The β2-adrenergic receptor activates pro-migratory and pro-proliferative pathways in dermal fibroblasts via divergent mechanisms

Christine E. Pullar* and R. Rivkah Isseroff
Department of Dermatology, University of California, Davis, TB 192, One Shields Avenue, CA 95616, USA
*Author for correspondence (e-mail: cepullar@ucdavis.edu)

Accepted 24 October 2005
Journal of Cell Science 119, 592-602 Published by The Company of Biologists 2006
doi:10.1242/jcs.02772

Summary
Dermal fibroblasts are required for skin wound repair; they migrate into the wound bed, proliferate, synthesize extracellular matrix components and contract the wound. Although fibroblasts express β2-adrenergic receptors (β2-AR) and cutaneous keratinocytes can synthesize β-AR agonists (catecholamines), the functional significance of this hormonal mediator network in the skin has not been addressed. Emerging studies from our laboratory demonstrate that β2-AR activation modulates keratinocyte migration, essential for wound re-epithelialization. Here we describe an investigation of the effects of β2-AR activation on the dermal component of wound healing. We examined β2-AR-mediated regulation of biological processes in dermal fibroblasts that are critical for wound repair: migration, proliferation, contractile ability and cytoskeletal conformation.

We provide evidence for the activation of at least two divergent β2-AR-mediated signaling pathways in dermal fibroblasts, a Src-dependent pro-migratory pathway, transduced through the epidermal growth factor receptor and extracellular signal-regulated kinase, and a PKA-dependent pro-proliferative pathway. β2-AR activation attenuates collagen gel contraction and alters the actin cytoskeleton and focal adhesion distribution through PKA-dependent mechanisms. Our work uncovers a previously unrecognized role for the adrenergic hormonal mediator network in the cutaneous wound repair process. Exploiting these divergent β2-AR agonist responses in cutaneous cells may generate novel therapeutic approaches for the control of wound healing.

Key words: Wound healing, EGFR transactivation, Src, cAMP, Motility, Skin

Introduction
The healing of cutaneous wounds is a dynamic, well-organized and complex process requiring the orchestration of many different cell types and cellular processes (Martin, 1997). Dermal fibroblasts are actively involved in this process. They migrate to the wound site, proliferate, synthesize extra-cellular matrix components, form granulation tissue (Grinnell, 1994), and generate mechanical forces within the wound to initiate wound contraction (Gabbiani et al., 1972). Wound contraction can be beneficial to overall wound healing by decreasing the wound area and forming a mechanically strong reparative scar.

In certain situations, however, fibroblast presence and activity can be deleterious to wound healing. Undesirable wound contracture can occur, particularly as a consequence of burn and trauma wounds, and can result in both cosmetic and functional problems (Fang and Alexander, 1990; Rudolph, 1992; Skalli, 1988; Vande Berg and Rudolph, 1985). Additionally, the accumulation of abnormally large numbers of fibroblasts within a healing wound can also result in a fibrotic, contracted scar (Redden and Doolin, 2003). Understanding the mechanisms that regulate dermal fibroblast migration, proliferation and wound contraction could, therefore, be beneficial for devising novel therapies to regulate fibrosis and wound contraction to ultimately improve the wound healing process.

β2-ARs are the only class of β-AR expressed on the three major cell types of human skin: keratinocytes (Steinkraus et al., 1996) dermal fibroblasts (McSwigan et al., 1981) and melanocytes (Gillbro et al., 2004). Emerging studies from our laboratory point to a role of the β2-adrenergic signaling pathway in wound healing. We recently showed that the ERK signaling pathway in keratinocytes is remarkably attenuated by β2-adrenergic receptor (β2-AR) activation, resulting in marked diminution of keratinocyte migration by a cAMP-independent (Chen et al., 2002), phosphatase PP2A-dependent mechanism (Pullar et al., 2003). These findings imply that β2-adrenergic signaling could impair wound re-epithelialization, essential for wound healing (Martin, 1997). Indeed, we observe a β-AR agonist-mediated delay in both human and murine skin wound healing (Pullar et al., 2006). However, as dermal fibroblast migration, proliferation and wound contraction are also required for wound repair, we sought to determine how β2-AR activation might affect these cells, which are so crucial to the repair process.

We demonstrate that β2-AR activation is both promotogenic and pro-mitogenic in dermal fibroblasts. Dermal fibroblast-mediated collagen gel contraction was attenuated upon β2-AR activation and we observed changes in the
conformation of the cytoskeletal proteins actin and vinculin. We discovered that divergent pathways transduced the signals from the β2-AR; the pro-migratory pathway was Src dependent, while all other effects investigated here were protein kinase A (PKA) dependent. Our work uncovers a novel, previously unrecognized role for the β2-AR in wound repair.

Results

β2-AR activation increased dermal fibroblast single cell migration

Adrenergic receptor activation can retard the migration of human keratinocytes (Chen et al., 2002; Pullar et al., 2003). Here, we investigated the effect of β2-AR activation on dermal fibroblast single cell migration. Studies were performed in the presence or absence of β-AR agonist. In stark contrast to the effect in keratinocytes, we observed a significant increase in migratory speed of isolated dermal fibroblasts upon β2-AR activation (1 μM β-AR agonist; Fig. 1A,B). To ensure that the β-AR-mediated increase in fibroblast migration rate was not limited to a specific concentration of β-AR agonist, we performed single cell migration experiments at a range of β-AR agonist concentrations from 10 nM to 100 μM (Fig. 1C). We observed a β-AR agonist-mediated dose-dependent increase in migration rate. β-AR activation was pro-motogenic at all concentrations of β-AR agonist tested.

β2-AR activation transactivated the EGFR in dermal fibroblasts

The activation of the epidermal growth factor receptor (EGFR) is required for cell motility (Glading et al., 2000) and β-AR activation can transactivate the EGFR in COS-7 cells (Maudsley et al., 2000; Pierce et al., 2000). We, therefore, reasoned that β2-AR activation may be stimulating fibroblast migration by transactivating the EGFR. The EGFR was immunoprecipitated from unstimulated and β2-AR-activated cell lysates and probed with either an anti-EGFR antibody or an anti-phosphotyrosine antibody. Roughly equal quantities of EGFR were immunoprecipitated from each lysate (Fig. 1D). Whereas tyrosine phosphorylation of the EGFR was undetectable in the absence of β-AR agonist, β2-AR activation phosphorylated the EGFR on tyrosine residues (Fig. 1D,E). Thus, β2-AR activation does transactivate the EGFR in human dermal fibroblasts.

Fig. 1. Dermal fibroblasts were plated onto collagen-coated glass coverslips at a concentration of 125 cells/mm² in FM for 3-6 hours at 37°C. Migration experiments were performed in FM in the presence or absence of β-AR agonist (10 nM-100 μM). The migration of each single cell was monitored over a 1-hour period. The speed and distance traveled, at a concentration of 1 μM β-AR agonist, are represented graphically in A and B, respectively. The β-AR-mediated dose-dependent increase in distance traveled is represented graphically in C (β-AR agonist 10 nM-100 μM). The data are representative of three independent experiments with three different fibroblast strains (n=50). Values plotted are mean ± s.e.m. *P<0.01 between β-AR agonist and controls. Cells were starved of growth factors in DMEM for 16 hours. 1-2×10⁷ cells were either left untreated or treated with 1 μM β-AR agonist in DMEM for 10 minutes at 37°C. After treatment, cell lysates were prepared and the EGFR was immunoprecipitated. EGFR antibody-associated proteins were electrophoresed on two separate 10% polyacrylamide gels at the same time and transferred to membranes. Membranes were immunoblotted with either an EGFR antibody (EGFR WB) or an anti-phosphotyrosine antibody (PY WB; D). Gels were aligned to allow correct identification of the EGFR protein. The data shown are representative of three independent experiments from three separate cell strains. Three blots from three separate experiments were scanned for EGFR tyrosine phosphorylation and densitometry was performed using a gel plotting macro in NIH Image 1.62. Data was averaged, statistically analyzed and represented graphically (Fig. 1E). Values plotted are mean ± s.e.m. (n=3). *P<0.01 between β-AR agonist and control.
β2-AR activation increased the phosphorylation of ERK in dermal fibroblasts

ERK is known to regulate cell motility (Klemke et al., 1997), therefore, we examined whether β2-AR activation increased ERK phosphorylation in human dermal fibroblasts. Equal protein loading was demonstrated by immunoblotting with an anti-ERK antibody (Fig. 2A). ERK was rapidly phosphorylated upon β2-AR activation, achieving a maximal increase of two- to threefold compared to unstimulated cells, 5 minutes after β-AR agonist addition. The phosphorylation of ERK remained significantly elevated for at least 30 minutes, returning to within basal levels after 1 hour (Fig. 2B). A time course of ERK phosphorylation in the absence of β-AR agonist confirmed that there was no change in phosphorylation during the time course of our experiment (results not shown).

The mechanism for the β2-AR-mediated increase in ERK phosphorylation was dependent on the Src-mediated transactivation of the EGFR

β2-AR-mediated ERK activation is dependent on the transactivation of the EGFR in COS-7 cells (Maudsley et al., 2000; Pierce et al., 2000). To determine whether a similar mechanism was responsible for the β2-AR-mediated ERK activation observed in dermal fibroblasts we pre-treated cells with the EGFR kinase inhibitor, AG1478 (10 μM) (Kim et al., 2003; Kim et al., 2002) for 90 minutes prior to β2-AR activation. The ERK immunoblot demonstrates equal protein loading in all lanes (Fig. 3A). Although the level of basal ERK phosphorylation appeared higher in cells pre-treated with AG1478 in the absence of β-AR agonist, we could no longer detect an EGF-mediated increase in ERK phosphorylation confirming complete inhibition of the EGFR kinase. AG1478 pre-treatment completely prevented the β2-AR-mediated increase in ERK phosphorylation demonstrating that EGFR transactivation is essential for β2-AR-mediated ERK phosphorylation in dermal fibroblasts (Fig. 3A,B).

Src has been suggested to play a role in β3-AR-mediated ERK activation in brown adipocytes (Lindquist et al., 2000) and a human salivary gland cell line (Yeh et al., 2005). To determine if Src played a role in β2-AR-mediated ERK activation in dermal fibroblasts we pre-treated cells with the Src inhibitor PP2 for 6 hours prior to β-AR agonist addition. The ERK immunoblot demonstrates equal protein loading in all lanes (Fig. 3C). PP2 pre-treatment completely prevented any β-AR agonist mediated increase in ERK phosphorylation (Fig. 3C,D). It therefore appears that β2-AR-mediated ERK phosphorylation was Src and EGFR transactivation-dependent in dermal fibroblasts.

β2-AR-mediated EGFR transactivation is Src-dependent in both COS-7 cells (Maudsley et al., 2000; Pierce et al., 2001) and a human salivary gland cell line (Yeh et al., 2005). As we have demonstrated that β2-AR-mediated ERK activation is both EGFR and Src dependent, we wondered if the β2-AR-mediated transactivation of the EGFR was also Src dependent. Dermal fibroblasts were pre-treated with the Src inhibitor, PP2 (10 μM) for 6 hours prior to β2-AR activation. The EGFR immunoblot demonstrates equal protein loading in all lanes (Fig. 3E). While the β2-AR-mediated transactivation of the EGFR receptor was observed within 15 minutes, as described above (in Fig. 1C) PP2 completely prevented its tyrosine phosphorylation, demonstrating that the β2-AR-mediated transactivation of the EGFR was also Src dependent (Fig. 3E,F).

The β2-AR-mediated increase in dermal fibroblast migration was Src dependent

As ERK plays a pivotal role in fibroblast motility (Glading et al., 2000) and we have demonstrated here that β2-AR-mediated ERK phosphorylation was dependent on Src activity, we hypothesized that the β2-AR-mediated increase in dermal fibroblast migration might also be Src dependent. Dermal fibroblasts were pre-treated with the Src inhibitor PP2 for 6 hours prior to observing single cell migration, in the presence or the absence of β-AR agonists. PP2 completely prevented the β2-AR-mediated augmentation of dermal fibroblast migration (Fig. 4) demonstrating that the β2-AR-mediated increase in dermal fibroblast migration was also Src-dependent.

β2-AR activation increased the proliferation of dermal fibroblasts via a cAMP-dependent mechanism

β-AR activation can augment (Colombo et al., 2001) or...
Fig. 3. Dermal fibroblasts were starved of growth factors in DMEM for 16 hours as described. 1-2×10⁵ cells were pre-incubated with either DMEM alone (0, 5-60 minutes ISO) or DMEM containing either 10 μM AG1478 (A,B) for 90 minutes or 10 μM PP2 for 6 hours at 37°C (C,D). Cells were either untreated (control, 0, 5-60 minutes ISO) or stimulated with DMEM containing inhibitor and 1 μM β-AR agonist for 5-60 minutes at 37°C, unless otherwise noted. After treatment, cell lysates from each experiment were prepared and electrophoresed on the same 10% polyacrylamide gels and transferred to membranes. Membranes were immunoblotted with either an anti-ERK antibody, a anti-phospho ERK antibody (P-ERK) an anti-EGFR antibody (EGFR) or an anti-phosphotyrosine antibody (PY). Three blots from separate AG1478 or PP2 experiments were scanned for p-ERK or PY and densitometry performed using a gel plotting macro in NIH Image 1.62. Data was averaged, statistically analyzed and represented graphically (B,D,F). Values plotted are means ± s.e.m. (n=3). *P<0.01 between conditions and controls. # no significant difference between AG1478/control and AG1478/β-AR agonist or PP2/control and PP2/β-AR agonist. The data shown are representative of three independent experiments from three separate cell strains.
conversely, decrease (Liu et al., 2004) cell proliferation, depending on the cell type studied. Thus it was important to determine what effect β2-AR activation would have on human dermal fibroblast proliferation. Therefore, human dermal fibroblasts were grown in the presence or absence of β-AR agonist (1 μM). β-AR activation significantly increased fibroblast proliferation, with a maximum augmentation of 55% at day 6 (Fig. 5A). To ensure that the β-AR-mediated increase in fibroblast proliferation rate was not limited to a specific concentration of β-AR agonist we performed proliferation experiments at a range of β-AR agonist concentrations from 10 nM to 10 μM. We observed a dose-dependent increase in proliferation rate. β-AR activation was pro-mitogenic at all concentrations of β-AR agonist tested (results not shown).

β2-AR can couple to Gi, (Xiao et al., 1999) increasing intracellular cAMP levels and activating downstream cAMP-dependent kinases such as PKA and EPAC (exchange proteins directly activated by cAMP) (Hanoune and Defer, 2001). To determine if the β2-AR-mediated increase in dermal fibroblast proliferation was cAMP-dependent we initially incubated cells in the presence of sp-cAMP, an active cAMP analog (Van Haastert et al., 1984), to increase the concentration of intracellular cAMP. The growth rate of fibroblasts maintained in the presence of β-AR agonist, sp-cAMP or both, were practically indistinguishable from each other, hinting that the β2-AR-mediated pro-mitogenic effects were cAMP-dependent (Fig. 5B).

The inactive cAMP analog, rp-cAMP (Van Haastert et al., 1984), a specific PKA inhibitor (de Wit et al., 1982) had no effect on proliferation alone, but when added to the dermal fibroblasts before the β-AR agonist it almost completely prevented the β2-AR-mediated augmentation of proliferation (Fig. 5C). The β2-AR-mediated augmentation of dermal fibroblast proliferation was, therefore, mediated by a cAMP/PKA-dependent mechanism.

β2-AR activation attenuated the dermal fibroblast-mediated contraction of collagen gels

Fibroblast-seeded collagen gels have been widely used experimentally as a wound contraction model because they simulate fibroblast behavior in the early phases of wound healing (Grinnell, 2000). To determine whether β2-AR activation would alter the contraction of dermal fibroblast-seeded collagen gels, collagen lattices populated with dermal fibroblasts were assembled in either the absence or presence of 10 μM β-AR agonist. After 24 hours the addition of β-AR agonist had markedly delayed gel contraction (Fig. 6). The delay was maintained throughout the 5 days of the experiment and could be prevented with antagonist pre-treatment (results not shown). We also observed an inhibition of collagen gel contraction at lower concentrations of β-AR agonist (10 nM and 1 μM, results not shown) with maximum inhibition observed at 10 μM β-AR agonist.

As we had determined that the β2-AR-mediated augmentation of dermal fibroblast proliferation was mediated by a cAMP/PKA-dependent mechanism we hypothesized that the mechanism for the β2-AR-mediated delay in dermal fibroblast-mediated gel contraction could also be cAMP dependent. We added the inactive cAMP analog, rp-cAMP (Dostmann et al., 1990), to the collagen gels before casting, at a concentration known to inactivate cAMP-mediated downstream signaling components (50 μM) (Dostmann et al., 1990; Hirshman et al., 2001). Rp-cAMP alone had no effect on the contraction of the collagen gels but partially prevented the β2-AR-mediated delay in contraction (Fig. 6). After 24 hours, β-AR agonist-treated gels were only 47% contracted, whereas gels cast with dermal fibroblasts pre-treated with rp-cAMP prior to β-agonist addition were 59% contracted, and untreated gels were 71% contracted, indicating that the mechanism for the β2-AR-mediated delay in dermal fibroblast collagen gel contraction was partly cAMP/PKA dependent.

At day 4, the gels were digested with collagenase, cells were counted and the viability of the fibroblasts was assessed by Trypan Blue exclusion. Cells were 95% viable and the cell number was found to be comparable between control, β-agonist-treated and rp-cAMP-treated gels and similar to seeding density (data not shown).
β2-AR activation alters the dermal fibroblast cytoskeleton

Actin remodeling plays an important role in cell motility (Pantaloni et al., 2001), proliferation (Blakesley et al., 1998; Cuadros et al., 2000; Ikeda et al., 2003; Joneson et al., 1996; Landriscina et al., 2000; Sastrodihardjo et al., 1987) and collagen gel contraction (Miki et al., 2000). Actin filaments terminate in focal adhesions, where several proteins, including vinculin, mediate interactions with the actin cytoskeleton (Burridge and Fath, 1989).

As we have demonstrated that β2-AR activation in dermal fibroblasts is pro-motogenic, pro-mitogenic and anti-contractive, we were interested to see if it also altered cytoskeletal F-actin and focal adhesion number and size using vinculin as a focal adhesion marker (Beningo et al., 2001; Burridge and Fath, 1989).

All cells plated in the absence of β-AR agonist showed pronounced transcytoplasmic actin stress fibers along the borders of the cells and multiple vinculin-containing focal adhesions (Fig. 7A). Pre-treating with β-AR agonist for 15 minutes (1 μM) markedly decreased actin staining in 90% of the cells, suggestive of β2-AR-mediated actin depolymerization (Hirshman et al., 2001), and also decreased the number and size of vinculin-containing focal adhesions (Fig. 7B). ImageJ was used to quantify the reduction in actin- and vinculin-associated fluorescence by measuring the mean pixel intensity of 25 cells from each condition. Control cells had a mean pixel intensity of 50.3±4.8. β-AR agonist treatment resulted in a 67% drop in mean pixel intensity to a level of 16.6±2.0.

Since we have determined that the β2-AR-mediated augmentation of proliferation and attenuation of collagen gel contraction of fibroblasts was graded — with fibroblasts plating in the absence of β-AR agonist showing pronounced transcytoplasmic actin stress fibers along the borders of the cells and multiple vinculin-containing focal adhesions. Pre-treating with β-AR agonist for 15 minutes (1 μM) markedly decreased actin staining in 90% of the cells, suggestive of β2-AR-mediated actin depolymerization (Hirshman et al., 2001). ImageJ was used to quantify the reduction in actin- and vinculin-associated fluorescence by measuring the mean pixel intensity of 25 cells from each condition. Control cells had a mean pixel intensity of 50.3±4.8. β-AR agonist treatment resulted in a 67% drop in mean pixel intensity to a level of 16.6±2.0.

Since we have determined that the β2-AR-mediated augmentation of proliferation and attenuation of collagen gel contraction of fibroblasts was graded — with fibroblasts plating in the absence of β-AR agonist showing pronounced transcytoplasmic actin stress fibers along the borders of the cells and multiple vinculin-containing focal adhesions. Pre-treating with β-AR agonist for 15 minutes (1 μM) markedly decreased actin staining in 90% of the cells, suggestive of β2-AR-mediated actin depolymerization (Hirshman et al., 2001). ImageJ was used to quantify the reduction in actin- and vinculin-associated fluorescence by measuring the mean pixel intensity of 25 cells from each condition. Control cells had a mean pixel intensity of 50.3±4.8. β-AR agonist treatment resulted in a 67% drop in mean pixel intensity to a level of 16.6±2.0.
contraction were both cAMP/PKA dependent we hypothesized that the β2-AR agonist-mediated change in actin stress fibers and vinculin-associated focal adhesions could also be cAMP/PKA dependent. We pre-treated dermal fibroblasts with the active cAMP analog, sp-cAMP (50 μM) for 45 minutes. 80% of sp-cAMP-treated cells had the same staining pattern as β-AR agonist-treated cells, with reduced actin and vinculin staining, indicating a cAMP-mediated mechanism (Fig. 7C). Adding both β-AR agonists and sp-cAMP simultaneously resulted in a similar staining pattern (Fig. 7D). ImageJ was used to quantify the reduction in actin- and vinculin-associated fluorescence by measuring the mean pixel intensity of 25 cells from each condition. The mean pixel intensity measured in sp-cAMP-treated cells was 55.7±7.1. Additionally, sp-cAMP pre-treatment prevented the β-AR-mediated decrease in actin/vinculin-associated immunofluorescent staining, the mean pixel intensity of sp-cAMP pre-treated, β-AR agonist-treated cells was 47.4±4.5, a level within the range of pixel intensity measured in control cells.

To confirm the role of cAMP/PKA in the β-AR agonist-mediated reduction in actin and vinculin staining, dermal fibroblasts were pre-treated with the inactive cAMP analog, rp-cAMP, to inhibit PKA (de Wit et al., 1982). There was no observed effect of rp-cAMP treatment alone on the actin or vinculin staining of dermal fibroblasts (Fig. 7E), all cells resembled untreated cells. Pre-treatment with rp-cAMP, however, prevented the β-AR agonist-mediated decrease in actin and vinculin staining in 90% of the cells, confirming that the mechanism for the β2-AR-mediated alteration of cytoskeletal conformation was cAMP/PKA dependent (Fig. 7F). ImageJ was used to quantify the reduction in actin- and vinculin-associated fluorescence by measuring the mean pixel intensity of 25 cells from each condition. Conversely, the inactive cAMP analog, rp-cAMP did not significantly alter the mean pixel intensity observed in control cells. The mean pixel intensity measured in rp-cAMP-treated cells was 55.7±7.1. Additionally, rp-cAMP pre-treatment prevented the β-AR-mediated decrease in actin/vinculin-associated immunofluorescent staining, the mean pixel intensity of rp-cAMP pre-treated, β-AR agonist-treated cells was 47.4±4.5, a level within the range of pixel intensity measured in control cells.

Discussion
Adrenergic receptors were identified in human skin over three decades ago (Tseraidis and Bavykina, 1972). Interestingly, β2-ARs are the only class of β-AR expressed on the three major cell types of human skin: keratinocytes (Steinkraus et al., 1996), dermal fibroblasts (McSwigan et al., 1981) and melanocytes (Gillbro et al., 2004). Keratinocytes also have the capacity to synthesize the catecholamines epinephrine and nor-epinephrine, both ligands for adrenergic receptors (Schallreuter, 1997; Schallreuter et al., 1995). With cells that express both receptors and ligands, it is becoming evident that the skin generates a localized hormonal mediator network, which has the potential to regulate its physiology.

Clues to the physiological role of the β2-AR/catecholamine network within skin have been previously uncovered by the demonstration of alterations within the components of this network in some epidermal skin diseases. In atopic eczema there is a point mutation in the β2-AR gene and a low β2-AR density on keratinocytes and peripheral blood lymphocytes (Schallreuter, 1997). In psoriasis, epidermal cells from psoriatic lesions demonstrate a low cAMP response to β2-AR activation (Eedy et al., 1990). Additionally, a paracrine role for the hormone mediator network in skin homeostasis has been demonstrated recently as keratinocyte catecholamine synthesis can regulate melanogenesis in melanocytes (Gillbro et al., 2004).

Our laboratory has discovered a novel role for the adrenergic hormonal mediator network in modulating skin wound repair. We
reported that β2-AR activation decreased keratinocyte migration and ERK phosphorylation in a cAMP-independent (Chen et al., 2002) and phosphatase PP2A-dependent manner (Pullar et al., 2003). β-AR agonists decrease the re-epithelialization of both human and murine skin wounds (Pullar et al., 2006). As multiple cell types contribute to cutaneous wound healing (Martin, 1997) and β-AR activation can result in diametrically opposing responses in different cell types (Masur et al., 2001; Murphy et al., 1998; Salathe, 2002; Spurzem et al., 2002), it was important to examine the response to β2-AR activation in other cutaneous cells. Here we demonstrate the unique effects of β2-AR activation on the physiological processes that contribute to the fibroblasts reparative role in the skin: migration, proliferation and contractile ability. Further, we elucidate the divergent signaling pathways by which these β2-AR-driven responses are generated.

We discovered that in contrast to the anti-motogenic effects of β2-AR activation in keratinocytes (Pullar et al., 2003; Pullar and Isseroff, 2005), the activation of β2-AR in dermal fibroblasts was both pro-mitogenic and pro-mitogenic. The diametrically opposite response to β2-AR activation in fibroblasts as compared to keratinocytes underscores the importance of evaluating the β2-AR-mediated responses in a specific cell type. For example: ERK phosphorylation was increased by β2-AR activation in dermal fibroblasts yet decreased in keratinocytes (Pullar et al., 2003).

We provide evidence for the activation of divergent pro-motogenic and pro-mitogenic β2-AR-mediated signaling pathways in dermal fibroblasts. β2-ARs are classical GPCRs, capable of coupling to Gs and increasing intracellular cAMP (Hurley, 1999; Xiao et al., 1999). Indeed, we discovered that a cAMP/PKA-dependent pathway mediated the β-AR agonist-induced increase in dermal fibroblast proliferation and decrease in contraction of collagen gels. On the other hand, the β2-AR-mediated transactivation of the EGFR, and increase in ERK phosphorylation and migration were Src-dependent. The mechanism for β2-AR-mediated Src-dependent EGFR transactivation could be dependent on the matrix metallloprotease-mediated release of heparin-binding EGF (Kim et al., 2002; Pierce et al., 2000), clathrin-mediated endocytosis (Maudsley et al., 2000) or both. Actin cytoskeletal remodeling (Pantalone et al., 2001) and focal adhesion turnover (Burridge and Fath, 1989) play an important role in migration. The β2-AR-mediated changes in cytoskeletal conformation were cAMP/PKA-dependent, however Src inhibition attenuated the β2-AR-mediated increase in migration, suggesting that Src could be upstream of cAMP/PKA in dermal fibroblasts. Indeed, murine embryonic fibroblasts overexpressing c-Src show enhanced β-AR-mediated cAMP accumulation (Bushman et al., 1990).

Thus in dermal fibroblasts, divergent signaling pathways control cellular responses to β2-AR activation: a PKA-dependent pathway controls proliferation, contractile ability and cytoskeletal conformation while a Src-dependent pathway regulates migration. These pathways are summarized in Fig. 8.

β2-AR activation can, therefore, modulate the behavior of both keratinocytes and dermal fibroblasts in vitro. Our previous work on keratinocytes and human and murine skin wound healing demonstrates that β2-AR activation is detrimental to skin re-epithelialization (Pullar et al., 2003; Pullar and Isseroff, 2005; Pullar et al., 2006) and the results presented here suggest that β2-AR activation on dermal fibroblasts may also contribute to a β2-AR-mediated delay in skin wound repair. The ability of β2-AR activation to delay fibroblast-mediated collagen gel contraction may translate into decreased wound contraction in vivo, which could be detrimental to wound healing as dermal fibroblasts form the granulation tissue in the wound generating the mechanical forces that initiate contraction to decrease wound size (Gabbiani et al., 1972). Additionally, the β-AR agonist-mediated increase in fibroblast migration rate and proliferation could result in the accumulation of abnormally large numbers of fibroblasts in the wound, which may also be deleterious to the wound repair process, resulting in unwanted fibrosis and scarring (Redden and Doolin, 2003). It would, however, be premature to conclude that the β-AR-mediated effects we observe in dermal fibroblasts in vitro would contribute to the attenuation of wound healing in vivo and experiments are presently underway in our lab to address these questions.

What evidence is there that endogenous cutaneous catecholamines could potentially regulate wound repair? Catecholamines form a critical component of the body’s response to stress (Nankova and Sabban, 1999), that can have a deleterious effect on wound healing (Detillion et al., 2004). Surgical stress can increase post-operative plasma levels of catecholamines and cortisol, the major stress-induced hormones and cortisol has long been correlated with impaired human skin wound healing (Ebrecht et al., 2004). Normal circulating levels of epinephrine are reported to be 0.3-0.8 nmol/l in human plasma (Sedowofia et al., 1998) but increase by greater than tenfold (3-12 nmol/l) within the first 6 hours following injury (Crum et al., 1990; Matsui et al., 1991; Sedowofia et al., 1998). Since, we observed pro-motogenic, anti-contractile and pro-mitogenic effects in the nanomolar range in vitro and as this corresponds to the circulating levels seen in post trauma, our results may indeed be physiologically relevant. It is also important to note that catecholamines are rapidly metabolized by the liver (Martel, 1998), therefore, we
would hypothesize that levels of hormone in the blood or plasma may not reflect the concentrations of catecholamines synthesized locally by the epidermis at sites of injury/stress, which could be higher.

Additionally, topical application of β2-AR agonists impaired human and murine wound re-epithelialization (Pullar et al., 2006) and a β2-AR antagonist improved barrier recovery, as evaluated by measuring transepidermal water loss (Denda et al., 2003). The current finding that β2-AR activation also regulates dermal fibroblast migration, proliferation and contractile ability, processes that are all critically required for wound repair, now provides mechanistic support for the regulatory role of the catecholamine hormonal network in the repair process. Further investigation of this regulatory pathway will improve our understanding of the wound healing process.

To summarize, we have identified novel, divergent, β2-AR-mediated pro-motogenic and pro-mitogenic mechanisms in dermal fibroblasts. The pro-motogenic pathway was Src dependent, while, the pro-mitogenic pathway, the attenuation of collagen gel contraction and alterations in cytoskeletal conformation were all cAMP/PKA dependent. Our work uncovers a previously unrecognized role for the adrenergic hormonal mediator network in cutaneous wound repair and provides tantalizing information to prompt further study of β2-AR modulation of the wound healing process.

Materials and Methods

Materials for cell treatments

The β2-AR agonist, isoproterenol, inhibitors, AG 1478, PP2 and the cAMP analogs, 8cAMP and 8cAMP, were purchased from Calbiochem (San Diego, CA).

Dermal fibroblast growth

Human dermal fibroblasts (HDF) were isolated from neonatal foreskins obtained by routine circumcision under an approved exemption from the University of California, Davis, Institutional Review Board, as previously described (Isseroff et al., 1987). At least three fibroblast strains, between passages 3 and 7, were used in all experiments. Stock cultures were maintained as monolayers in plastic cell culture dishes from Falcon Labware (BD Biosciences, San Jose, CA) using fibroblast growth medium (FM: Dulbecco’s modified Eagle’s medium (DMEM, basal medium), 1% antibiotic solution from Gibco (Grand Island, NY), and 10% calf serum (Tissue Culture Biologicals, Tulare, CA)). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO2.

Single cell migration assay

All single cell migration assays were performed on cells plated on glass coverslips (Eppendorf, Hamburg, Germany) that had been coated for 1 hour at 37°C in a humidified atmosphere of 5% CO2. The mixture gelled within 30 minutes upon incubation at 37°C in a humidified atmosphere of 5% air and 5% CO2. To assure even contraction, lattices were detached from the sides of the dishes after 2 hours by rimming the edges of the dishes using a sterile 100 μl tip and gently shaking the dishes until the gels slid freely. Lattice retraction was measured every day by placing the dishes over a flat ruler on a black background. After maximum retraction the lattices were digested with collagenase I (1,000 U/ml; Worthington Biochemicals, Freehold, NJ) and removed at 30 minutes for assessment of cell number and viability by Trypan Blue exclusion. Statistical analysis was performed using the two-tailed Student’s t-test packaged with

μM β-AR agonist alone or DMEM alone (control) for 5-60 minutes at 37°C, unless otherwise noted. Inhibitor-treated cells were then stimulated with DMEM containing inhibitor and 1 μM β-AR agonist or inhibitor and DMEM alone (control) for 5-60 minutes at 37°C, unless otherwise noted. Cells were plated immediately on ice, washed twice with ice-cold phosphate-buffered saline (PBS) containing phosphatase inhibitors (50 mM NaF and 1 mM Na3VO4) and scraped in 50 mM lysis buffer (PBS containing 0.5% Triton X-100, 50 mM NaF, 1 mM Na3VO4, leupeptin 10 μg/ml, aprotinin 30 μg/ml, PMSF 200 μg/ml, pepstatin A 10 μg/ml). The lysates were transferred into 1.5 ml tubes, incubated on ice for 20 minutes and then centrifuged at 14,000 g for 10 minutes at 4°C. The protein concentration of the samples was determined using the Bradford Assay (Bio-Rad Laboratories, Hercules, CA).

Immunoblotting

Each protein sample (5 μg) was added to an equal volume of 2X reducing sample loading buffer (Bio-Rad, Hercules, CA) and electrophoresed on 10% polyacrylamide Tris-HCl gels (Bio-Rad, Hercules, CA). Proteins were transferred to Immobilon membranes (Bio-Rad, Hercules, CA) and immunoblotted with either an anti-ERK antibody (#9102), an anti-phospho-ERK antibody (#9101; Cell Signaling Technology, Beverly, MA), an anti-phosphotyrosine antibody (Ab-4; Oncogene, Boston, MA) or an anti-EGFR antibody (1005; Santa Cruz Biotechnology, Santa Cruz, CA). The immunoblots were developed by enhanced chemiluminescence (ECL) according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometry was performed on scanned images using NIH Image 1.6.

Immunoprecipitation

Anti-EGFR antibody (1005; 5 μg) (Santa Cruz Biotechnology, Santa Cruz, CA) linked to 30 μl of pre-washed protein A/G Sepharose beads (Amersham Pharmacia, Piscataway, NJ) was used to immunoprecipitate the desired proteins from 1 ml of lysate prepared from 1-2x106 cells, either untreated or pre-treated with 1 μM β-AR agonist for 10 minutes, as described in the cell treatments section. Lysates were initially pre-cleared with 150 μl of pre-washed beads for 30 minutes at room temperature and then incubated with the antibody-bound beads at 4°C overnight on a rotary mixer. The beads were washed five times with lysis buffer, 1X reducing sample loading buffer was added (Bio-Rad, Hercules, CA), the samples were boiled for 3 minutes and centrifuged to pellet the beads. The supernatants were loaded onto two 10% polyacrylamide Tris-HCl gels (Bio-Rad, Hercules, CA) and the proteins were separated electrophoretically followed by transfer to Immobilon membrane for immunoblotting with either an anti-phosphotyrosine antibody (Ab-4) (Oncogene, Boston, MA) or an anti-EGFR antibody (1005; Santa Cruz Biotechnology, Santa Cruz, CA), as outlined above.

Proliferation assay

Dermal fibroblasts were released from the tissue culture plate by treatment with 0.25% trypsin/EDTA (Gibco, Grand Island, NY), resuspended in FM and counted using a haemocytometer. Cells were either untreated or pre-treated with 50 μM 8sp-cAMP or 50 μM 8p-cAMP for 30 minutes prior to β-AR agonist addition in FM. 5×103 cells were plated per well in a 12-well plate in triplicate in FM in the presence or absence of 10 μM β-AR agonist, 30 μM 8sp-cAMP or 50 μM 8p-cAMP. Triplicate wells were harvested and counted on days 2, 4 and 8. The medium was changed every day.

Collagen gel contraction assay

A solution of bovine collagen types I (97%) and III (3 mg collagen/ml Vitrogen 100; Collagen Corp., Palo Alto, CA) was mixed with triple strength DMEM, containing 20 mM Hepes buffer (Gibco, Grand Island, NY) to maintain neutral pH, calf serum (Tissue Culture Biologicals, Tulare, CA), cells (detached by trypsin from monolayer confluent cultures) and β-2-adrenergic receptor agonists or cAMP analogs if required. The immediate solutions were prepared and cooled to 4°C prior to mixing to prevent premature gelation. The final solution contained, by volume: 40% DMEM, 33% Hepes buffer, 25% calf serum, and 2% β-2-AR agonist. Cells were either untreated or pre-incubated with either 50 μM 8sp-cAMP or 50 μM 8p-cAMP for 30 minutes at 37°C and added to the collagen gel mix, in the presence or absence of 10 nM-10 μM β-AR agonist, 30 μM 8sp-cAMP or 50 μM 8p-cAMP. Triplicate wells were harvested and counted on days 2, 4 and 5. Treatment was monitored by placing the dishes over a flat ruler on a black background. After maximum retraction the lattices were digested with collagenase I (1,000 U/ml; Worthington Biochemicals, Freehold, NJ) and removed at 30 minutes for assessment of cell number and viability by Trypan Blue exclusion. Statistical analysis was performed using the two-tailed Student’s t-test packaged with
β2-AR is motogenic and mitogenic in dermal fibroblasts

KaleidaGraph software (Synergy Software, Reading, PA). Significance was ascribed to the addition of 1 × 10⁴ H9252-AR agonist for 15 minutes in FM. All steps were performed at room temperature unless otherwise noted. Coverslips were washed twice in phosphate-buffered saline (PBS) and fixed for 10 minutes in 4% paraformaldehyde. Coverslips were washed twice in PBS between each step. Cells were permeabilized for 5 minutes with 0.1% Triton X-100/PBS, blocked with 5% goat serum/PBS for 20 minutes, then primary monomolecular anti-vinculin antibody (Sigma, St Louis, MO) was added drop-wise in 1% goat serum/PBS (1:100) and incubated for 1 hour at 37°C. A goat anti-mouse Cy3 (Jackson Labs, West Grove, PA) (1:100) antibody was then added in 1% goat serum/PBS for 1 hour at 37°C. Alexa Fluor 488 phalloidin (Molecular Probes, Eugene, OR; 1:40) in PBS was added for 20 minutes and finally Prolong anti-fade reagent (Molecular Probes) was used according to manufacturer's instructions to mount the coverslips onto glass microscope slides. Slides were viewed on an inverted fluorescence Nikon Diaphot microscope using a 40× pan fluor objective. Images were captured using Q-imaging Retiga-EX cameras (Burnaby, BC, Canada) and pseudo-colored for Alexa Fluor 488 phalloidin (actin) and red for Cy3 (vinculin) using Improvision Openlab software (Lexington, MA). ImageJ was used to measure the mean pixel intensity of the actin and vinculin-associated fluorescent staining on 25 individual cells from each group. In each case, a background pixel reading was subtracted from the mean pixel intensity of each cell. The data was averaged and statistically analyzed using Student's t-test.

This work was supported in part by National Institutes of Health grants AR 48827 (C.E.P.) and AR 44518 (R.L.I.). NIH Image and ImageJ are public domain image processing and analysis programs for the Macintosh (developed at the US National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/ and http://rsb.info.nih.gov/ij/).

References

Beningo, K. A., Dembo, M., Kaverina, I., Small, J. V. and Wang, Y. L. (2003). Beta2-adrenergic receptor antagonist disrupts the actin cytoskeleton and inhibits proliferation and migration of dermal fibroblasts. J. Cell Biol. 161, 1241-1248.

de Wit, R. J., Hoppe, J., Sterk, P. F., Canavan, J. P., Shaw, C. and Trimble, E. R. (1991). The endocrine and paracrine effects of beta2-adrenoceptor agonists on human bronchial smooth muscle. J. Clin. Invest. 88, 2004-2016.

Fang, C. H. and Alexander, J. W. (1990). Wound contraction following transplantation of microskin autografts with overlaid skin allograft in experimental animals. Burns 16, 180-192.

Gabbiani, G., Hirschel, B. J., Ryan, G. B., Statkov, P. R. and Majno, G. (1972). Granulation tissue as a contractile organ. A study of structure and function. J. Exp. Med. 135, 725-743.

Glading, A., Chang, P., Lauffenburger, D. A. and Wells, A. (2000). Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway. J. Biol. Chem. 275, 2390-2398.

Grinnell, F. (1994). Fibroblasts, myofibroblasts, and wound contraction. J. Cell Biol. 124, 401-406.

Grinnell, F. (2000). Fibroblast-collagen-matrix contraction: growth-factor signalling and mechanical loading. Trends Cell Biol. 10, 362-365.

Hanoune, J. and Defer, N. (2001). Regulation and role of adenyl cyclase isoforms. Annu. Rev. Pharmacol. Toxicol. 41, 145-174.

Birnbaumer, L., Zhu, D., Panettieri, R. A. and Emala, C. W. (2001). Alpha2-adrenoceptor signaling in smooth muscle cells: a novel PKA-independent pathway. Am. J. Physiol. Cell Physiol. 281, C1468-C1476.

Birnbaumer, L., Zhu, D., Panettieri, R. A. and Emala, C. W. (2001). Alpha2-adrenoceptor signaling in smooth muscle cells: a novel PKA-independent pathway. Am. J. Physiol. Cell Physiol. 281, C1468-C1476.
Nankova, B. B. and Sabban, E. L. (1999). Multiple signalling pathways exist in the stress-triggered regulation of gene expression for catecholamine biosynthetic enzymes and several neuropeptides in the rat adrenal medulla. Acta Physiol. Scand. 167, 1-9.

Pantaloni, D., Le Clainche, C. and Carlier, M. F. (2001). Mechanism of actin-based motility. Science 292, 1502-1506.

Pierce, K. L., Maudsley, S., Daaka, Y., Luttrell, L. M. and Lefkowitz, R. J. (2000). Role of endocytosis in the activation of the extracellular signal-regulated kinase cascade by sequestering and nonsequestering G protein-coupled receptors. Proc. Natl. Acad. Sci. USA 97, 1489-1494.

Pierce, K. L., Tohgo, A., Ahn, S., Field, M. E., Luttrell, L. M. and Lefkowitz, R. J. (2001). Epidermal growth factor (EGF) Receptor-dependent ERK Activation by G Protein-coupled receptors. A co-culture system for identifying intermediates upstream and downstream of heparin-binding EGF shedding. J. Biol. Chem. 276, 23155-23160.

Pullar, C. E. and Isseroff, R. R. (2005). Cyclic AMP mediates keratinocyte directional migration in an electric field. J. Cell Sci. 118, 2023-2034.

Pullar, C. E., Grahn, J. C., Liu, W. and Isseroff, R. R. (2006). β2 adrenergic receptor activation delays wound healing. PNAS J. 20, 76-86.

Redden, R. A. and Doolin, E. J. (2003). Collagen crosslinking and cell density have distinct effects on fibroblast-mediated contraction of collagen gels. Skin Res. Technol. 9, 290-293.

Rudolph, R., Berg, J. V. and Ehrlich, H. P. (1992). Wound contraction and scar formation in Wound Healing: Biochemical and Clinical Aspects. Vol. 96 (ed. I. K. Cohen, R. F. Diegelmann and W. J. Lindblad), pp. 114. Philadelphia: W. B. Saunders.

Sastrodihardjo, S., Sasaki, Y., Shiba, Y. and Kanno, Y. (1987). Possible involvement of reorganization of actin filaments, induced by tumor-promoting phorbol esters, in changes in colony shape and enhancement of proliferation of cultured epithelial cells. J. Cell Physiol. 132, 49-56.

Schallreuter, K. U. (1997). Epidermal adrenergic signal transduction as part of the neuronal network in the human epidermis. J. Invest. Dermatol. Symp. Proc. 2, 37-40.

Schallreuter, K. U., Lempke, K. R., Pittelkow, M. R., Wood, J. M., Korner, C. and Malik, R. (1995). Catecholamines in human keratinocyte differentiation. J. Invest. Dermatol. 104, 953-957.

Sedowofia, K., Barclay, C., Quaba, A., Smith, A., Stephen, R., Thomson, M., Watson, A. and McIntosh, N. (1998). The systemic stress response to thermal injury in children. Clin. Endocrinol. (Oxford) 49, 335-341.

Skalli, O. and Gabbiiani, G. (1988). The biology of the myofibroblast relationship to wound contraction and fibrocontractive disease. In The Molecular and Cellular Biology of Wound Repair (ed. R. A. F. Clark and P. M. Henson), pp. 373-404. New York: Plenum Press.

Spurzem, J. R., Gupta, J., Veys, T., Kneiff, K. R., Rennard, S. I. and Wyatt, T. A. (2002). Activation of protein kinase A accelerates bovine bronchial epithelial cell migration. Am. J. Physiol. Lung Cell Mol. Physiol. 282, L1108-L1116.

Steinkraus, V., Mak, J. C., Pichlmeier, U., Xensing, H., Ring, J. and Barnes, P. J. (1996). Autoradiographic mapping of beta-adreceptors in human skin. Arch. Dermatol. Res. 288, 549-553.

Tseraidsis, G. S. and Bavykina, E. A. (1972). Adrenergic innervation of normal human skin. Vests. Dermatol. Venerol. 46, 40-55.

Van Haastert, P. J., Van Driel, R., Jastorff, B., Baraniak, J., Stec, W. J. and De Wit, R. J. (1984). Competitive CAMP antagonists for cAMP-receptor proteins. J. Biol. Chem. 259, 10020-10024.

Vande Berg, J. S. and Rudolph, R. (1985). Cultured myofibroblasts: a useful model to study wound contraction and pathological contracture. Ann. Plast. Surg. 14, 111-120.

Xiao, R. P., Cheng, H., Zhou, Y. Y., Kuschel, M. and Lakatta, E. G. (1999). Recent advances in cardiac beta(2)-adrenergic signal transduction. Circ. Res. 85, 1092-1100.

Yeh, C. K., Ghosh, P. M., Dang, H., Liu, Q., Lin, A. L., Zhang, B. X. and Katz, M. S. (2005). (beta) Adrenergic responsive activation of extracellular signal-regulated protein kinases in salivary cells. Role of epidermal growth factor receptor and cAMP. Am J. Physiol. Cell Physiol. 288, C1357-C1366.