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Stress Signals, Mediated by Membranous Glucocorticoid Receptor, Activate PLC/PKC/GSK-3β/β-catenin Pathway to Inhibit Wound Closure

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Abstract

Glucocorticoids (GCs), key mediators of stress signals, are also potent wound healing inhibitors. To understand how stress signals inhibit wound healing, we investigated the role of membranous glucocorticoid receptor (mbGR) by using cell-impermeable BSA-conjugated dexamethasone. We found that mbGR inhibits keratinocyte migration and wound closure by activating a Wnt-like phospholipase (PLC)/ protein kinase C (PKC) signaling cascade. Rapid activation of mbGR/PLC/PKC further leads to activation of known biomarkers of nonhealing found in patients, β-catenin and c-myc. Conversely, a selective inhibitor of PKC, calphostin C, blocks mbGR/PKC pathway, and rescues GC-mediated inhibition of keratinocyte migration in vitro and accelerates wound epithelialization of human wounds ex vivo. This novel signaling mechanism may have a major impact on understanding how stress response via GC signaling regulates homeostasis and its role in development and treatments of skin diseases, including wound healing. To test tissue specificity of this nongenomic signaling mechanism, we tested retinal and bronchial human epithelial cells and fibroblasts. We found that mbGR/PLC/PKC signaling cascade exists in all cell

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CONFLICT OF INTEREST

Dr. Tomic-Canic is listed as an inventor of a patent PCT/US2010/062361 “Composition and methods for promoting epithelialization and wound closure” issued to the New York University based on the data presented, in part, in the study and stands to potentially gain royalties from future commercialization.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.11.036.

types tested, suggesting a more general role. The discovery of this nongenomic signaling pathway, in which glucocorticoids activate Wnt pathway via mbGR, provides new insights into how stress-mediated signals may activate growth signals in various epithelial and mesenchymal tissues.

INTRODUCTION

In addition to being major stress signals, glucocorticoids (GCs) are widely used therapeutic agents in treatment of both local and systemic inflammatory disorders. Prolonged therapeutic use of GCs has numerous side effects including potent inhibition of wound healing (Lee et al., 2005; Stojadinovic et al., 2007; Vukelic et al., 2011). Wound healing involves a complex, multistep process that requires an intricate balance of various signaling pathways to restore epidermal barrier. Aberrant signaling in response to injury leads to impairment of epithelialization, common for major types of chronic wounds (Brem et al., 2007; Pastar et al., 2014; Stojadinovic et al., 2005). In spite of epidemic proportions of nonhealing wounds, development of new therapies is impeded by lack of understanding regarding mechanisms that inhibit healing (Eming et al., 2014).

We found that GCs are synthesized in epidermis and regulate inflammatory response during acute injury (Slominski et al., 2007; Slominski et al., 2005a; Slominski et al., 2005b; Slominski et al., 2004; Stojadinovic et al., 2007; Vukelic et al., 2011), whereas stress was found to impair wound healing (Stojadinovic et al., 2012). Emerging evidence that local production in skin may contribute to systemic GCs and vice versa suggests substantial cross-talk between local and systemic hypothalamic-pituitary-adrenal axis that may influence healing outcomes (Jozic et al., 2014, Jozic et al., 2015; Skobowiat and Slominski, 2015; Slominski et al., 2015).

The membranous fraction of glucocorticoid receptor (mbGR) was found to regulate the activity of many signaling molecules (Almawi and Melemedjian, 2002; Chen and Farese, 1999; Samarasinghe et al., 2012; Strehl and Buttgereit, 2014; Strehl et al., 2011; Vernocchi et al., 2013). These nongenomic changes do not require direct interaction of glucocorticoid receptor (GR) with a promoter. Instead, they activate secondary messenger systems to generate biological responses within minutes, serving as a priming event to prepare cells for subsequent genomic activity (Almawi and Melemedjian, 2002; Chen and Farese, 1999; Strehl and Buttgereit, 2014; Strehl et al., 2011; Vernocchi et al., 2013). Our recent discovery that GR localizes to the plasma membrane of keratinocytes adds to the complexity and suggests presence of additional pathways in skin (Stojadinovic et al., 2013).

Wnt pathway is essential for many aspects of skin development, physiology, and pathology (He et al., 1998). Previous microarray analyses of GC-treated keratinocytes, demonstrated upregulation of protein kinase C (PKC) (Stojadinovic et al., 2007), which regulates glycogen synthase kinase 3-β (GSK-3β) activity (Christian et al., 2002; Goode et al., 1992; Hart et al., 1998; Hinoi et al., 2000; Nakamura et al., 1998). We found that β-catenin acts as a coregulator of GR-mediated transcriptional regulation of wound-inducible keratin genes and EGF-mediated migration (Lee et al., 2005; Radoja et al., 2000; Stojadinovic et al., 2005; Stojadinovic et al., 2007). Furthermore, we found that in epidermis of chronic wounds, activation of β-catenin, and consequentially c-myc, is associated with a nonhealing

phenotype (Stojadinovic et al., 2005). Thus, we postulate that activation of mbGR may activate a Wnt-like pathway, contributing to inhibition of wound closure. Indeed, we found that targeting mbGR by dexamethasone (Dex)-BSA leads to rapid activation of phospholipase-γ (PLCγ) and protein kinase C (PKC) that, in turn, leads to phosphorylation of GSK-3β, activation of nuclear β-catenin and subsequent overexpression of c-myc. This nongenomic signaling functionally impairs keratinocytes resulting in inhibition of migration and wound closure that can be reversed by selective inhibitors of PKC. Furthermore, we found that this signaling pathway functions in various cells and tissues, suggesting its more general role in integrating stress signals (mediated by GCs) into growth signals (mediated by Wnt pathway).

RESULTS

GCs promote nuclear localization of β-catenin through protein kinase C pathway

To test if GCs activate β-catenin, we stimulated primary human keratinocytes (HEKs) with Dex in the presence/absence of GR antagonist, Ru486, and evaluated localization of the phosphorylated β-catenin through indirect immunofluorescence (Ogiwara et al., 1998; Stojadinovic et al., 2005). As expected, treatment with lithium chloride resulted in activation and nuclearization of β-catenin (Figure 1a and b). Similarly, treatment with Dex led to a robust nuclearization of β-catenin, which could be blocked by pretreatment with GR antagonist, Ru486, suggesting that GCs may activate β-catenin pathway (Figure 1c and d). To determine if observed activation of β-catenin is mediated by PKC, we pretreated HEKs in the presence or absence of calphostin C (CC), a known inhibitor of PKC (Ogiwara et al., 1998). Pretreatment with CC blocked the Dex-mediated activation of β-catenin, as evidenced by its absence from the nucleus of HEKs (Figure 1e). These findings suggest that GCs can mediate rapid effects, resulting in nuclear localization of β-catenin through activation of the PKC pathway.

Selective activation of membranous glucocorticoid receptor by BSA-conjugated dexamethasone does not contribute to transcriptional regulation of genomic glucocorticoid receptor

We and others found previously that a subpopulation of the GR is localized at the plasma membrane (Bartholome et al., 2004; Stojadinovic et al., 2013; Strehl et al., 2011). We used Dex-BSA to functionally characterize the mbGR because the large BSA moiety prevents entry into cells and biding to their intracellular receptors (Hu et al., 2010; Nahar et al., 2015; Samarasinghe et al., 2012). To test if mbGR is involved in activating β-catenin, we stimulated HEKs with Dex-BSA in the presence/absence of Ru486 and CC (GR and PKC antagonists, respectively) and evaluated localization of the phosphorylated β-catenin through indirect immunofluorescence. Dex-BSA treatment led to nuclearization of β-catenin, which was reversed by GR and PKC antagonists (Figure 1, f–h). We then treated HEKs with 1 μM Dex (D) or 100 nM Dex-BSA (DB) for 10, 15, 30, 45, 60, or 120 minutes and assessed activation of GR by immunoblotting of GR phosphorylation at S211 (Wang et al., 2002). We observed rapid activation of GR, within first 10 minutes, that reached its peak between 30 and 45 minutes after stimulation with either ligand (Supplementary Figure S1a online). To discern genomic from nongenomic effects, we treated HEKs with either Dex or Dex-BSA in

the presence or absence of a transcriptional inhibitor, actinomycin D, and tested the expression of GILZ (GC response element-induced leucine zipper) via real-time PCR, which we found to be among the first transcriptionally regulated genes by GCs in HEKs (Stojadinovic et al., 2007). As expected, Dex stimulated induction of GILZ, whereas Dex-BSA showed no change in GILZ expression (Supplementary Figure S1b online), suggesting that Dex-BSA-mbGR does not contribute to genomic effects by directly regulating gene expression. Furthermore, we treated keratinocytes with Dex or Dex-BSA and analyzed phosphorylated GR (S211) localization after subcellular fractionation and observed that treatment of keratinocytes with Dex stimulated nuclear translocation of phosphorylated GR, whereas treatment with Dex-BSA did not. There were no detectable changes in nuclearization of total GR as shown by immunofluorescence staining of HEKs after treatment with Dex or Dex-BSA (Supplementary Figure S1c online). Collectively, these results suggest that Dex-BSA selectively activates membranous GR in HEKs and does not contribute to transcriptional regulation of genomic GR.

Membranous glucocorticoid receptor activates phospholipase C/protein kinase C/ glycogen synthase kinase 3 beta pathway that induces c-myc

To assess if mbGR activates β-catenin and by which mechanism, we first examined the phosphorylation state of GSK-3β (Goode et al., 1992) by treating with either Dex or Dex-BSA for 30 minutes and immunoblotting against phosphorylated GSK-3β (Supplementary Figure S1d online). We observed a clear induction of GSK-3β (S9) phosphorylation in keratinocytes treated with Dex-BSA (comparable to Dex), indicating rapid GSK-3β phosphorylation mediated by the mbGR. Because inhibition of PKC by treatment with CC resulted in a significant reduction of nuclear β-catenin (see Figure 1), we focused on testing whether PKC pathway was essential for mbGR-mediated induction of GSK-3β phospho-inactivation and nuclear translocation of β-catenin. We treated keratinocytes with either Dex or Dex-BSA, and tested for activation of PKC and its upstream regulator PLCγ via immunoblotting. We observed a clear phosphorylation of both PKC (pan S660) and its upstream regulator PLCγ1 (Y783) (Supplementary Figure S1e online).

Next, we used a series of small enzymatic inhibitors of each step of the signaling pathway. HEKs were incubated with Dex-BSA alone or in combination with either GR-antagonist Ru486, PKC inhibitors CC or Go6976, and PLC inhibitor, genistein. Pretreatment of cells with Ru486 selectively inhibited the Dex-BSAemediated phosphorylation of GR (S211) shown by immunoblotting (Figure 2a). We observed that Dex-BSA induced phosphorylation of PLCγ1, whereas genistein pretreatment inhibited it (Figure 2b). Because genistein is a nonspecific tyrosine kinase inhibitor with multiple effects in the cell, we also performed experiments using a PLC-specific inhibitor, U73122, and found similar results (Supplementary Figure S1h online). Furthermore, Dex-BSA induced phosphorylation of PKC, which was ameliorated by pretreatment of cells with CC (Figure 2c). Lastly, we pretreated cells with either CC (general PKC inhibitor) or Go6976 (selective PKCα/β1 inhibitor) and assessed phosphorylation of GSK-3β (S9) by immunoblotting. We observed that Dex-BSA induced phosphorylation of GSK-3β; however, pretreatment of cells with either CC or Go6976 selectively inhibited Dex-BSAemediated phosphorylation of GSK-3β (Figure 2d). Taken together, these results show that GCs mediate GSK-3β phosphorylation

by activating PLC/PKC pathway, thus resulting in nuclearization of β-catenin via a pathway that uses mbGR. To confirm that indeed Dex-BSAemediated activation of the PLC/PKC pathway results in transcriptionally active β-catenin, we tested the expression of c-myc (a known β-catenin target gene) (Stojadinovic et al., 2005) by incubating HEKs with Dex-BSA for 4 hours in the presence or absence of PKC inhibitor, CC. We confirmed that Dex-BSAemediated induction of c-myc expression is effectively blocked by CC (Figure 2e). Altogether, we conclude that activation of mbGR induces c-myc expression in a PLC/PKC-dependent manner.

Membranous glucocorticoid receptor/phospholipase C/protein kinase C pathway is not restricted to skin or epithelial cells

Because we observed a novel mbGR-mediated signaling mechanism in HEKs, we then examined whether the observed mbGR/PLC/PKC/GSK-3β signaling pathway is restricted to skin epithelium or whether it is found in cells of other epithelial origin. We treated D407 human retinal epithelial cells (Figure 3a), undifferentiated or differentiated primary human bronchial epithelial cells (Figure 3b; Supplementary Figure S1f online) in presence or absence of 100 nM Dex-BSA for 30 minutes and assayed existence of the mbGR/PLC/PKC/GSK-3β signaling cascade by immunoblotting. We observed a clear induction in phosphorylation of GR (S211), PLCγ1 (Y783), PKC pan (S660), and GSK-3β (S9) in both eye and lung epithelia (Figure 3a and b; Supplementary Figure S1f) as well as the downstream target c-myc in D407 human retinal epithelial cells (Supplementary Figure S1g online). Interestingly, we also observed the presence of the same signaling cascade in primary human fibroblasts (Figure 3c), leading us to conclude that mbGR signaling cascade is conserved not only in various epithelial cell types but also in other cells of mesenchymal origin, like fibroblasts.

Inhibition of protein kinase C restores keratinocyte migration in vitro and accelerates wound closure ex vivo

We found previously that activated GR inhibits wound healing by disrupting migration of keratinocytes (Lee et al., 2005; Stojadinovic et al., 2007). To functionally test whether activation of the PLC/PKC pathway by mbGR inhibits epithelialization and wound closure, migration of keratinocytes was quantified by a wound-scratch assay at 0 hours, 24 hours, and 48 hours. Keratinocytes were treated with Dex or Dex-BSA in the presence or absence of either inhibitors of GR (Ru486), PLCγ (genistein), and PKC (CC) or EGF, a known stimulator. We found that Dex-BSA inhibits keratinocyte migration similarly to Dex, suggesting that mbGR regulates inhibition of migration in HEK (Figure 4; Video 1). Furthermore, inhibition of either GR, PLCγ, or PKC (by Ru486, genistein, and CC, respectively) reversed the Dex-BSAemediated inhibition (Figure 4; Video 1). Similar effects can be observed as early as 4 hours after treatment with Dex-BSA (data not shown). We conclude that sustained stimulation of mbGR by GCs elicits activation of mbGR/PLCγ/PKC signaling cascade and leads to inhibition of keratinocyte migration.

To test functional implications of the mbGR nongenomic pathway, we used two established models: human skin ex vivo and organotypic culture wound model (Ojeh et al., 2014; Stojadinovic and Tomic-Canic, 2013). In both models, skin was wounded by 3-mm punch

biopsies and treated with Dex, Dex-BSA, and EGF as positive control. Histologic assessments at day 4 postwounding show that Dex-BSA treatment inhibited epithelialization similarly to Dex in both models (Figure 5; Supplementary Figure S2 online). Furthermore, pretreatment of skin with CC prevented Dex-BSAemediated inhibition of wound healing. When present alone, CC accelerated wound closure (Figure 5). This acceleration of wound closure by CC can be contributed by blocking of activation of mbGR by endogenously synthesized cortisol (Vukelic et al, 2011).

Taken together, our data show how a new molecular mechanism of activation of the PLC/PKC by GCs via the mbGR results in inhibition of keratinocyte migration and epithelialization, thus contributing to wound healing impairment. Conversely, inhibition of this pathway accelerates wound closure, providing new therapeutic approaches to stimulate wound healing.

DISCUSSION

Here we show a novel mechanism that converts incoming stress signal, mediated by mbGR, into growth signals similar to those of Wnt, by activation of the β-catenin (Figure 6). We also show that stress mediators, GCs, trigger fast-acting nongenomic effects via mbGR that activate the PLC/PKC/β-catenin pathway, induce c-myc, and contribute to impairment of healing. Furthermore, we show that inhibition of PKC restores keratinocyte migration and accelerates epithelialization. Importantly, this novel signaling pathway is functional in many cell types and tissues, suggesting a more general impact. The nongenomic GC signaling that activates PKC and merges multiple major pathways may have significant impacts on understanding molecular mechanisms that govern homeostasis or pathophysiology of cutaneous (and other) diseases. The potential implications of such a concept are immense, as components of the Wnt signaling are key regulators of cell differentiation and proliferation and have, thus, been found to be deregulated in many diseases ranging from skin, cardiovascular, and neuronal disorders to various forms of cancer (Abrahamsson et al., 2009; Clements et al., 2003; Tian et al., 2003; van Gijn et al., 2002). It also underscores the potential role of stress-related signaling in these diseases. However, the contribution of GC-mediated activation of β-catenin in these processes remains to be elucidated.

In the context of skin, Wnt signaling controls development, stem cell cycling, hair follicle expansion, and terminal differentiation of the hair lineage (Fuchs et al., 2004; Zhou et al., 1995). Although β-catenin promotes the recruitment of fibroblast to the wound site, uncontrolled Wnt signaling may lead to aggressive fibromatosis and resemble a hyperactive wound. In most types of chronic wounds, impairment of epithelialization arises from aberrant signaling that governs activity of keratinocytes in response to injury (Brem et al., 2007; Brem et al., 2003; Pastar et al., 2014; Stojadinovic et al., 2005). A hyperproliferative epidermis is a hallmark of the nonhealing epidermal edge of chronic wounds (Nunan et al., 2014; Stojadinovic et al., 2008) that was documented by activation of Wnt pathway and overexpression of c-myc (Eming et al., 2014; Stojadinovic et al., 2005). In spite of the activation of these growth signals, keratinocytes in chronic wounds are not epithelializing because of impairment in migration. In this report, we show a similar paradox. Although stimulation of the membranous fraction of GR results in activation of the same growth

signals (β-catenin nuclearization and subsequent induction of c-myc), it results in inhibition of wound closure. All these findings suggest that GCs, in part via mbGR, may play an important role in inhibition of epithelialization and pathophysiology of chronic wounds.

The identification of PKC as a downstream target of mbGR has multiple implications, especially if one takes into account the relative abundance of PKC signaling in tissues and systemic circulating presence of GC (Czerwinski et al., 2005; Helfrich et al., 2007; Mellor and Parker, 1998; Nishizuka, 1988; Selbie et al., 1993). The PKC family is involved in signals that control cellular survival, proliferation, and differentiation (Denning et al., 1998; Farese et al., 1992; Nishizuka, 1988; Nishizuka, 1995; Wertheimer et al., 2001), and therefore plays a major role in development of diseases (Braiman et al., 1999; Dlugosz and Yuspa, 1993; Gordge et al., 1996; Idris et al., 2001; Prevostel et al., 1995; Standaert et al., 1997; Wallace et al., 2014). Our discovery of PKC activation by mbGR provides new insights into mechanisms by which systemic and local corticosteroids may contribute to PKC-mediated effects. Although we are currently investigating whether mbGR exhibits selective activation of PKC isoenzymes in both skin and other epithelia, further understanding of greater physiologic/pathologic implications of this mechanism that extend beyond skin will provide important insights into systemic GC action and its contribution to various diseases.

Deregulation of physiologic pathways underlies mechanisms of how psychosocial stressors are hypothesized to influence biology of chronic disease. Psychosocial stressors have been known to influence the hypothalamic-pituitary-adrenal axis, one of the major physiologic pathways controlling neuroendrocrine response. GCs are also commonly used as antiemetics in various forms of cancer therapy (Keith, 2008; Mitre-Aguilar et al., 2015; Rutz, 2002). Moreover, cancer patients frequently exhibit alterations in cortisol regulation (Sephton et al., 2000). The discovery that a physiologically relevant dose of glucocorticoids in epithelial and mesenchymal cells stimulates a Wnt-like signaling cascade, which culminates in elevated expression of c-myc, a regulator of cell cycle, may have profound effects on how to potentially mitigate adverse effects of both endogenous and exogenous GCs in cancer patients and opens up many avenues to pharmaceutically target mbGR in conjunction with already established inducers of tumor cell death.

Selective targeting of mbGR, as we have shown above, could prove invaluable in not only understanding various disease development but also targeted steroid therapies and their side effects. This is particularly important in the context of the endogenous constitutive synthesis of cortisol by keratinocytes (Slominski et al., 2015; Slominski et al., 2013). Topical CC, by blocking mbGR activation triggered by endogenously synthesized cortisol, resulted in accelerated epithelialization and subsequent wound healing, suggesting that selective targeting of subsets of GR, such as mbGR, may have therapeutic effects or may alleviate multiple side effects of prolonged GC therapy. It is not surprising that CC shows less-effective rescue of Dex-mediated wound healing inhibition when compared with Dex-BSA. Unlike Dex-BSA, which acts only on mbGR, Dex can stimulate multiple fractions of the receptor and simultaneously activates non-genomic and genomic GR effects.

We identified a novel molecular mechanism by which mbGR exerts a rapid, nongenomic effect via PLC/PKC cascade, leading to activation of β-catenin and c-myc. A functional consequence of mbGR/PLC/PKC pathway is inhibition of keratinocyte migration and wound closure. This mechanism may have major effects on the understanding of how GC signaling regulates homeostasis and its role in development of skin diseases that possibly extends beyond to other tissues.

MATERIALS AND METHODS

Immunofluorescence

Immunofluorescence protocol was followed as previously described (Jozic et al., 2012). Cells were grown to 65% confluency. After washing with phosphate buffered saline, fixing in 5% formalin for 5 minutes, and being permeabilized with 0.1% Triton X-100 (Sigma, St. Louis, MO) for 10 minutes, cells were blocked with 1% BSA in phosphate buffered saline for 30 minutes and then incubated in β-catenin Ab in 1% BSA for 24 hours. This was followed by phosphate buffered saline washes and incubation with Alexa Flour Ab (Thermo Fisher Scientific, Waltham, MA) in 1% BSA for 1 hour. Cells were mounted and nuclei were visualized with propidium iodide.

Immunoblotting

Cell lysates for immunoblotting and cell fractionation were prepared from a subconfluent 10-cm plate of HEKs following published protocol (Jozic et al., 2011; Jozic et al., 2012). Protein from each sample was resolved on 4 to 20% gradient Tris-Glycine gels (Bio-Rad Laboratories, Hercules, CA) and transferred onto polyvinylidene difluoride membranes. A list of antibodies used is provided in Supplementary Materials.

Quantitative PCR

RNA isolation and purification were performed as previously described (Ramirez et al., 2015). A total of 1.0 μg of total RNA from HEK was reverse transcribed using a qScript cDNA kit and real-time PCR was performed in triplicate using the Bio-Rad CFX Connect thermal cycler and detection system (Bio-Rad Laboratories) and a PerfeCTa SYBR Green Supermix (QuantaBio, Beverly, MA). Relative expression was normalized for levels of HPRT1. The primer sequences are provided in Supplementary Materials. Statistical comparisons were performed using Student *t* test.

Wound migration assay

HEK were grown to confluence in a 96-well ImageLock plates (Essen Bioscience, Ann Arbor, MI), treated with 4cμg/ml mitomycin-C, and wounded by scratch with a 96-pin wound making tool (Wound-Maker, Essen Bioscience). Cells were incubated for 48 hours, and two representative images from each well of relative migration were taken every 2 hours after the initial wound using IncuCyte Zoom system (Essen Bioscience) and quantified using Cell Migration Analysis software module (Essen Bioscience).

Ex vivo wound closure

Human skin specimens from reduction surgery were used to generate acute wounds as previously described (Ojeh et al., 2014; Stojadinovic and Tomic-Canic, 2013). Briefly, a 3-mm biopsy punch was used to create acute wounds (n = 9 per treatment), which were treated at the time of wounding in presence, absence, or combination of 1 μM Dex, 0.1 μM Dex-BSA, and 0.2 μM CC and treated daily for 4 days on air-liquid interface with DMEM, 1% antibiotic-antimycotic (Invitrogen) and 10% fetal bovine serum (Lonza, Basel, Switzerland) at 37°C, 5% CO_2, and 95% relative humidity. Ex vivo acute wound specimens were frozen in optimal cutting temperature compound, and rate of healing was analyzed for epithelialization by histology assessment using a Nikon eclipse E400 microscope and NIS Elements software.

Statistics

Results from quantitative experiments are expressed as mean ± standard error of the mean. For pairwise comparisons we used the Student *t* test, and significance was accepted at a $P < 0.05$. Where appropriate, two-way analysis of variance was also used via the Bonferroni multiple comparison procedure (GraphPad Prism; GraphPad Software, La Jolla, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

CC	calphostin C
Dex	dexamethasone
Dex-BSA	BSA-conjugated dexamethasone
GC	glucocorticoid
GR	glucocorticoid receptor
GILZ	glucocorticoid response element-induced leucine zipper
GSK-3β	glycogen synthase kinase 3 beta
HEK	human keratinocyte
mbGR	membranous glucocorticoid receptor

PKC	protein kinase C
PLC	phospholipase C

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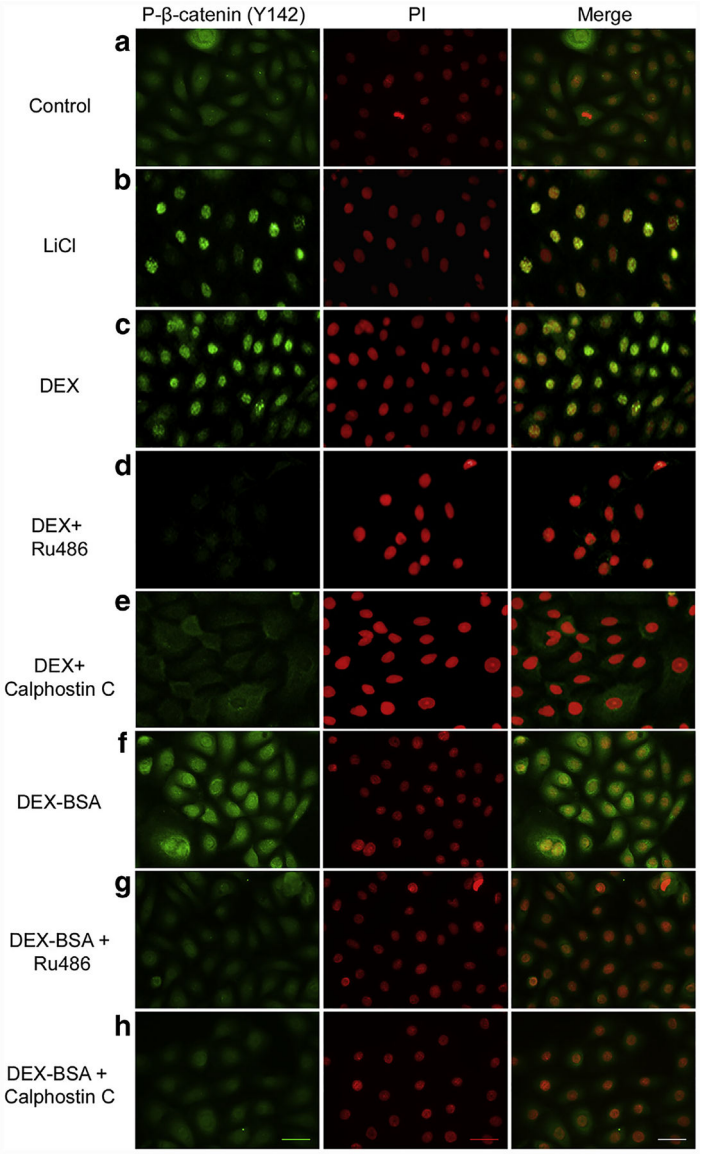

Figure 1. GCs promote nuclearization of β-catenin through PKC.

Primary human keratinocytes control (**a**) or stimulated with either 30 mM LiCl (**b**), 1 μM Dex (**c**) or 100 nM Dex-BSA (**f**) for 24 hours in presence of 1 μM Ru486 (**d** and **g**) or 0.2 μM CC (**e** and **h**) are shown. Presence of phospho-β-catenin (Y142) was visualized by immunofluorescence (green; left panels). Nuclei were visualized by staining with PI (red; middle panels) and relative nuclearization was assessed by merging the two images using ImageJ (with co-localization appearing yellow; right panels). CC, calphostin C; Dex, dexamethasone; Dex-BSA, BSA-conjugated dexamethasone; GC, glucocorticoid; LiCl, lithium chloride; PI, propidium iodide; PKC, protein kinase C. Scale bar = 50 μm.

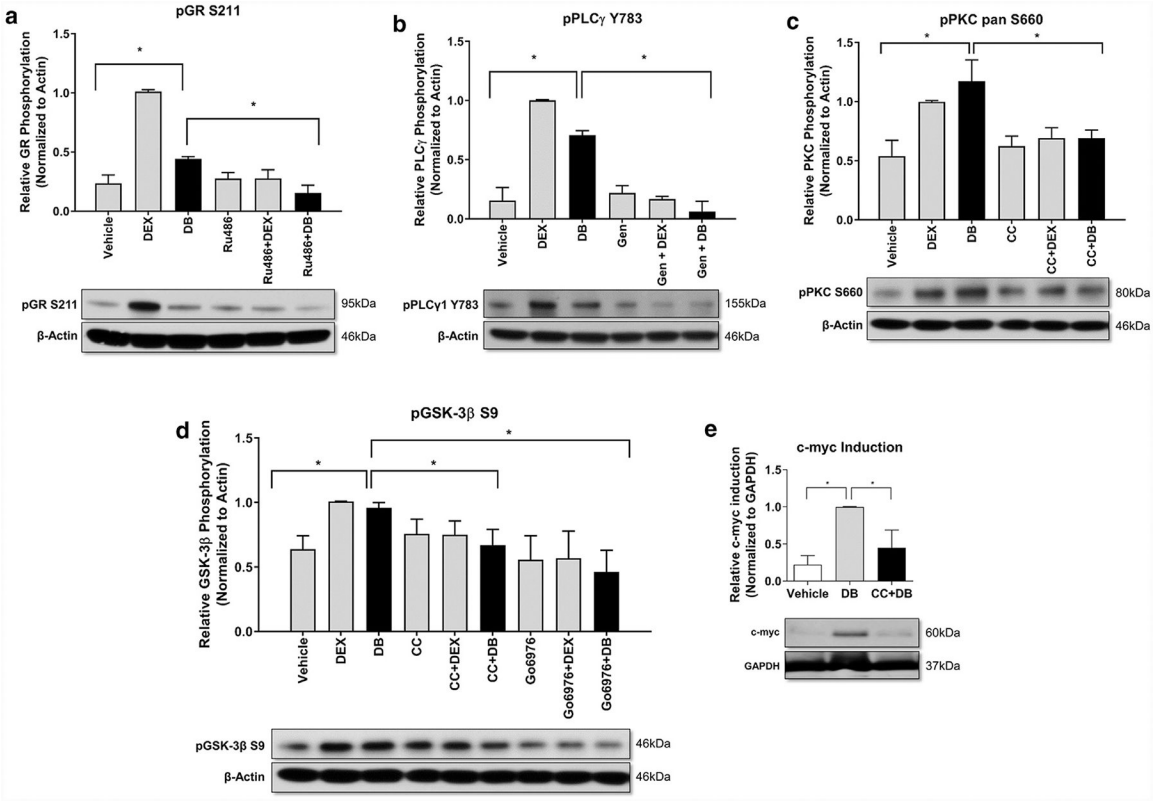

Figure 2. mbGR stimulation activates PLC/PKC signaling cascade and induces phosphorylation and subsequent inactivation of GSK-3β resulting in c-myc induction.

Cells were stimulated with vehicle (DMSO), 1 μM Dex, or 100 nM Dex-BSA (DB) for 30 minutes upon treatment with GR antagonist Ru486 (1 μM), PLCγ inhibitor, genistein (50 μM) or PKC inhibitors CC (200 nM) or Go6976 (4μM). Phosphorylation of GR (**a**), PLCγ (**b**), PKC (**c**), and GSK-3β (**d**) were assessed by western blot. (**e**) Cells were stimulated with vehicle (DMSO) or 100 nM Dex-BSA for 4 hours upon treatment with 200 nM CC, and induction of c-myc was assessed by western blotting. All quantifications were performed using ImageJ with error bars corresponding to standard deviation from n = 3. CC, calphostin C; Dex, dexamethasone; Dex-BSA, BSA-conjugated dexamethasone (DB); GR, glucocorticoid receptor; GSK-3β, glycogen synthase kinase 3 beta; mbGR, membranous glucocorticoid receptor; PKC, protein kinase C; PLC, phospholipase C. $*P \leq 0.05$ (Student *t* test).

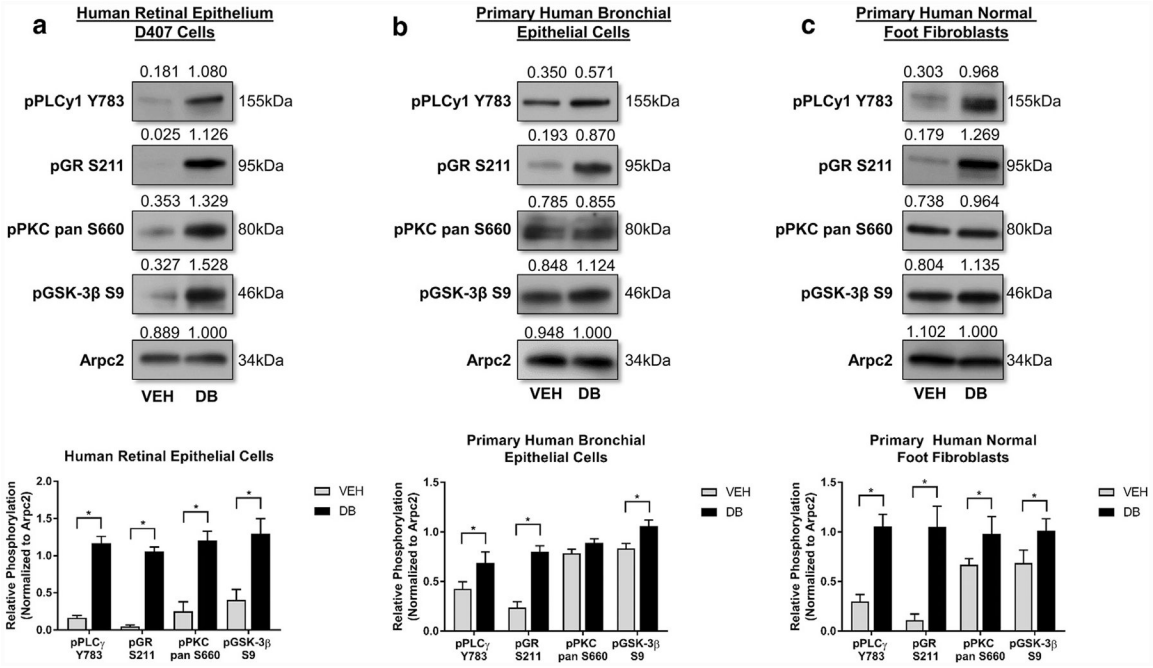

Figure 3. mbGR mediated activation of PLCγ/PKC/GSK-3β is present not only in cells of epithelial origin (eye and lung), but also in cells of mesenchymal origin (foot fibroblasts).

Presence of mbGR mediated activation of PLCy/PKC/GSK-3B signaling cascade was assessed in (**a**) D407-human retinal epithelial cells and (**b**) primary human bronchial epithelial cells, as well as in cells of nonepithelial origin like (**c**) primary human foot fibroblasts. Cells were stimulated with vehicle (DMSO) or 100 nM Dex-BSA (DB) for 30 minutes, and phosphorylation of PLCγ, GR, PKC pan, and GSK-3β were assessed by western blot, with Arpc2 serving as loading control. All quantifications were performed using ImageJ with error bars corresponding to standard deviation from n = 3. Dex-BSA, BSA-conjugated dexamethasone (DB); GR, glucocorticoid receptor; GSK-3β, glycogen synthase kinase 3 beta; mbGR, membranous glucocorticoid receptor; PKC, protein kinase C; PLC, phospholipase C. $*P \leq 0.05$ (Student *t* test).

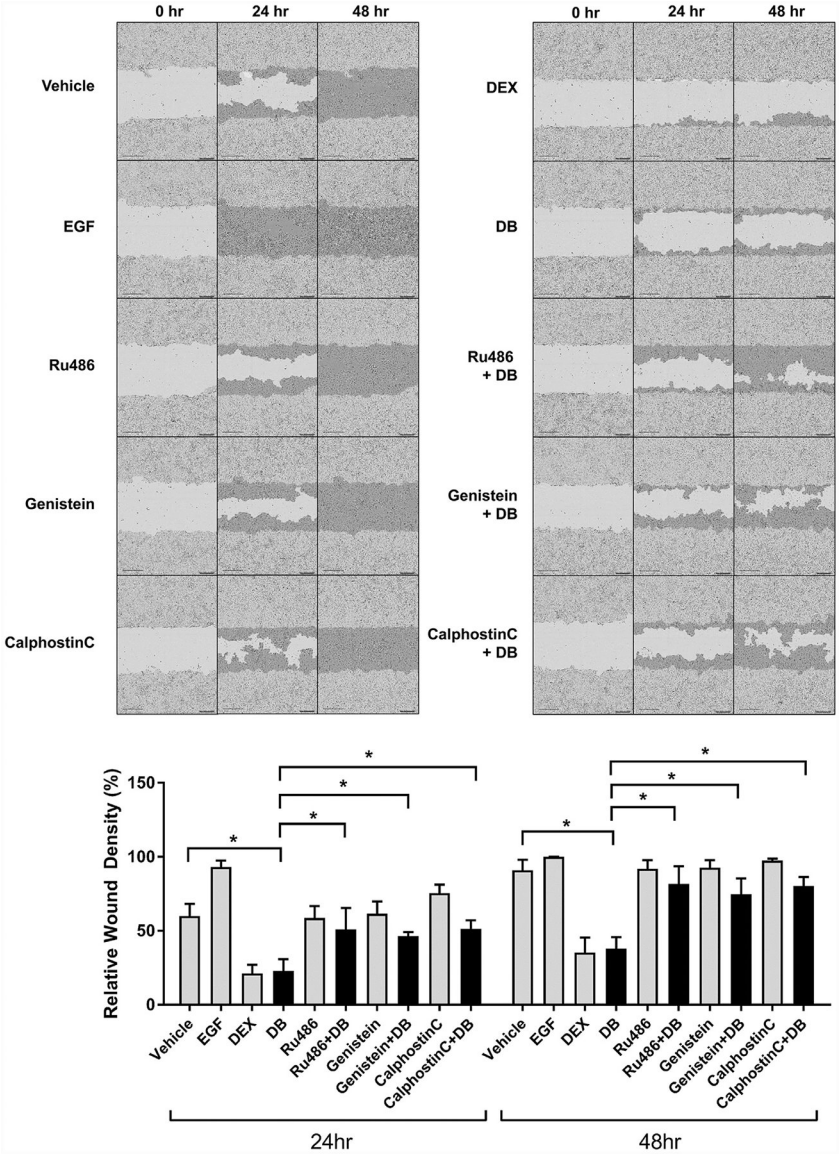

Figure 4. Selective activation of the mbGR results in inhibition of keratinocyte migration.

Primary human keratinocytes were pretreated with 4 μg/ml mitomycin-C and stimulated with vehicle (DMSO), 25 ng/ml EGF (positive control), 1 μM Dex or 100 nM Dex-BSA (DB). Cells were wounded by a scratch and their migration was assessed at the time of the scratch (0 h) every 2 hours for 48 hours. Representative images at 0, 24, and 48 hours after the initial scratch were used to quantify migration by using Cell Migration Analysis software module (Essen Bioscience) comparing relative wound density, with light gray corresponding to initial wound scratch and dark gray corresponding to repopulation of the wound over time. Error bars correspond to standard deviation from n = 16. $*P \leq 0.05$ (Student *t* test). Dex, dexamethasone; Dex-BSA, BSA-conjugated dexamethasone (DB); mbGR, membranous glucocorticoid receptor.

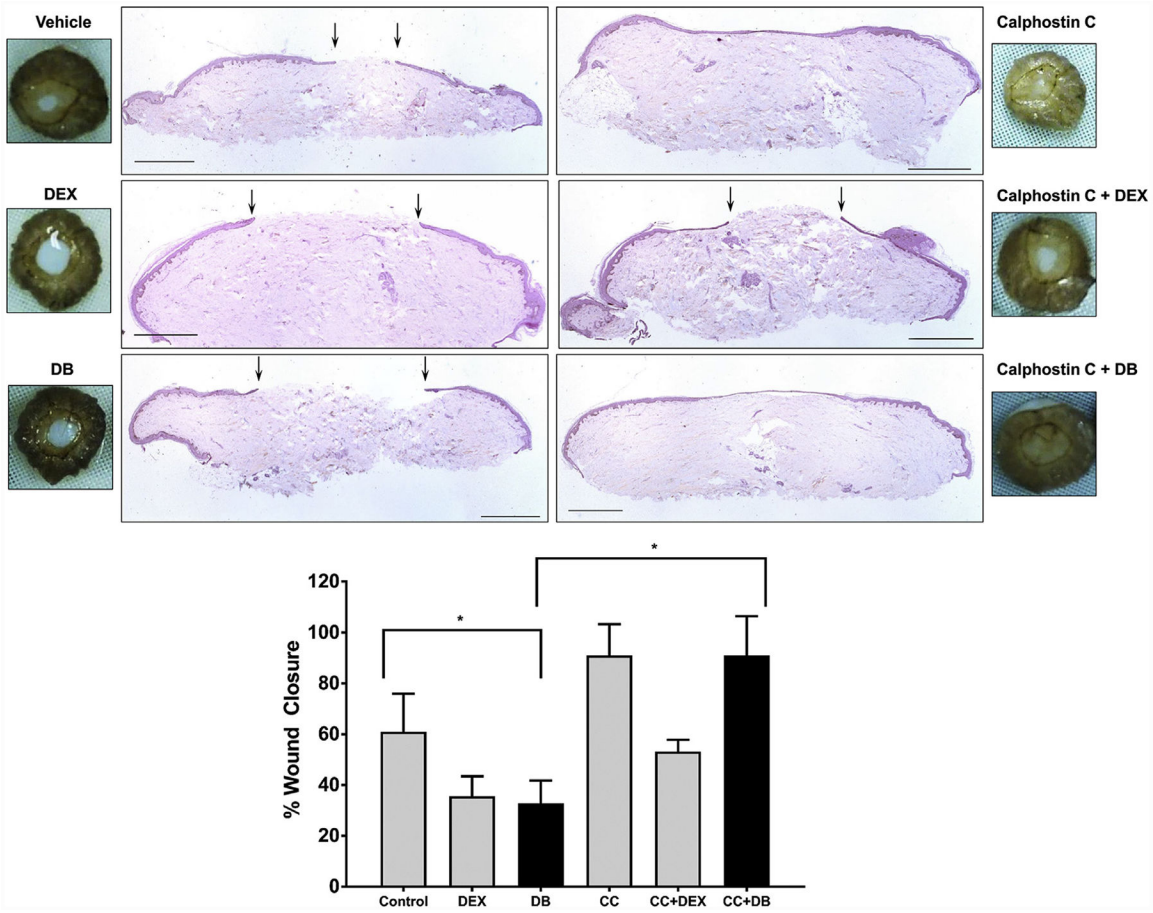

Figure 5. Selective targeting of mbGR by Dex-BSA impedes epithelialization and can be ameliorated by inhibiting PKC in an ex vivo model of wound closure.

Normal human skin was wounded using a 3-mm biopsy punch and maintained at the air-liquid interface in presence or absence of 1 μM Dex, 100 nM Dex-BSA (DB), or PKC inhibitor CC (0.2 μM). Wound healing was assessed at day 4 after wounding, a time when exponential epithelialization occurs. Wound closure was quantified by histology using ImageJ software. Gross photos show visual signs of closure and correspond to the histology assessments with black arrows pointing to epithelial tongue location at day 4. Error bars correspond to standard deviation from n = 4. *$P \leq 0.05$ (Student *t* test). Scale bar 1 = mm. Dex, dexamethasone; Dex-BSA, BSA-conjugated dexamethasone (DB); mbGR, membranous glucocorticoid receptor; PKC, protein kinase C.

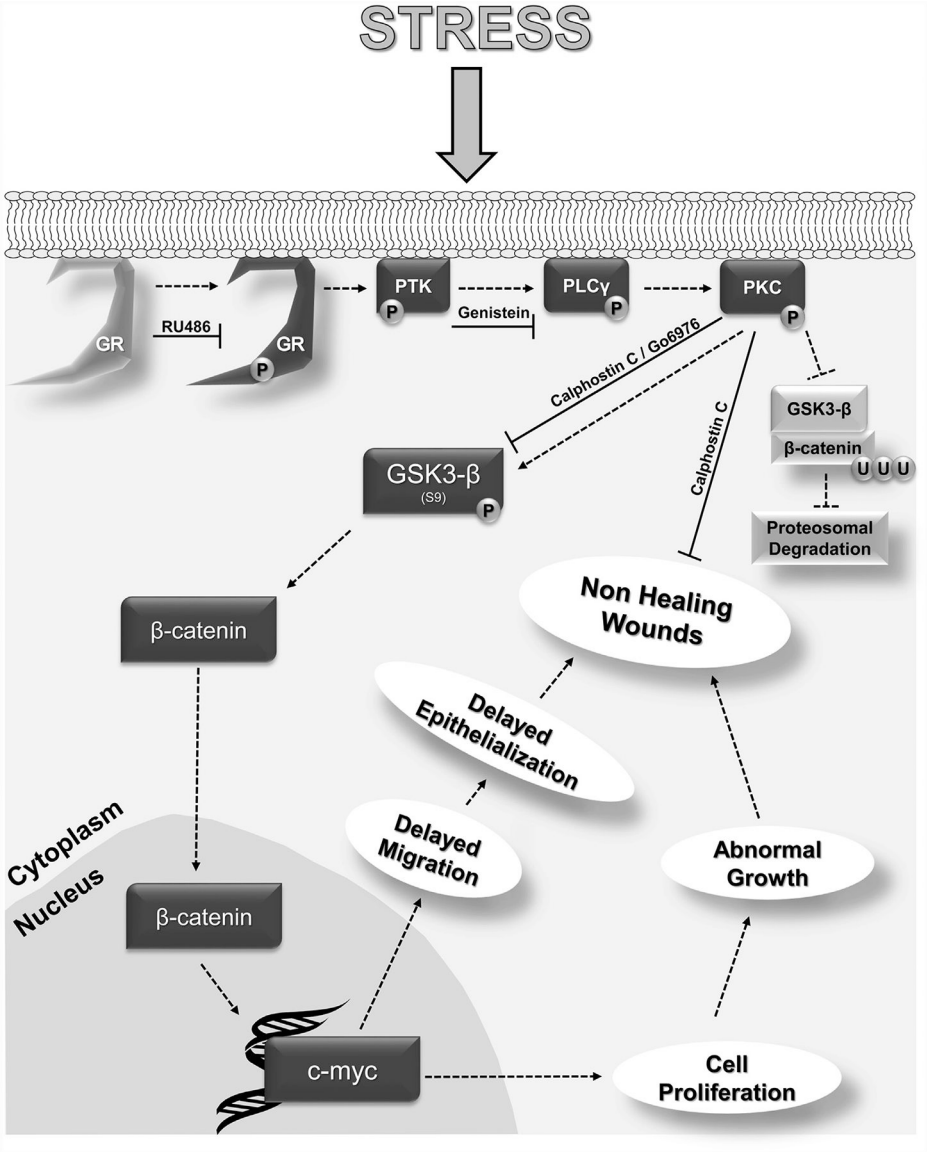

Figure 6. Proposed mechanism by which GC-mediated activation of mbGR contributes to inhibition of keratinocyte migration and wound healing.

A diagram summarizing findings from this study is shown. Upon binding to the membranous fraction of GR, GCs induce a phosphorylation and activation of GR, followed by activation PLCγ (via PTK), PKC, and subsequent phospho-inactivation of GSK-3β. This in turn liberates β-catenin from the inactivation complex thereby allowing it to translocate into the nucleus and induce c-myc expression, thus inducing cell proliferation while delaying keratinocyte migration and subsequent epithelialization. GC, glucocorticoid; GR, glucocorticoid receptor; GSK-3β, glycogen synthase kinase 3 beta; mbGR, membranous glucocorticoid receptor; P, phosphorylation; PKC, protein kinase C; PLC, phospholipase C; PTK, protein tyrosine kinase; U, ubiquitination.