Acute Wounding Alters the Beta2-Adrenergic Signaling and Catecholamine Synthetic Pathways in Keratinocytes

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Keratinocyte migration is critical for wound re-epithelialization. Previous studies showed that epinephrine activates the beta2-adrenergic receptor (B2AR), impairing keratinocyte migration. Here, we investigated the keratinocyte catecholamine synthetic pathway in response to acute trauma. Cultured keratinocytes were scratch wounded and expression levels of the B2AR and catecholamine synthetic enzymes tyrosine hydroxylase and phenylethanolamine-N-methyltransferase were assayed. The binding affinity of the B2AR was measured. Wounding downregulated B2AR, tyrosine hydroxylase, and phenylethanolamine-N-methyltransferase expression, but pre-exposure to timolol, a beta-adrenergic receptor antagonist, delayed this effect. In wounded keratinocytes, B2AR-binding affinity remained depressed even after its expression returned to prewounding levels. Keratinocyte-derived norepinephrine increased after wounding. Norepinephrine impaired keratinocyte migration; this effect was abrogated with B2AR-selective antagonist ICI-118,551 but not with B1AR-selective antagonist bisoprolol. Finally, for clinical relevance, we determined that norepinephrine was present in freshly wounded skin, thus providing a potential mechanism for impaired healing by local B2AR activation in wound-edge keratinocytes. Taken together, the data show that keratinocytes modulate catecholamine synthetic enzymes and release norepinephrine after scratch wounding. Norepinephrine appears to be a stress-related mediator that impairs keratinocyte migration through activation of the B2AR. Future therapeutic strategies evaluating modulation of norepinephrine-related effects in the wound are warranted.

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INTRODUCTION

The directed migration of keratinocytes is critical to wound re-epithelialization and is controlled by many factors (reviewed in (Santoro and Gaudino, 2005; Sivamani *et al.*, 2007)). One important modulator is the beta-adrenergic receptor.

The beta-adrenergic receptor signaling system has an important role in epidermal wound physiology. Activation of the beta2-adrenergic receptor (B2AR) delays epidermal barrier repair, whereas blockade of this receptor increases it (Denda *et al.*, 2003). Furthermore, isoproterenol, a synthetic pharmacological agonist for the (B2AR) decreases keratinocyte migratory speed, reduces *in vitro* scratch-wound closure, and delays *ex vivo* human wound re-epithelialization (Pullar *et al.*, 2003, 2006a). Conversely, blockade of the keratinocyte B2AR increases migratory speed and accelerates wound re-epithelialization (Pullar *et al.*, 2006b; Sivamani *et al.*, 2009b; Pullar *et al.*, 2012). Thus, wound re-epithelialization can be modulated through the B2AR through its control of keratinocyte migration. Catecholamines are the endogenous agonists for the adrenergic family of receptors and epinephrine has the highest selectivity and affinity for the B2AR (Stiles *et al.*, 1984; Frielle *et al.*, 1988). Earlier work has demonstrated that epinephrine inhibits keratinocyte migration through the B2AR (Donaldson and Mahan, 1984) and more recent work has shown that keratinocytes themselves possess the enzymes necessary for the synthesis of epinephrine (Schallreuter *et al.*, 1992; Pullar *et al.*, 2006b; Sivamani *et al.*, 2009b), providing the potential for an autocrine inhibitory loop.

Stress, such as UVB irradiation (Iizuka *et al.*, 1985; Chai *et al.*, 1996) or thermal injury (Sivamani *et al.*, 2009b) modulates keratinocyte expression of B2AR and cyclic AMP. However, acute (non-thermal) wounding of the skin is much more common, and the effects of this trauma on catecholamine

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Abbreviations: B1AR, beta1-adrenergic receptor; B2AR, beta2-adrenergic receptor; DOPEG, 3,4-dihydroxyphenylethylene glycol; PNMT, phenylethanolamine-N-methyltransferase; TH, tyrosine hydroxylase

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generation, B2AR expression, pathway signaling, and healing of the epidermis has not been studied. Assays that evaluate the *en masse* movement of sheets of cells from the free edge, such as the scratch-wound assay, have served as a surrogate for investigating the modulation of wound healing (Sivamani *et al.*, 2009b; Melchionna *et al.*, 2012; Meyer *et al.*, 2012; Rotty and Coulombe, 2012; Shibata *et al.*, 2012; Chen *et al.*, 2013; Pan *et al.*, 2013) and was utilized here to fill the information gap regarding the influence of catecholamines and the B2AR in epithelial wound healing.

RESULTS

Keratinocytes decrease B2AR expression in response to wounding

Confluent keratinocyte monolayers were scratch wounded and assessed for the expression of the B2AR at various times after scratch wounding. Keratinocytes decreased B2AR expression by 1 hour after wounding, but this expression recovered toward unwounded levels by 6 hours after wounding (Figure 1a). Downregulation of the B2AR is well described in response to ligand binding of the B2AR (Shenoy *et al.*, 2001; Johnson, 2006), but it should be noted that this downregulation occurred in the absence of exogenously added epinephrine. However, keratinocytes can synthesize epinephrine (Schallreuter *et al.*, 1992; Pullar *et al.*, 2006b) that could be responsible for the observed downregulation. We therefore investigated whether this downregulation of the B2AR could be delayed by incubating the scratch wounds with a beta-adrenergic antagonist. We incubated keratinocytes with 10 μM timolol, a non-specific BAR antagonist, to block the B2AR. The addition of timolol delayed the downregulation of B2AR expression (Figure 1b).

Scratch wounding decreases the affinity of the keratinocyte BAR

Keratinocytes in confluent cultures that were scratch wounded showed decreased affinity of the beta-adrenergic receptor at 24 hours after wounding as evidenced by binding affinity curves and a Scatchard plot (Figure 2a and b). The overall binding sites increased from control, $B_{max} = 32.44$, to 24 hours post-scratch, $B_{max} = 52.01$ (Figure 2c). The overall binding affinity decreased from control, $K_d = 0.2862$, to 24 hours post-scratch, $K_d = 0.43$ (Figure 2c).

Keratinocytes decrease synthesis of catecholamines in response to scratch wounding

Previous reports have shown that keratinocytes express enzymes necessary for the synthesis of catecholamines (Schallreuter *et al.*, 1992; Pullar *et al.*, 2006b; Sivamani *et al.*, 2009a, 2009b) and we investigated how scratch wounding modulates the expression of these enzymes. Among the catecholamines, epinephrine has a much greater affinity for the B2AR in comparison to norepinephrine. As such, we investigated the expression of both tyrosine hydroxylase (TH), which is the rate-limiting enzyme in the catecholamine synthesis pathway, and phenylethanolamine-N-methyltransferase (PNMT), which is the enzyme required for the final conversion of norepinephrine into epinephrine. We observed that both TH and PNMT transiently decreased by 1 hour after scratch wounding and returned to baseline expression by 24 hours (Figure 3a and b), similar to the B2AR. The addition of timolol-abrogated modulation of TH expression and delayed the decrease in PNMT expression such that it decreased at 2 hours instead of at 1 hour after the scratch wound (Figure 3c and d). Both TH and PNMT expression decreased during the post-scratch time in a similar fashion as the B2AR expression (compare with Figure 1).

Scratch wounding increases extracellular epinephrine and norepinephrine levels

After scratch wounding, epinephrine levels in the medium did not change after 24 hours (Figure 4a). However, norepinephrine levels were elevated at 1 hour and approximately 3.7-fold at 24 hours after scratch wounding (Figure 4b). To assess for the breakdown product of epinephrine and norepinephrine, we assayed for the levels of 3,4-dihydroxyphenylethylene glycol (DOPEG) and found that this compound

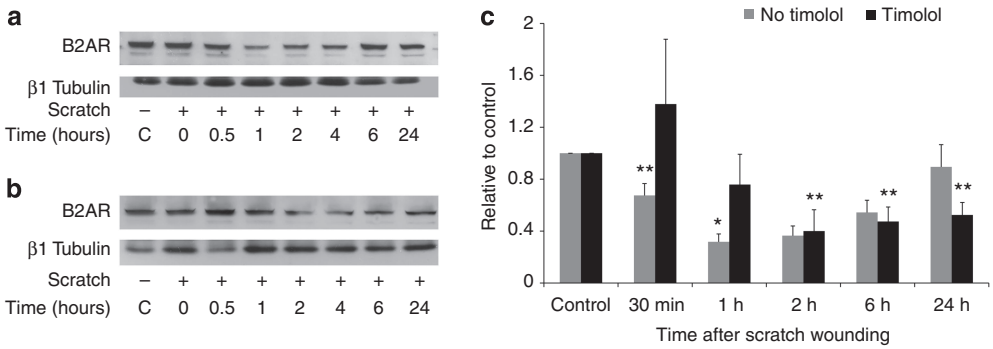
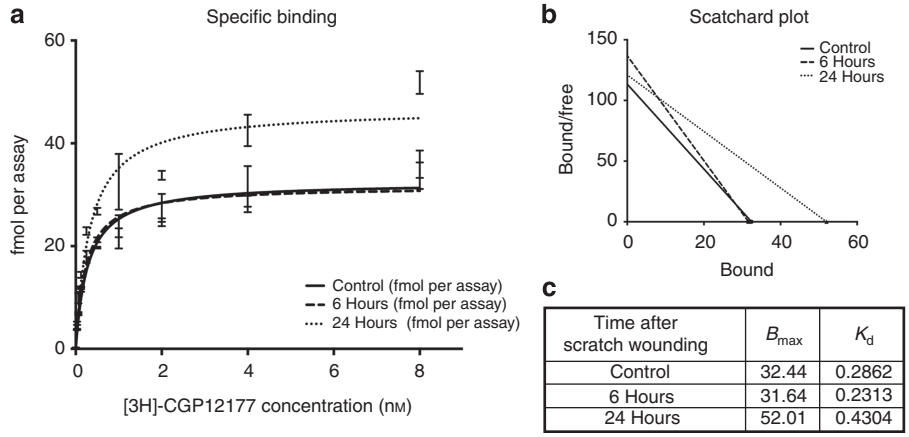


Figure 1. Keratinocyte beta2-adrenergic receptor (B2AR) expression transiently decreases after scratch wounding. (**a**) Confluent keratinocyte cultures were scratch-wounded and allowed to recover in keratinocyte growth medium. The expression of the beta2AR (B2AR) was monitored over time and was decreased by 30 minutes after wounding. Expression of the B2AR recovered toward control keratinocyte expression levels by 6 and 24 hours after wounding. (**b**) Pretreatment with 10 μM timolol (betaAR antagonist) delayed the decrease in B2AR expression in response to scratch wounding to 2 hours, with no evidence of recovery of B2AR expression by 24 hours. Unscratched confluent keratinocytes served as controls (C). (**c**) Graphical depiction of the timeline for the decrease of B2AR with and without timolol. Data are representative of three repeat experiments in two different keratinocyte strains. Similar temporal results were obtained in the two strains examined. Error bars represent mean ± SEM; $^*P < 0.01$; $^{**}P < 0.05$.



c

Time after scratch wounding	B_{max}	K_d
Control	32.44	0.2862
6 Hours	31.64	0.2313
24 Hours	52.01	0.4304

Figure 2. Scratch wounding decreases the affinity of the keratinocyte beta-adrenergic receptor for its ligand. (**a**) Confluent keratinocytes were scratch wounded and then collected at 6 and 24 hours after wounding. The cells were exposed to [3H]-CGP12177, a radionuclide and beta-adrenergic receptor ligand, in the absence (total binding) or presence of 5 μM propranolol (non-specific binding). (**b**) Binding saturation curves were linearized by plotting the bound radioligand to unbound radioligand against the bound ligand concentration. The linear curves were generated with a least-squares fit to the data. The data represent the results of three replicates. (**c**) Keratinocytes had decreased binding affinity for the beta-adrenergic receptor ligand 24 hours after wounding in comparison with control unwounded keratinocytes or keratinocytes 6 hours after wounding. Error bars represent mean ± SEM.

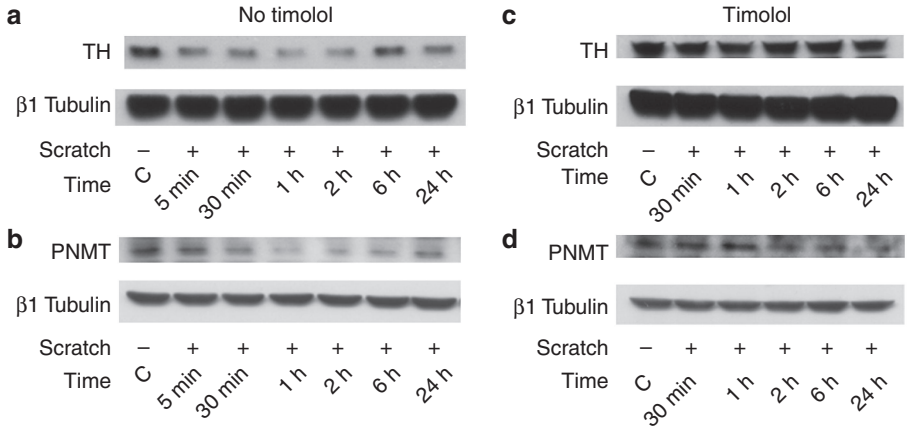


Figure 3. Scratch wounding transiently decreases keratinocyte expression of catecholamine-synthesizing enzymes and is delayed by timolol (betaAR antagonist). Confluent keratinocytes were scratch wounded and allowed to recover in keratinocyte growth medium. (**a**) Tyrosine hydroxylase (TH), the rate-limiting enzyme of the catecholamine synthesis pathway, decreased in expression by 30 minutes after scratch wounding. By 6 hours after scratch wounding, the expression of TH returned to baseline. (**b**) The expression of phenylethanolamine-N-methyl transferase (PNMT), the enzyme required for the synthesis of epinephrine, decreased by 1 hour, in a similar manner as the TH expression. PNMT expression returned toward baseline by 24 hours after scratch wounding. Unscratched confluent keratinocytes served as controls (C). (**c**) Confluent keratinocytes were scratch wounded and allowed to recover in keratinocyte growth medium incubated with 10 mM timolol. Pretreatment with timolol completely abrogates any modulation of the TH expression after scratch wounding. (**d**) The presence of timolol delays and mitigates the downregulation of PNMT. Unscratched confluent keratinocytes served as controls (C). Data are representative of two repeat experiments in at least two different keratinocyte strains.

was 2.2-fold increased at 1 hour and 1.9-fold increase at 24 hours after wounding (Figure 4b), suggesting potential metabolic breakdown of released epinephrine to this compound.

Scratch wounding decreases intracellular epinephrine and norepinephrine levels

Both epinephrine and norepinephrine were at the highest intracellular levels prior to wounding and then subsequently decreased 30 minutes and 1 hour after wounding (Figure 4b). However, intracellular levels of epinephrine and norepinephrine both began to increase again by 24 hours. No intracellular DOPEG was detected.

Norepinephrine reduces keratinocyte motility via the B2AR

Norepinephrine reduced keratinocyte cell motility in a dose-dependant manner (Figure 5a). The cells were then co-incubated with selective B1AR or B2AR antagonists to further evaluate norepinephrine's BAR-mediated effects. Co-incubation of the selective B1AR antagonist bisoprolol did not mitigate norepinephrine-induced reduction in keratinocyte motility (Figure 5b). However, co-incubation with the

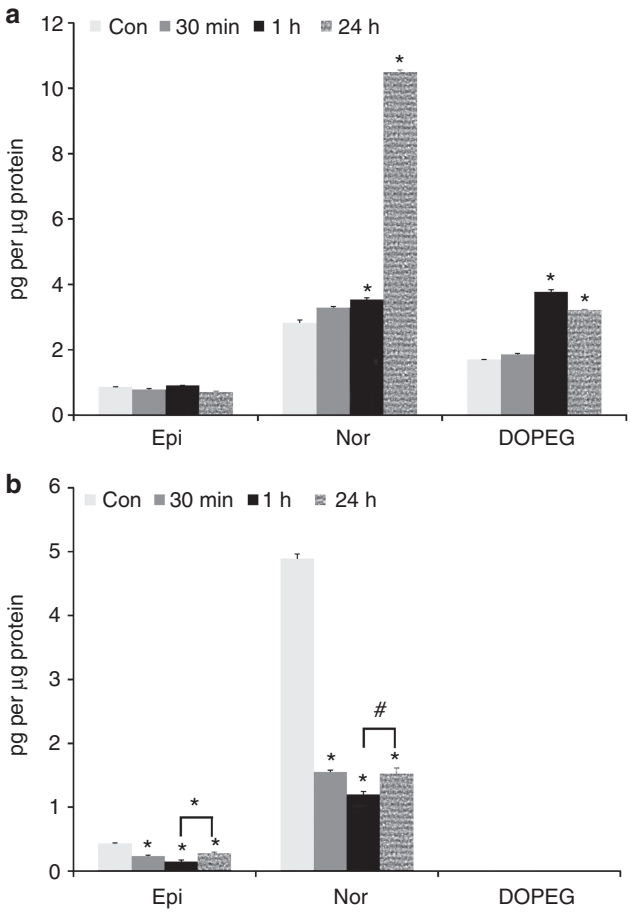


Figure 4. Scratch wounding leads to the release of catecholamines. (**a**) Scratch wounding of confluent normal human keratinocytes (NHKs) leads to the release of norepinephrine and 3,4-dihydroxyphenylethylene glycol (DOPEG), a breakdown product of epinephrine and norepinephrine, at 1 hour and 24 hours after scratching. However, no change in epinephrine was measured in the supernatant. (**b**) Intracellular catecholamines. No intracellular DOPEG was measured. Epi, epinephrine; Nor, norepinephrine; DOPEG, 3,4-dihydroxyphenylethylene glycol. $^{*}P<0.01$; $^{\#}P<0.05$. The units pg µg protein^{-1} refers to pg per µg of cellular protein. Error bars represent mean ± SEM; $n=4$ samples from two different keratinocyte strains.

selective B2AR antagonist ICI-118,551 abrogated norepinephrine-mediated reduction in keratinocyte motility (Figure 5b).

Noerpinephrine is present in human cutaneous wounds

Human skin tissue from acutely generated surgical wounds was harvested within 30 minutes of surgical wounding from five different donors, and processed for extraction and quantification of catecholamines. Norepinephrine was present in the wounds (Figure 5c).

DISCUSSION

The catecholamine network and the B2AR have an important role in keratinocyte wound-healing physiology (reviewed in (Sivamani *et al.*, 2007)). Activation of the B2AR delays epidermal barrier homeostasis (Denda *et al.*, 2003), reduces keratinocyte motility (Pullar *et al.*, 2003), and delays wound re-epithelialization (Pullar *et al.*, 2006a). Conversely, blockade of the B2AR leads to increased keratinocyte motility and accelerates wound re-epithelialization (Pullar *et al.*, 2006b; Sivamani *et al.*, 2009b). Notably, blockade of the B2AR increases keratinocyte motility and wound re-epithelialization even in the absence of an exogenous agonist, suggesting an autocrine mechanism for activation of the keratinocyte B2AR (Pullar *et al.*, 2006b; Sivamani *et al.*, 2009b). Indeed, keratinocytes possess the capacity to synthesize catecholamines (Schallreuter *et al.*, 1992; Pullar *et al.*, 2006b), providing the potential for a negative feedback loop.

Here, we used the wounding of a confluent sheet of human keratinocytes as the experimental paradigm in which to evaluate the effects of acute wounding of the epidermis on the B2AR pathway. In doing so, we demonstrate the utility of the scratch-wound assay to model epithelial wound healing, similar to previous investigations (Sivamani *et al.*, 2009b; Melchionna *et al.*, 2012; Meyer *et al.*, 2012; Rotty and Coulombe, 2012; Shibata *et al.*, 2012; Chen *et al.*, 2013). We show that cultured keratinocytes transiently reduce their expression of the B2AR and that the B2AR has decreased affinity for its ligand in response to scratch wounding. As activation of the B2AR decreases migratory rate, the downregulation of the B2AR expression and affinity could represent a negative feedback mechanism where keratinocytes shift to a pro-migratory phenotype. Our finding that the keratinocyte BAR has lowered affinity for its ligand even when levels have returned to pre-scratch levels at 24 hours further support that keratinocytes have shifted into a pro-migratory phenotype. This is in agreement with previous reports that have shown that keratinocytes respond to scratch wounding by transiently shifting to a more migratory phenotype through upregulation of multiple genes such as BCAR3 and MAP4K, although the influence of the B2AR was not noted (Fitsialos *et al.*, 2007). We observed that blockade of the B2AR delays its downregulation, highly suggestive of a negative feedback mechanism involving activation of this receptor.

Our results also demonstrate that norepinephrine is an important component of the acute *in vitro* epithelial wound microenvironment. Norepinephrine levels were increased by 1 hour after wounding keratinocyte sheets and markedly increased 24 hours after wounding. In addition to epinephrine, norepinephrine binds, activates, and modulates the expression of the B2AR (Swaminath *et al.*, 2004; Wang *et al.*, 2008; Weitl and Seifert, 2008; Reiner *et al.*, 2010; Masuda *et al.*, 2012). In this study, we noted that at 24 hours after wounding, the norepinephrine levels were an order of magnitude (14-fold) higher than epinephrine levels, and may contribute to B2AR stimulation, especially as keratinocytes have been shown to relatively express almost 100-fold less B1AR than B2AR (Steenhuis *et al.*, 2011). Interestingly, although previous work has focused on effects of synthetic catecholamines (e.g., isoproterenol), our finding is of particular significance in we demonstrate that keratinocytes are able to modulate B2AR

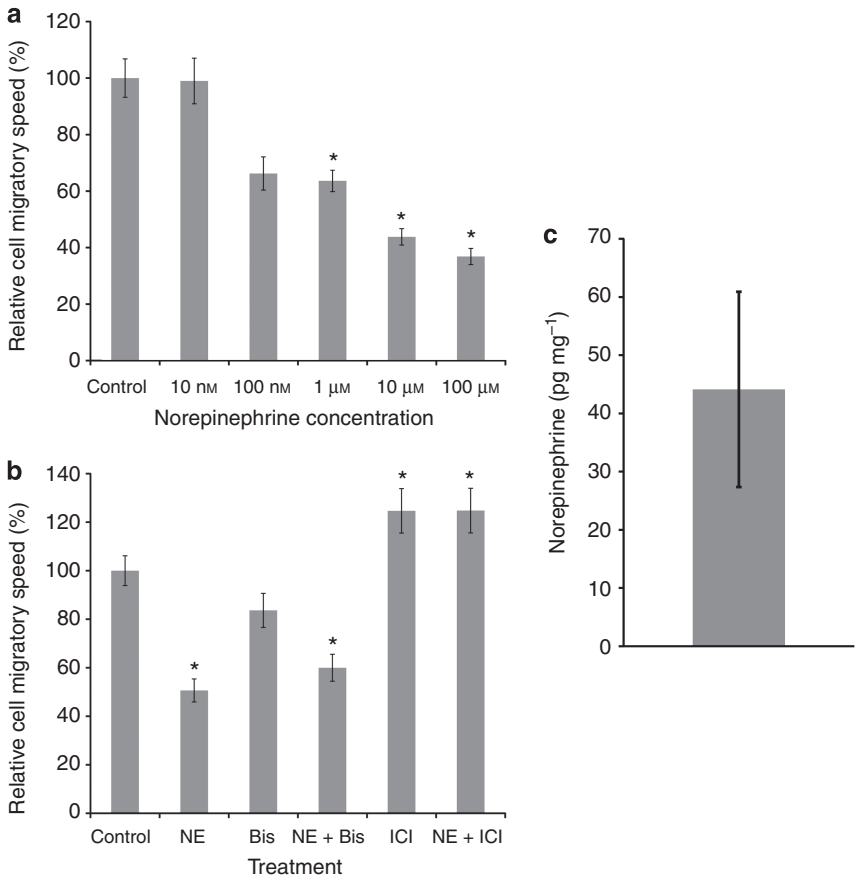


Figure 5. Norepinephrine reduces the migratory rate of cultured keratinocytes via the beta2-adrenergic receptor (B2AR) and is found in human wounds. (**a**) Norepinephrine impairs single-cell migration in a dose-dependent manner. *$P<0.01$; $n=40$. Data are representative of triplicate experiments in two strains. (**b**) To test for receptor-specific-mediated effects, experiments were performed where 10 mM of norepinephrine (NE) was either co-incubated with the B1AR-selective antagonist, 10 mM bisoprolol (Bis), or with the B2AR-selective antagonist, 10 mM ICI-118,551 (ICI). The addition of bisoprolol did not reverse the impaired motility induced by norepinephrine. However, the addition of ICI-118,551, reversed the impaired motility induced by NE. *$P<0.01$; $n=45$. Data are representative of triplicate experiments in two strains. (**c**) Tissue collected from human skin wounds revealed the presence of norepinephrine. Norepinephrine amounts were normalized to the weight of removed wound tissue. $n=5$ wounds from five different individuals. Error bars represent mean ± SEM.

expression through an autocrine mechanism involving the activation of the B2AR with endogenously generated norepinephrine. Our results are likely not limited to skin keratinocytes, as other epithelia, such as the corneal and oral epithelium predominantly express B2AR (Ghoghawala *et al.*, 2008; Steenhuis *et al.*, 2011). As norepinrphine is also found in these tissues (Murphy *et al.*, 1998; Kennedy *et al.*, 2001; Ghoghawala *et al.*, 2008; Steenhuis *et al.*, 2011), it is quite possible that norepinephrine has a significant role in wound healing of these epithelia as well. Additional studies are needed to evaluate this possibility.

Activation-mediated downregulation of B2AR is a well-known phenomenon (Barak and Caron, 1995). Although keratinocytes are capable of synthesizing epinephrine (Pullar *et al.*, 2006b; Sivamani *et al.*, 2009b), an endogenous ligand to the B2AR, it is unclear which population of keratinocytes is responsible for the release of the B2AR ligand in the scratch-wound assay. Several populations of keratinocytes are present in the scratch wound including those that are acutely injured during the scratch wound and the cells that are found away from the wound edge that were not directly injured by the scratch wound itself. It remains to be determined whether the acutely injured cells or the cells at the wound edge are responsible for the release of the keratinocyte-secreted B2AR ligand. In the particular assay used in this study, the majority of the damaged cells were removed as part of the scratch wounding procedure, and our subsequent analyses are more likely representative of the physiological responses of the wound-edge keratinocytes rather than the acutely injured keratinocytes. Regardless, our results clearly show that B2AR expression is modulated through activation of the receptor. Interestingly, we found that blockade of the B2AR delayed but did not completely block downregulation of the B2AR, suggesting that a mechanism independent of activation of the receptor may also be involved in downregulation of the B2AR. Indeed, keratinocytes have previously been shown to decrease their expression of the B2AR in response to chemical irritation (Chang *et al.*, 1998) or ultraviolet injury (Chai *et al.*, 1996). A similar B2AR-independent injury-related mechanism may be involved in the late decrease in B2AR expression.

We found that the changes in the expression of the catecholamine synthetic enzymes, TH and PNMT, followed the pattern of modulation of the B2AR. Our results suggest that expression of the B2AR is important for TH and PNMT expression. In agreement with this, basal and inducible TH transcription is regulated by a cAMP response element (Kim *et al.*, 1993; Sabban, 1997; Lim *et al.*, 2000; Lewis-Tuffin *et al.*, 2004). Increases in cAMP, irrespective of the inducing stimuli, lead to increased TH expression (Pliego Rivero *et al.*, 1999; Lewis-Tuffin *et al.*, 2004; DeCastro *et al.*, 2005; Maharjan *et al.*, 2005). Also, it has been shown that increases in intracellular concentrations of Ca^{2+} also increase TH expression through the cAMP response element-dependent and independent mechanisms (Nagamoto-Combs *et al.*, 1997). Moreover, catecholaminergic activation of the B2AR potentiates glucocorticoid receptor activation (Schmidt *et al.*, 2001) and the glucocorticoid receptor element is a noted inducer of TH gene expression (Hagerty *et al.*, 2001a, b) and PNMT expression (Wong *et al.*, 1998; Tai *et al.*, 2002). Therefore, it is not unexpected that we observe TH and PNMT expression to transiently decrease and return to baseline in concordance with the B2AR expression. However, we find that activation of the B2AR is needed for downregulation of TH as TH expression did not modulate in scratch-wounded cultures where B2AR activation was blocked. However, when the B2AR eventually decreased in the presence of timolol, the TH levels continued to remain unchanged from baseline suggesting that there may be a B2AR-independent pathway that keeps TH elevated when the B2AR is blocked, even when the B2AR expression eventually decreased. Apparently, although a post-scratch wound decrease in TH was completely blocked with the use of B2AR antagonists, post-scratch decrease in the PNMT expression was only delayed and not completely blocked with the addition of timolol. This suggests that the keratinocyte may depend on the presence of the B2AR for the subsequent synthesis of epinephrine, as PNMT is the enzyme that generates epinephrine from norepinephrine. However, as TH levels remained elevated in the presence of an antagonist to the B2AR, the keratinocyte seems to selectively prefer downregulation of the capability to synthesize epinephrine as opposed to norepinephrine. This is further supported by our finding that norepinephrine levels preferentially elevate 24 hours after scratch wounding, whereas epinephrine does not (Figure 4a).

Our data demonstrate modulation of norepinephrine, its receptor, and synthetic enzymes in cultured human keratinocytes. To extend the translational significance of these findings, we sampled human skin wound tissue. Skin tissue harvested in the absence of injected epinephrine, and within 30 minutes of surgical injury, was processed for catecholamine analysis. Levels range from those reported in non-stressed muscle ($20\,pg\,mg^{-1}$, (Ohkuwa *et al.*, 2005)) to levels that approximate those found in human serum ($\sim 700\,ng\,ml^{-1}$, (Kuebler *et al.*, 2013)). Thus, it is clear that the wound microenvironment is one wherein norepinephrine is present, and levels may vary widely. As our data support a role for norepinephrine in modulating (at least) the keratinocyte component of healing, it is tempting to suggest that the variation in norepinephrine within the wound niche may impact upon healing. We are currently evaluating this possibility.

Catecholamines generated locally in the wound appear to impair keratinocyte migration as shown here and previously (Sivamani *et al.*, 2009b). Elevations in systemic epinephrine, relative to normal levels, have been found to impair wound healing in mice (Romana-Souza *et al.*, 2010; Kim *et al.*, 2014). However, the role of systemic elevation of norepinephrine is less clear. Although depletion of systemic norepinephrine impairs healing (Gosain *et al.*, 2006), acute increases in norepinephrine could impair healing by decreasing macrophage efficiency in generating microbicidal oxidizing agents (Kuebler *et al.*, 2013). The differences in the magnitude of the systemic elevation and the persistence of this elevation may explain the discrepancy, and future studies will be needed to determine the effects of stress levels of systemic norepinephrine.

Keratinocytes tightly coordinate their response to wound stimuli in an autocrine fashion through the B2AR and the production of catecholamines. Regulation and activation of the catecholamine pathway and the B2AR occurs through both B2AR-mediated and B2AR-independent pathways. In response to the stress of wounding, keratinocytes acutely decrease their expression of both the B2AR and the catecholamine-synthesizing enzymes, and this may serve as a protective response to acutely improve keratinocytes migratory rate and re-epithelialization. Our results suggest that the catecholamine network in keratinocytes is an important autocrine pathway in wound-healing physiology, which plays an active role in the keratinocyte acute wound-healing response. Norepinephrine, in particular, appears to be an important endogenous component of the stress-induced wound microenvironment. The presence and actions of catecholamines in the wound microenvironment is supported by reports of therapeutic success with the use of topical timolol, a beta-adrenergic receptor antagonist (Tang *et al.*, 2012; Lev-Tov *et al.*, 2013). Fully characterizing the B2AR autocrine pathway, and now, its newly discovered wound mediator, norepinephrine, may provide new insights into therapeutic approaches for improving healing.

MATERIALS AND METHODS

Materials

Isoproterenol was purchased from Calbiochem (San Diego, CA). Antibodies against the B2AR (sc-569) and phenylethanolamine-N-methyltransferase (sc-16458) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against TH (#2792) was purchased from Cell Signaling Technology (Danvers, MA) and the antibody against β1 tublin was purchased from Sigma (St Louis, MO). ICI-118,551, norepinephrine, and bisoprolol were purchased from Sigma.

Cell culture and wounding

Human keratinocytes were derived from neonatal foreskins, obtained with a human subjects protocol approved by the UC Davis Institutional Review Board. Keratinocytes were cultured in Epilife medium (Cascade Biologics, Portland, OR) that was supplemented with Human Keratinocyte Growth Supplement (Cascade Biologics) as

described (Pullar *et al.*, 2006b) and incubated at 37 °C in a humidified atmosphere of 5% CO_2.

After cells reached confluence in a 100-mm culture dish, multiple 1-mm wide scratches were made with a sterile pipette. After wounding, cells were washed with sterile Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) multiple times to remove damaged cells, and then returned to the incubator, and allowed to recover for various times. Timolol pretreatments were performed for one hour prior to scratch wounding. Culture dishes of sham control were used to exclude any effects of the stresses other than wounding, and they were treated similarly to the experimental cells, except that they were not scratch-wounded.

Cell lysis and western blot analysis

Cells were lysed as described (McClaren and Isseroff, 1994; Shi and Isseroff, 1996). Briefly, after treatments the cultures were rinsed with PBS and scraped in a lysis buffer (10 mM Tris, 20 mM NaCl, 25 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 2 mM Na_3VO_4, 1 μM phenylmethylsulfonyl fluoride, protease inhibitors, and pH 7.5). Cell lysates were rocked on ice for 30 minutes, and pelleted at 14,000*g* for 20 minutes. The supernatant was aliquoted and stored at –80 °C. Protein concentrations in the cell lysates were determined using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA).

For western blot analysis, samples with equal amounts of protein were loaded into a 10 or 7.5% acrylamide gel, and SDS-PAGE was run at 150 mV. The proteins on the gel were transferred onto a polyvinylidene difluoride membrane at 100 mV for 60 or 75 minutes. The membranes were blocked with 5% non-fat dry milk in PBS, and then probed with appropriate primary antibodies, and also with antibody against β1 tublin as a protein-loading reference. The immunoreactive proteins were detected using horseradish peroxidase-linked anti-IgG, and then stained with enhanced chemiluminescent western blotting detection reagents (Amersham Life Science, St Louis, MO). Images were obtained by scanning the films, and the optical density of each band was determined, and normalized with the optical density of the loading control β1 tublin using NIH Image 1.61 (Scion, Bethesda, MD).

Cell migration studies

Cell migration was monitored microscopically as previously described (Pullar *et al.*, 2003). Briefly, keratinocytes were plated at a subconfluent density of 50 cells mm^{-2} onto collagen-coated multi-well plates (60 μg ml^{-1}) (Sigma) for 2 hours at 37 °C. The multi-well plate was then placed into a migration chamber to monitor individual cell migration in each well over a 1-hour period at 37 °C. The migration chamber was placed on an inverted Nikon Diaphot (Nikon, Garden City, NJ) microscope. Time lapse images of the cell migratory paths were digitally captured by Velocity imaging software (PerkinElmer, Waltham, MA) every 10 minutes for a 1-hour period. Each cell's center of mass was tracked in OpenLab (PerkinElmer), the data were automatically exported to FileMaker Pro 3.0 (FileMaker, Santa Clara, CA). Data on migration speed for each slide were averaged across cells measured, so that the data analyzed are statistically independent. Untreated cells served as the control.

Collection of human wound tissue

Human wound tissue was collected from five subjects who were 52, 60, 63, 64, and 65 years old. The wounds were collected from the edges of surgical toe amputation sites at the time of the standard surgical repair, in procedures that had not included any injected epinephrine prior to excision. Tissue was collected at the viable wound margin, within 30 minutes of skin injury. Immediately upon release from the wound, tissue samples were snap-frozen in liquid nitrogen and stored at − 80 °C All tissue collection procedures were approved by the institutional review board at the Veterans Affairs Medical Center in Mather, CA.

HPLC-elecctrochemical detection analysis of catecholamines

After scratch assays were performed, aspirated cell culture supernatants and cell lysates were separately acidified with perchloric acid to a final concentration of 0.2 N prior to storage at − 80 °C for future analysis. After thawing 1 ml of supernatant, 400 μl of HEPES pH 9.0 buffer, 600 μl of neutralization solution (0.33 N NaOH; 0.4% sodium sulfite; 0.67 mM EDTA), and 5 ng of internal standard 3, 4-Dihdroxybenzylamine (DHBA) was added and then centrifuged at 14,000*g* for 5 minutes. For cell lysates, 70 μl of thawed lysate was treated with 120 μl HEPES pH 9.0 buffer, 1 ml of sulfite solution (1.25% sodium sulfite; 2 mM EDTA), and 5 ng of DHBA. After centrifugation, supernatant or lysate solutions were applied to conditioned MonoSpin PBA solid phase extraction spin columns (GL Sciences, Torrance, CA). Spin column purifications were performed according to the manufacturer's specifications and catecholamines were eluted in 200 μl of 2% acetic acid. Eluents were then stored at − 80 °C for future HPLC electrochemical detection analyses.

For human wound tissue samples, tissues were removed from a − 80 °C freezer and pulverized using a tissue pulverizer (Cole Parmer, Vernon Hills, IL) under liquid nitrogen. Pulverized tissue was then weighed and acidified by adding 250 μl of 0.2 N perchloric acid. Acidified solutions were then further homogenized by sonication (Qsonica, Newtown, CT) and stored at − 80 °C for catecholamine analysis. After thawing, 1.4 ml sulfite solution (1.25% sodium sulfite; 2 mM EDTA) was added to homogenized tissue and centrifuged for 5 minutes at 3,000*g*. The supernatant was aspirated and then treated with 500 μl HEPES pH 9.0 buffer and 5 ng of DHBA prior to filtering through a 0.22-um syringe filter. Filtered tissue homogenate was then applied to conditioned MonoSpin PBA spin columns and eluted as above.

HPLC separation of samples was performed using a Synergi 4-um Fusion-reverse phase 250 × 4.6-mm column (Phenomenex, Torrance, CA) and a Hewlett Packard series 1050 pump and auto injector system. The column temperature was maintained at 28 ° C and data were acquired using Chem Station software. Chromatographic separation was performed using a mobile phase that was modified from Leis *et al.* (Leis *et al.*, 2004) and consisted of 50 mM citric acid, 0.1 M sodium acetate, 0.15 mM EDTA, 1 mM sodium 1-octanesulfonate, and 4% methanol, pH 4 and was pumped at a flow rate of 1 ml per min. Detection of catecholamine compounds was performed using a LC-4C amperometric detector (Bioanalytical Systems, West Lafayette, IN) using potential of − 700 mV. Commercially purchased standards were used for calibration of the instrument (norepinephrine, Sigma; epinephrine, MP Biomedicals (Santa Ana, CA); 3,4-dihydroxyphenyl glycol or DOPEG; Sigma; and DHBA, Sigma). Standard curves were generated for each compound (ranging from 4,000 to 4 pg) with correlation coefficients (R^2) near 1.0 for all compounds.

Radioligand binding

The hydrophilic non-selective beta-adrenergic antagonist [3H]CGP12177 (PerkinElmer) was used as radioligand for whole-cell binding assays. All assays were carried out in triplicate. Normal human keratinocytes from neonates were cultured to confluency in 500-cm^2 square dishes (Sigma-Aldrich, Catalog #CLS431110-16EA). Once confluent, cultures were scratch wounded with a comb, washed three times with PBS followed by replacement of medium until time for cell harvest with 0.25% trypsin/EDTA. Control cultures were similarly washed with PBS followed by addition of fresh medium. Cells were counted using trypan blue exclusion and resuspended in medium at 5×10^6 cells per ml. Binding was measured by a modified method as previously reported (Steinkraus *et al.*, 1991). Total binding was determined by aliquoting 150 µl of cell suspension (5×10^6 cells per ml), 50 µl of [3H]CGP12177, and 50 µl of medium. Non-specific binding was determined by aliquoting 150 µl of cell suspension (5×10^6 cells per ml), 50 µl of [3H]CGP12177, and 50 µl of propranolol at a final volume of 5 µM. Both curves yield 250 µl total volume for each varying hot compound concentration. [3H]-CGP12177 was serially diluted in a range from 0.03 to 8 nM in 50 µl within the 250 µl total volume. Incubation was for 90 minutes in a shaking water bath at 37 °C. Termination of reaction was performed using rapid vaccum filtation using Whatman GF/C glass fiber filter and ice-cold harvest buffer (50 mM Tris HCl, 10 mM MgCl2: pH = 7.4). Radioactivity was determined by liquid scintillation spectrometry. Results were analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA) software to determine specific binding and Scatchard transformation.

Statistical analysis

These data were analyzed using one-way analysis of variane and *P*-values less than 0.05 were taken to be statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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