothesis that irritants such as SLS induce cell activation and inflammation via a protease-dependent EGFR pathway. Future studies will focus on identifying the specific metalloproteases and EGFR ligands responsible.

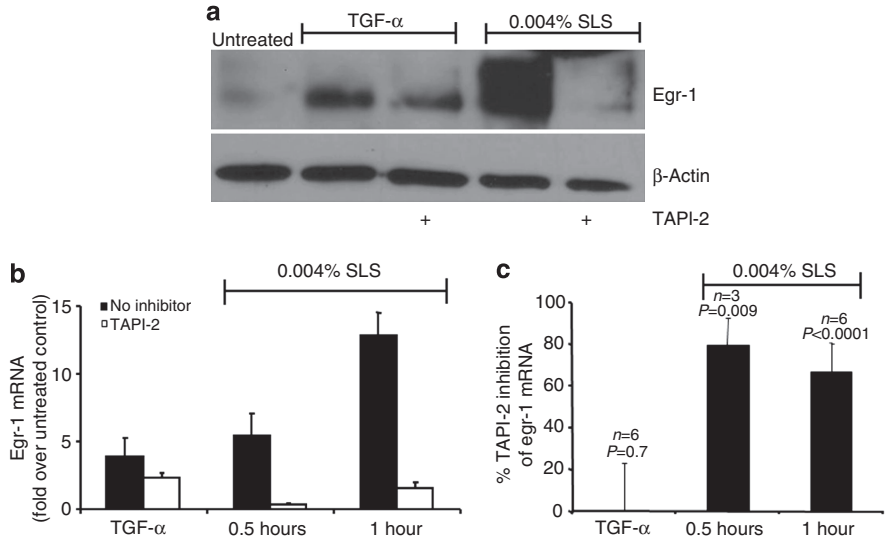

Figure 4. Sodium lauryl sulfate (SLS)-induced early growth response-1 (egr-1) expression is sensitive to the metalloprotease inhibitor TAPI-2. Serum-starved HaCaT cells were pretreated for 30 minutes with TAPI-2 (25 μM) before stimulation. (**a**) Changes to nuclear egr-1 protein at 2 hours after SLS or transforming growth factor alpha (TGF-α) (5 ng ml^{-1}) treatment were ascertained via western analysis (**b**) RNA was isolated and processed as described in Materials and Methods. Inhibition of SLS-induced egr-1 mRNA was determined using real-time PCR primers specific for human egr-1 cDNA. TGF-α (25 ng ml^{-1}, 2 hours) acts as a negative control for protease effect. Representative data are expressed as fold increase of input RNA from treated samples over untreated control ± SD. Input was normalized using 18S. (**c**) Average inhibition of egr-1 mRNA by TAPI-2 ± SD; statistical significance was determined using a paired Student's *t*-test (two tailed).

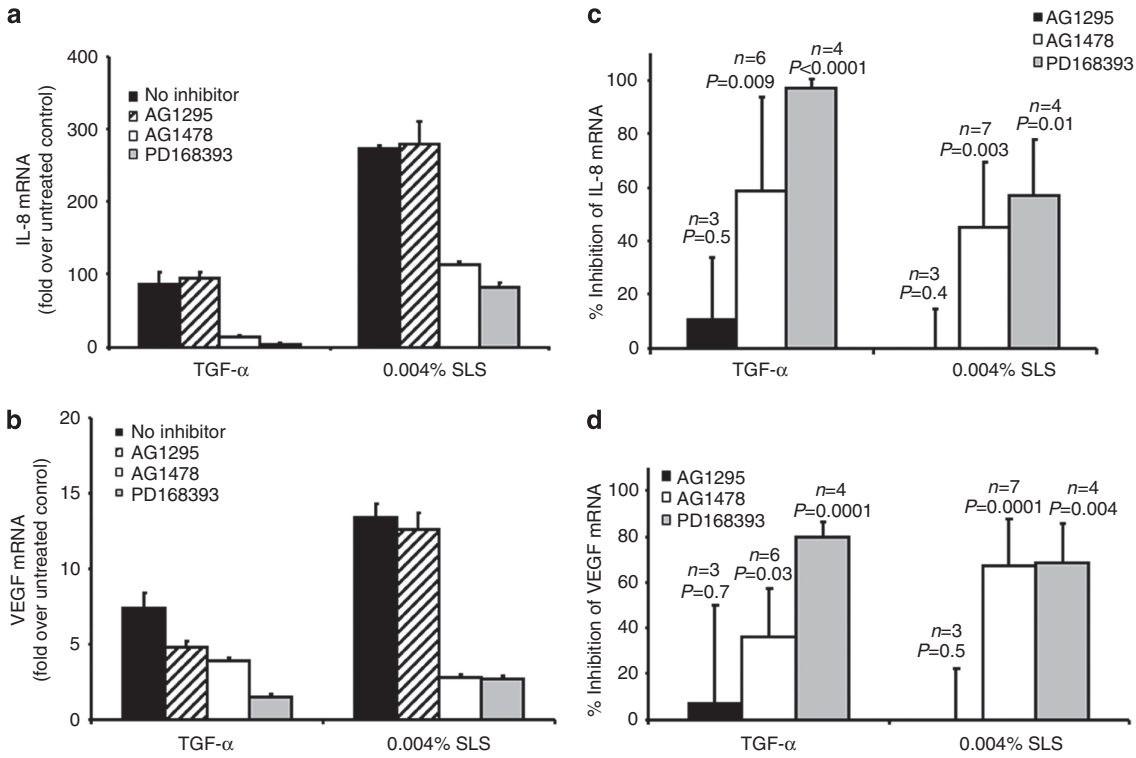

Figure 5. EGFR inhibitors diminish sodium lauryl sulfate (SLS)-induced IL-8 and vascular endothelial growth factor (VEGF) mRNAs. Serum-starved HaCaT cells were pretreated for 30 minutes with 62.5 nM AG1478, PD168393, or AG1295 before adding 0.004% SLS or transforming growth factor alpha (TGF-α) (5 ng ml^{-1}) for an additional hour. Total RNA was isolated and processed as described in Materials and Methods. Primers specific for human (**a**) IL-8 and (**b**) VEGF cDNAs were utilized in real-time PCR for quantification of mRNA induced by SLS treatment. Representative results are expressed as fold increase in input RNA in treated samples over untreated control cells ± SD. Input was normalized using 18S. Data were combined to determine average inhibition of (**c**) IL-8 and (**d**) VEGF mRNAs ± SD; statistical significance was determined using a paired Student's *t*-test (two tailed).

SLS induces IL-8 and VEGF mRNAs through activation of EGFR

Following identification of components involved in SLS signaling upstream of egr-1, we looked for additional relevant protease-sensitive, EGFR-dependent SLS-inducible genes. SLS and other irritants have previously been shown to induce VEGF and IL-8 in keratinocytes both *in vivo* and *in vitro* (Wilmer *et al.*, 1994; Grangsjo *et al.*, 1996; Torma *et al.*, 2006; de Jongh *et al.*, 2006b). These studies varied significantly in SLS concentration; moreover, 4 hours post-treatment was the earliest time point examined (Torma *et al.*, 2006), and no mechanisms for VEGF induction were proposed. We hypothesized that SLS treatment induces these pro-inflammatory agents via release of EGF-like ligands and engagement of EGFR.

As previously demonstrated, SLS treatment of HaCaT cells showed a time-dependent increase in both IL-8 and VEGF mRNAs (data not shown). Consistent with our hypothesis, the upregulation of these pro-inflammatory mRNAs was inhibited by the MEK inhibitor PD98059 (data not shown), the protease inhibitor TAPI-2 (Supplementary Figure S8 online), and the EGFR inhibitors AG1478 and PD168393 (Figure 5), but not AG1295 (Figure 5). Furthermore, PD168393, but not AG1295 also significantly inhibited SLS-induced IL-8 mRNA in PHK (Supplementary Figure S9 online); induction of VEGF was minimal due to high baseline levels of VEGF mRNA (data not shown). These data support that SLS induction of VEGF and IL-8 mRNAs appears to depend on both EGFR and metalloproteases.

SLS induces egr-1 and IL-8 in *in vivo* tissue

As metabolically active epidermal cells are ultimately responsible for irritant response and inflammatory mediator production, SLS exposure in our *in vitro* model may not be predictive of the mediators induced *in vivo*. In order to examine whether *in vivo* SLS treatment of skin resulted in induction of the same genes observed *in vitro*, we measured egr-1, IL-8, and VEGF mRNAs in normal human skin treated for 6 hours with 20% SLS. Treatment of intact skin with SLS induced statistically significant upregulation of egr-1 mRNA (Supplementary Table S1 online). In addition, SLS treatment caused variable induction of IL-8 mRNA (Supplementary Table S1 online), but no consistent increase of VEGF mRNA (Supplementary Table S1 online). These data demonstrate a similar pattern of gene activation induced *in vivo* as what we observed *in vitro*.

DISCUSSION

Irritant injury is one of the most common skin afflictions, yet has been subjected to only modest basic research. Searching the Medline database (1950-present) using the term irritant dermatitis yields slightly more than 3000 references, most of which focus on clinical aspects of this condition. Studies

examining the mechanisms involved in the elicitation of ICD have demonstrated the expression of a host of pro-inflammatory cytokines, which is not surprising given the nature of the disorder. The mechanisms linking the exposure of skin to the irritant and the resulting gene activations remain incompletely defined.

At the most basic level induction of pro-inflammatory genes generally requires induction and/or activation of transcription factors. We initially focused on such factors as targets for irritant effects and found that SLS rapidly induces the expression of the transcription factor egr-1. Previous studies linking egr-1 induction to EGFR activation encouraged us to explore the role of EGFR in this induction. Our studies demonstrated unambiguous protease-dependent activation of EGFR and resultant production of egr-1 mRNA and protein as well as IL-8 and VEGF mRNAs *in vitro*.

This irritant-mediated protease-dependent mechanism of EGFR activation shares distinct similarities with what has previously been described in wound healing models (Pastore *et al.*, 2008). Physical injury to keratinocytes results in protease-dependent release of EGFR ligands. Thus, irritant effects appear to function within the continuum of mechanisms previously described for focal epithelial injury. This is not entirely surprising, and perhaps irritant effects should be classified as field injury in contrast to focal injury.

Despite the findings in this study, additional questions remain. Previous studies examining targets for irritant injury suggested that irritant-mediated disruption of barrier function, via effects on the stratum corneum (SC), is the exclusive target and mechanism for irritant-induced inflammation; these conclusions relied on models that used either tape stripping or acetone treatment of human or mouse skin (Wood *et al.*, 1992, 1996; Rissmann *et al.*, 2009). However, the data generated in these models discount the possibility of direct effects of irritants on skin cells including keratinocytes *in vivo*. Studies of SLS penetration into human and rodent skin clearly demonstrate penetration of SLS into the epidermis at concentrations consistent with those used in our *in vitro* studies (Fullerton *et al.*, 1994; Patil *et al.*, 1995; de Jongh *et al.*, 2006a). Furthermore, studies that purport exclusive SC targeting used models that neglect potential mediation of effects via non-SC targets. Tape stripping is not a standardized technique, and the mechanical stretching involved may have effects on skin components other than the SC. Additionally, acetone likely penetrates deeper than the SC, and previous studies fail to address this effect. Whether or not irritants such as SLS can activate signaling pathways independent of SC perturbation *in vivo* is still an open question. When we compared genes activated by SLS in a simplified system *in vitro* and compared them with genes activated *in vivo*, we were able to identify almost 50 genes that were upregulated in both models (Supplementary Table S2 online). The overlapping pattern of gene activation between HaCaT cells *in vitro* and patch tests *in vivo* suggests strongly that inflammatory gene activation *in vivo* after SLS exposure may not be exclusively dependent upon SC perturbation.

Although our data demonstrate some role of injury-induced, protease-dependent EGFR activation, the mechanisms mediating protease activation by irritants are yet undefined. G-protein-linked receptor activation has been associated with protease activation. Despite many applicable keratinocyte G-protein receptors that could initiate this cascade, Yin *et al.* (2007) reported that wounding of epithelial cultures releases adenosine triphosphate, which activates G-protein-linked purinergic receptors that subsequently releases EGF-like ligands by protease activity. Additionally, adenosine triphosphate release has been linked to irritant effects both *in vitro* and *in vivo* (Mizumoto *et al.*, 2002, 2003).

The role of EGFR activation in cutaneous inflammation is still not completely defined (Pastore *et al.*, 2008). Although we have identified EGFR activation in keratinocytes following SLS treatment, inhibition of EGFR for therapeutic benefits for ICD remains controversial. Mascia *et al.* (2003) reported that blocking EGFR or MEK1/p44/42 ERK with pharmacological inhibitors increased hypersensitivity in mice , suggesting that EGFR functions as an anti-inflammatory mediator. Conversely, EGFR activation was suggested to be pro-inflammatory by Cook *et al.* (1997) who generated mice overexpressing amphiregulin that exhibited profound skin inflammation and characteristics of psoriasis. The discrepancies in these data suggest that the effects of EGFR activation may be context- and cell-specific, which rely on specifics such as the EGF-like ligand(s) released, ligand concentration, signaling kinetics, and specific ErbB receptor subtype activated. EGFR activation may be capable of both pro- and anti-inflammatory roles.

Although the data presented in this manuscript focus on EGFR signaling as an early event in response to SLS-mediated injury, additional activation of pathways upstream of protease activation are also relevant and require additional study. The ability to study irritant-mediated events occurring rapidly after irritant exposure will facilitate discovery of, to our knowledge, previously unreported pathways relevant to clinical observations of skin irritation.

MATERIALS AND METHODS

Reagents

SLS and AG1478 were obtained from Sigma-Aldrich (St Louis, MO). Anti-egr-1 (C-19), anti-EGFR, and anti-phospho-Y1173 EGFR were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-p44/42 ERK, anti-GAPDH, and anti-p44/42 ERK were from Cell Signaling Technology (Danvers, MA). The inhibitors AG1295, PD168393, PD98059, SP600125, and SB203580 were purchased from Calbiochem (San Diego, CA). Human recombinant TGF-α was from PeproTech (Rocky Hill, NJ). PowerSYBR was purchased from Applied Biosystems (Foster City, CA). All other reagents were the best grade possible and are commercially available.

Cell culture

HaCaT cells are non-tumorigenic and form an almost normal epidermis *in vivo* (Boukamp *et al.*, 1988), suggesting that the cells are a reasonable *in vitro* system for studying changes in epidermal cells. HaCaT cells were maintained in DMEM (Mediatech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1 × antibiotic/

anti-mycotic solution (Mediatech) in 5% CO_2 and 37 °C. Primary keratinocytes isolated from neonatal foreskin were obtained from Emory University Skin Diseases Research Center or Lifeline Cell Technology (Walkersville, MD). Cells were grown in KC growth (KGM bullet kit, Lonza, Walkersville, MD) or DermaLife K Cell Culture Medium (Lifeline Cell Technology) and maintained at 37 °C and 5% CO_2. Before experimentation, confluent PHK (p2-6) were starved for 24 hours in KC growth media devoid of bovine pituitary extract and EGF.

Western blot analysis

HaCaT cells were grown as described above in 60-mm dishes. At 24 hours before treatment, the media was changed to 0.5% fetal bovine serum DMEM or KGM without EGF. Cytoplasmic and nuclear proteins isolated in the presence of protease and phosphatase inhibitors were resolved using SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-egr-1, anti-phospho-Y1173 EGFR, or anti-phospho-p44/42 ERK overnight at 4 °C. All antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA). Blots were stripped using Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) per manufacturer's directions before reprobing with anti-EGFR or anti-p44/42 ERK. To confirm even loading, blots were reprobed with anti-β-actin (Stratagene, La Jolla, CA).

Real-time quantitative PCR

Before treatment, HaCaT cells were starved with 0.5% fetal bovine serum DMEM. Cells (90–100% confluent) were treated at times and doses indicated before total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) with on-column DNase I (Qiagen) treatment to remove genomic DNA. cDNA was generated using Superscript III Reverse Transcriptase per manufacturer's protocol (Invitrogen, Carlsbad, CA). Changes to gene expression were determined using specific primers for human egr-1, EGFR, VEGF, and IL-8 cDNAs in triplicate wells. To establish whether the increases in egr-1 protein levels were the result of increased transcription or translation, hnRNA reverse transcriptase-PCR primers that overlapped an intron/exon interface on the *egr-1* gene (F-5′-CTCCGTTGCGTCACTGTTG-3′, R-5′-ACTCCCGAGGTAGTTA ATAATTGAC-3′) were employed. Statistical significance was determined using two-tailed paired Student's *t*-test; *n* represents the number of independent experiments.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank the Emory University Department of Surgery Transplantation Group for use of their Agilent 2100 Bioanalyzer, Gregory Doho and the Emory Biomarker Service Center at Emory University's Winship Cancer Institute for assistance with the array data, and Dr Brian P. Pollack for experimental assistance and critical reading of this manuscript. This work was supported, in part, by grants from the Dermatology Foundation (KJW), NIAMS Research Training Grant (5T32 AR007585, KJW), NIOSH/CDC (NIOSH R21 OH07340, RAS), the Veterans Administration, and the Rich Foundation.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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