β-Adrenergic Receptor Trafficking, Degradation, and Cell Surface Expression Are Altered in Dermal Fibroblasts from Hypertrophic Scars

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Burn trauma elevates catecholamines for up to 2 years and causes hypertrophic scarring. Propranolol, a nonspecific β₁-, β₂-adrenergic receptor (AR) inverse agonist, counters the hypermetabolic response to elevated catecholamines and may decrease hypertrophic scarring by an unknown mechanism. We investigated the effect of burn injury on β₁- and β₂-AR expression, trafficking, and degradation in human dermal fibroblasts from hypertrophic scar [HSF], non-scar fibroblasts, and normal fibroblasts. We also investigated the modulation of these events by propranolol. Catecholamine-stimulated cAMP production was lower in HSFs and non-scar fibroblasts than in normal fibroblasts. β₁- and β₂-AR cell surface expression was lowest in HSFs, but propranolol increased cell surface expression of these receptors. Basal β₂-AR ubiquitination was higher in HSFs than non-scar or normal fibroblasts, suggesting accelerated receptor degradation. β-AR degradation was mainly driven by lysosomal-specific polyubiquitination at Lys-63 in normal fibroblasts and HSFs, which was abrogated by propranolol. Propranolol also targeted β-AR to the proteasome in HSFs. Confocal imaging showed a lack of β₂-AR-GFP trafficking to lysosomal compartments in catecholamine-stimulated HSFs. These data suggest that burn trauma alters the expression, trafficking, and degradation of β-ARs in dermal fibroblasts, which may then affect fibroblast responses to propranolol.


INTRODUCTION

Burn trauma is characterized by a massive increase in catecholamines (Jeschke et al., 2011) accompanied by increased inflammation and poor wound healing. These occurrences contribute to the formation of hypertrophic scars, which are painful and itchy, compromise function, and reduce the patient’s quality of life (Finnerty et al., 2016). Propranolol, a nonspecific β₁- and β₂-adrenergic receptor (AR) inverse agonist, counters postburn stress responses driven by the massive increase in systemic catecholamines, including the hypermetabolic response (Finnerty and Herndon, 2013; Herndon et al., 2001). We found that propranolol speeds burn wound healing (Ali et al., 2015), which we hypothesize to be associated with decreased hypertrophic scarring; a preliminary review of patient data showed a reduction in postburn scarring with propranolol (Finnerty, Herndon, & El Ayadi, unpublished data). Nevertheless, the mechanisms underlying scar reduction in propranolol-treated patients are unclear.

β-ARs are members of the GPCR family. This family of cell surface proteins facilitates physiological responses to hormones, neurotransmitters, lipids, and peptides. β-AR signaling depends on the subcellular localization of these receptors and the number present on the cell surface at the time of stimulus. After activation, GPCRs activate adenylylate cyclase to generate cAMP, which subsequently releases the catalytic subunits of protein kinase A (PKA). PKA is responsible for activating many downstream targets and enzymes involved in cell proliferation and migration, two processes that are highly implicated in hypertrophic scarring. GPCR signal transduction is also controlled by agonist-induced desensitization through uncoupling of G-protein activation and by internalization—the process whereby receptors undergo endocytosis and are either recycled to the cell surface or degraded internally (Drake et al., 2006; Hanyaloglu and von Zastrow, 2008).

After receptor activation, agonist-bound β₂-AR is phosphorylated on the carboxyl tail through activation of the GPCR kinases (GRKs) and engagement of β-arrestins, leading to short-term β₂-AR desensitization (Sarker et al., 2011). GRK-phosphorylated, β-arrestin–bound β₂-AR is ubiquitinated on two different domains, the intracellular loop (lys-63) and the carboxyl tail. This ubiquitination targets β₂-ARs.

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Abbreviations: β-AR, adrenergic receptor; HSF, hypertrophic scar fibroblast; GRK, GPCR kinase; NSF, non-scar fibroblast; NF, normal fibroblast; PKA, protein kinase A

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for lysosomal degradation, explaining the global reduction in cellular receptor levels that characterizes long-term desensitization of GPCRs upon prolonged agonist stimulation (Sarker et al., 2011).

Here, we determined the effect of burn trauma on β-AR expression, ubiquitination, trafficking, and degradation. Given our observation that propranolol decreases hypertrophic scarring in burn patients, we also investigated whether propranolol may ameliorate scarring by altering these events. We used primary dermal fibroblasts isolated from hypertrophic scar (hypertrophic scar fibroblast [HSF]) and non-scarred skin (non-scar fibroblast [NSF]) biopsy samples from several pediatric burn patients. Normal dermal fibroblasts (NFs) served as a control for burn-induced alterations. To mimic the postburn increase in catecholamines, we stimulated fibroblasts with the β-AR agonist epinephrine. We also used the β-AR agonist isoproterenol, which works similarly to some β2-AR-specific agonists and induces receptor internalization and desensitization. We assessed mRNA transcript expression, receptor distribution, receptor ubiquitination status with hemagglutinin-tagged wild type (Lauwers et al., 2009) and mutant ubiquitin constructs (Newton et al., 2008), and receptor trafficking with confocal imaging of GFP-tagged β2-AR.

RESULTS
To determine whether isolated human skin fibroblasts exhibited established markers of fibroblasts, we conducted flow cytometry and found that NFs, HSFs, and NSFs all expressed the anti-fibroblast marker CD90, fibronectin, and hyaluronic synthase-2, a critical enzyme for the biosynthesis of the fibroblast marker hyaluronan (see Supplementary Figure S1a online). These markers confirmed the fibroblast identity, as opposed to monocyte-derived fibrocytes, monocytes, and macrophages (Pilling et al., 2009).

Messenger RNA expression for β1-, β2-, and β3-ARs was similar in HSFs, NSFs, and NFs (Figure 1a). Primer specificity was confirmed in lung and heart tissue by determining enrichment in β1- and β2-ARs (see Supplementary Figure S1b). Given the known effects of propranolol, isoproterenol, or isoproterenol plus propranolol on β-AR activity, we determined the effects of these compounds on β-AR mRNA expression in these cells. Expression of β1-AR mRNA transcripts was not affected by propranolol, isoproterenol, or isoproterenol plus propranolol in any of the fibroblast lines (Figure 1b).

β2-AR mRNA transcripts were decreased in HSFs 1 hour after treatment with isoproterenol or propranolol plus isoproterenol (both P < 0.05) and remained decreased at 24 hours with propranolol plus isoproterenol (P < 0.05). Likewise, β2-AR mRNA expression in NSFs was decreased 1 hour after
isoproterenol treatment ($P < 0.05$) compared with vehicle-treated control. These levels were also decreased 24 hours after treatment with isoproterenol plus propranolol ($P < 0.05$). 

$\beta$-AR mRNA expression was decreased in HSFs 24 hours after treatment with isoproterenol or isoproterenol plus propranolol (both $P < 0.05$). Propranolol did not reverse the effects of isoproterenol on $\beta$-AR mRNA transcripts in any of the cell lines examined.

Catecholamine-induced cAMP generation and PKA activation are dampened in HSFs and can be blocked by propranolol

At 24 hours after plating, dermal fibroblasts were treated with epinephrine or isoproterenol with or without propranolol for 20 minutes. In all three cell types, epinephrine increased cAMP generation, and this was blocked by propranolol (Figure 2a–c). Epinephrine-induced cAMP generation was higher in NFs than in burn patient-derived fibroblasts (HSFs and NSFs). Similarly, isoproterenol induced a greater increase in cAMP in NFs than in HSFs and NSFs, and this was blocked by propranolol.

The cAMP-dependent protein kinase, PKA, is activated by the release of the catalytic subunit after an increase in cAMP levels. Basal PKA activity was lower in HSFs and NSFs than NFs (both $P < 0.01$) (Figure 2d). In NFs, PKA activity was increased by epinephrine ($P < 0.05$) and isoproterenol ($P < 0.01$). These effects were reversed by propranolol. In HSFs, epinephrine plus propranolol elevated PKA activity ($P < 0.001$).

Propranolol increases cell surface expression of $\beta$1- and $\beta$2-AR in HSFs

$\beta$1- and $\beta$2-AR cell surface expression was lower in HSFs than in NFs (Figure 3a and b). Propranolol significantly increased $\beta$1- and $\beta$2-AR cell surface expression in HSFs (both $P < 0.05$). Propranolol also increased $\beta$1- and $\beta$2-AR levels in the total fraction ($P < 0.05$) without affecting these levels in the cytosolic fraction (Figure 3a and b). In all dermal fibroblasts, propranolol failed to alter $\beta$3-AR levels in the cell surface fraction, the cytosolic fraction, or total homogenates.

Flow cytometric analysis showed decreased $\beta$1-AR expression in NFs 15 minutes after epinephrine treatment ($P < 0.05$) and 3 hours after epinephrine plus propranolol treatment ($P < 0.05$). $\beta$2-AR expression in NFs was decreased at 15 minutes after treatment with epinephrine ($P < 0.05$) (Figure 3c) and at 15 minutes and 3 hours after treatment with propranolol (both $P < 0.05$). $\beta$3-AR expression in NFs was decreased at 1 hour after treatment with epinephrine, propranolol (both $P < 0.001$), and epinephrine plus propranolol ($P < 0.05$). $\beta$3-AR expression was still lowered 3 hours after

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**Figure 2.** cAMP generation and PKA activation are reduced in burn patient-derived fibroblasts after stimulation with epinephrine (EPI) and isoproterenol (ISO). At 24 hours after being plated, cells were treated with the indicated combination of EPI (10 μmol/L), ISO (10 μmol/L), and propranolol (PPL) (10 μmol/L). At 20 minutes after treatment, cells were collected, and cyclic AMP levels were measured in (a) NFs, (b) HSFs, and (c) NSFs. *$P < 0.05$, **$P < 0.01$ compared with vehicle-treated fibroblasts. (d) PKA activity was analyzed in HSFs, NSFs, and NFs as described in the Methods section. Data are presented as PKA activity per nanogram of tissue. ***$P < 0.01$ compared with vehicle-treated NFs, **$P < 0.05$ compared with vehicle-treated NFs, *$P < 0.05$ compared with vehicle-treated NSFs, and ****$P < 0.001$ compared with vehicle-treated HSFs. For each experiment, all cell lines were used at the same passage, which ranged from passages 6 to 9. Data represent the mean ± standard error of the mean of three independent experiments. CTR, control; HSF, hypertrophic scar fibroblast; M, mol/L; NF, normal fibroblast; NSF, non-scar fibroblast; PKA, protein kinase A.
treatment with propranolol \((P < 0.001)\) and epinephrine plus propranolol \((P < 0.01)\).

In HSFs, none of the treatments altered the expression of \(\beta_1\)-AR. \(\beta_2\)-AR expression in HSFs increased with propranolol at 1 and 3 hours (both \(P < 0.05\)) (Figure 3c), whereas \(\beta_3\)-AR expression was decreased with propranolol at 1 hour \((P < 0.05)\) and with epinephrine at 1 and 3 hours \((P < 0.05)\). In NSFs, epinephrine treatment increased \(\beta_1\)-AR at 15 minutes and 1 hour (both \(P < 0.05\)) and decreased \(\beta_2\)-AR at 15 minutes \((P < 0.01)\). None of the treatments affected \(\beta_3\)-AR expression in NSFs (Figure 3c).

Endogenous \(\beta\)-AR ubiquitination is altered in HSFs and can be modulated by propranolol

Ubiquitination regulates \(\beta\)-AR trafficking and degradation (Xiao and Shenoy, 2011). The nature of the polyubiquitin
chain dictates protein targeting to the proteasome, lysosomes, or endocytic system. Basal endogenous β2-AR ubiquitination was higher in HSFs than NSFs and NFs (Figure 4a). To determine the nature of polyubiquitin chains regulating β2-AR trafficking, we used Lys-specific polyubiquitin constructs. As shown in Figure 4b, basal total ubiquitination was higher in HSFs than in NFs and NSFs (lane 7 vs. lanes 1 and 13, Figure 4b and c, both \( P < 0.001 \)). Propranolol increased total ubiquitination in NFs (lane 2 vs. lane 1, \( P < 0.01 \)) but not HSFs (lane 8 vs. lane 7) or NSFs (lane 14 vs. lane 13) (Figure 4b and c).

Lys-48 polyubiquitination indicative of proteasome-dependent degradation was decreased by propranolol in NFs (lane 3 vs. lane 4, \( P < 0.05 \)) and slightly increased in HSFs (lane 9 vs. lane 10). Lys-63 polyubiquitination indicative of lysosomal-dependent degradation was decreased by propranolol in NFs (lane 5 vs. lane 6, \( P < 0.05 \)) and HSFs (lane 11 vs. lane 12, \( P < 0.001 \)) (Figure 4b and c), suggesting that propranolol may decrease β2-AR trafficking to the endocytic pathway.

**Propranolol modulates the degradation of β-AR in human dermal fibroblasts**

To analyze the proteasomal- and lysosomal-dependent degradation of β1-, β2-, and β3-AR in burn patient-derived dermal fibroblasts, we treated fibroblasts with the proteasome inhibitor MG132 or the lysosomal inhibitor leupeptin for 3 or 6 hours (Figure 5a—e). In NFs, HSFs, and NSFs, β1-AR degradation was reduced by MG132 or leupeptin (all \( P < 0.05 \)), suggesting the involvement of both systems in β1-AR degradation. With propranolol, β1-AR degradation through the proteasome was reduced in NFs but not in HSFs or NSFs (both \( P < 0.05 \)) (Figure 5b and c).

Basal β2-AR degradation was slower in HSFs and NSFs than in NFs (Figure 5a and d). Propranolol decreased lysosomal β2-AR degradation in NFs and NSFs, as shown by β2-AR accumulation in these cells (both \( P < 0.05 \)) (Figure 5b and d). Propranolol shifted β3-AR degradation to the lysosomes in NFs, as shown by an increase in β3-AR protein levels with leupeptin (\( P < 0.05 \)) (Figure 5b and e). In NSFs, propranolol directed β3-AR degradation toward the proteasome, as seen by an increase in β3-AR levels with MG132 (\( P < 0.05 \)) (Figure 5b and e).

**β2-AR–GFP translocates to lysosomal compartments in NFs and NSFs**

β2-AR trafficking was monitored by transfecting fibroblasts with GFP-tagged β2-AR constructs (Han et al., 2013). β2-AR was expressed mostly at the cell surface of NFs (Figure 6a, left top panel). Under basal conditions, β2-AR–GFP co-localization to lysosomal compartments was higher in HSFs than in NSFs or NFs (Figure 6a, top middle panel). Six hours of isoproterenol (10μmol/L) stimulation of fibroblasts resulted in translocation of β2-AR to the lysosomal compartments, as shown by the increased correlation index in NFs (\( P < 0.01 \)) and NSFs (\( P < 0.05 \)) but not HSFs (Figure 6a [second row] and b). Propranolol decreased these effects, as shown by decreased co-localization to the lysosomes in NFs and NSFs treated with isoproterenol with or without propranolol (both \( P < 0.05 \)) (Figure 6a [third row] and b), suggesting trafficking of β2-ARs to other degradation pathways.
To test such a possibility, co-localization studies were repeated in fibroblasts treated with isoproterenol with or without lysosome or proteasome inhibitors. In NFs, leupeptin resulted in lower lysosomal localization of β2-AR-eGFP than the proteasome inhibitor, confirming the canonical lysosomal-targeting of β2-AR after isoproterenol stimulation in control cells (Figure 6a, fourth row, left panel). In NSFs, leupeptin decreased isoproterenol-induced β2-AR trafficking to lysosomes compared with isoproterenol alone (Figure 6a, bottom row, right panel), whereas MG132 increased it (Figure 6a, fifth row, right panel). In HSFs, MG132 did not target β2-AR to the lysosomes (Figure 6b, bottom row, middle panel). This result was reproduced using Lysotracker (Molecular Probes, Eugene, OR) instead of LAMP-2A (data not shown). The β2-AR trafficking pattern was consistently seen in HSFs from several burn patients, suggesting that β2-AR is targeted to different degradation machinery after isoproterenol stimulation in these cells and therefore, recycling these receptors to the cell surface takes longer in HSFs than in normal fibroblasts.

DISCUSSION

We have shown that wound healing in burn patients is accelerated by propranolol (Ali et al., 2015). In addition, the severity of hypertrophic scarring, as assessed by the Vancouver scar scale, is significantly lower in propranolol-treated patients than in control individuals (Finnerty, Herndon, El Ayadi, unpublished data). To understand the mechanism by which propranolol decreases hypertrophic scarring, we analyzed the expression, ubiquitination, trafficking, and degradation of β-ARs in dermal fibroblasts from burn patient skin and scar and in normal neonatal human fibroblasts, all of which were found to express established fibroblast markers (Pilling et al., 2009). Together, our data show that burn trauma

Figure 4. Effect of burn trauma on endogenous β-AR ubiquitination in human dermal fibroblasts treated with or without propranolol (PPL). Cells were seeded on 10-cm plates in serum media and 24 hours later were transfected with 10 μg/ml HA-ubiquitin DNA in Opti-MEM reduced serum media using Lipofectamine 2000 reagent (Invitrogen, Life Technologies, Waltham, MA) following the manufacturer’s protocol. Five hours after transfection, Opti-MEM was replaced with full serum media. (a) Co-immunoprecipitation/Western blot analysis of ubiquitinated β2-AR in fibroblasts expressing wild-type HA-ubiquitin. (b) Representative blots showing ubiquitination of β2-AR in the presence or absence of PPL in cells expressing wild-type HA-ubiquitin constructs or mutant HA-ubiquitin constructs in which all lysine residues were mutated to arginine except Lys-63 or Lys-48. All lanes were preincubated with the proteasome inhibitor MG132 as described in the Methods section. (c) Densitometric analysis of β2-AR ubiquitination. Data are mean ± standard error of the mean of three independent experiments. * P < 0.05, *** P < 0.001. Dermal fibroblasts from different patients were used between passages 6 and 9. AR, adrenergic receptor; Ctrl, control; HA, human influenza hemagglutinin; HSF, hypertrophic scar fibroblast; NF, normal fibroblast; NSF, non-scar fibroblast; WT, wild type.
alters the expression, trafficking, and degradation of β1- and β2-AR but not β3-AR in dermal fibroblasts and that these changes affect fibroblast responses to propranolol.

Basal expression of β-AR mRNA transcripts was comparable in HSFs, NSFs, and NFs. Epinephrine- and isoproterenol-induced cAMP generation was lower in burn-derived HSFs and NSFs than NFs. Propranolol significantly decreased epinephrine- and isoproterenol-induced cAMP release from NFs and NSFs and, to a lesser extent, from HSFs. Basal PKA activity was also lower in burn patient-derived fibroblasts than NFs and was not increased by epinephrine or isoproterenol. These data suggest that expression of β-ARs at the cell surface may be involved in the decreased response to stimuli. However, this may not be the sole culprit for decreased cAMP and PKA activity in response to receptor stimulation. Although burn patient-derived fibroblasts do express β-ARs, some GPCR signaling components may not function properly. A similar notion was introduced by Reed and others after showing that decreased cAMP in atopic dermatitis may be due to a deficiency in adenylate cyclase (Reed et al., 1976; Wadskov et al., 1979). The levels of cAMP and cAMP effector proteins (e.g., PKA) are decreased in inflammatory diseases such as psoriasis and atopic dermatitis (Brion et al., 1986; Wadskov et al., 1979). Similar alterations may be occurring in dermal fibroblasts derived from burn patients because of the hyperinflammatory environment. Sustained exposure to catecholamines, mimicking the clinical presentation after severe burn injury, alters cAMP/PKA signaling in ventricular myocytes from rats, leading to dysfunction and hypertrophy (Fields et al., 2016). Decreased cAMP/PKA activity also results from an overactivation of β2-AR—coupled Gi proteins, thereby preventing the positive inotropic effect of agonist-induced β-AR stimulation (Xiao et al., 1999).

We investigated the possibility that HSFs may have fewer β-ARs at the cell surface. We found that propranolol significantly increased cell surface and total β1- and β2-AR in HSFs but not in NSFs and NFs. This suggests that the receptor is not internalized in HSFs to act on intracellular targets. As a result, cAMP generation and PKA activity are both decreased. Propranolol did not significantly affect the expression of cell surface β3-AR, which mainly activates noncanonical Gi/nitric oxide synthase pathways when exposed to high levels of catecholamines (Gauthier et al., 1998).

Decreased cell surface expression of β2-AR in HSFs may arise from greater degradation of receptors after internalization rather than reduced recycling to the cell surface. Trafficking of β2-AR to endocytic compartments, the proteasome system, or lysosomes for degradation is partially controlled by ubiquitination (Drake et al., 2006; Xiao and Shenoy, 2011). We found that basal β2-AR ubiquitination was higher in HSFs than other dermal fibroblast types, suggesting that these receptors are tagged for degradation. We determined which proteolytic system participates in β-AR degradation using hemagglutinin-tagged ubiquitin mutant constructs that recognize specific polyubiquitin chains (Lys-48 polyubiquitin or Lys-63 polyubiquitin). Polyubiquitin chains conjugated to Lys-29 or

Figure 5. Propranolol (PPL) modulates β-AR degradation in human dermal fibroblasts. At 24 hours after being plated, NFs, HSFs, and NSFs were pretreated for 30 minutes with PPL or vehicle and then treated for the indicated time with a proteasome inhibitor (MG132) (10 μmol/L) or lysosomal inhibitor (leupeptin) (50 μmol/L). Western blotting was performed to determine the expression of β1-, β2-, and β3-ARs in the presence of (a) vehicle or (b) PPL (10 μmol/L). (c) Quantification of β1-AR band intensity. (d) Quantification of β2-AR band intensity. (e) Quantification of β3-AR band intensity. Data were normalized to control and are presented as mean ± standard error of the mean of at least three independent experiments. *P < 0.05. AR, adrenergic receptor; HSF, hypertrophic scar fibroblast; Leup, leupeptin; NF, normal fibroblast; NSF, non-scar fibroblast.
Lys-48 of ubiquitin direct substrate proteins to the proteasome for degradation. Polyubiquitination on Lys-63 and monoubiquitination are implicated in receptor endocytosis, trafficking, sorting, translation, and DNA repair (Chiu et al., 2006; Lauwers et al., 2009). Propranolol decreased WT ubiquitination and Lys-63-linked polyubiquitination in HSFs and increased Lys-48-linked polyubiquitination. Thus, propranolol may help clear accumulated and possibly aggregated $\beta_2$-AR in these cells by targeting them to the proteasome for a quick recycling.

Imaging of GFP-tagged $\beta_2$-AR was consistent with protein expression, cAMP, and PKA activity data, suggesting that HSFs have fewer cell surface receptors. HSFs expressed more $\beta_2$-AR in the lysosomal compartments than NFs and NSFs. In the presence of isoproterenol, this expression pattern did not change, consistent with the idea that these cells have fewer receptors at the cell surface and therefore are less responsive to isoproterenol. Propranolol restored $\beta_2$-AR expression in NFs and NSFs but not HSFs.

In summary, decreased cell surface expression and increased ubiquitination of $\beta_2$-AR in HSFs suggests that $\beta_2$-AR is differently modulated by ubiquitination in these cells than in NFs or NSFs. The postburn catecholamine surge and subsequent hypertrophic scar development may alter the distribution and trafficking of dermal fibroblast $\beta$-ARs and the response of these receptors to propranolol or other beta blockers. Our data show that propranolol increases cell surface expression and decreases the general ubiquitination of $\beta_2$-AR in an attempt to make more receptors available at the cell surface for drug-mediated signaling. Decreased cell surface expression leads to decreased cAMP generation, PKA activity, and consequent engagement of downstream signaling pathways. Ongoing studies are investigating the effects of propranolol on signaling pathways involved in propranolol-induced reduction of hypertrophic scarring.

MATERIALS AND METHODS

Cell culture

Skin and scar biopsy samples were collected from patients at 6 or 12 months postburn, and fibroblast isolation was conducted in serum media as previously described (Zhang et al., 2012). Patients enrolled in the clinical trial were between 0 and 18 years of age at the time of injury, had at least 30% of the total body surface area burned, and required at least one operative intervention. This study was part of a large clinical trial (www.clinicaltrials.gov, NCT00675714) evaluating the outcomes of burn survivors. The study protocol and informed consent forms were in compliance with Declaration of Helsinki principles and were approved by the institutional review board of the University of Texas Medical Branch (Galveston, TX). In this study, we used skin and scar biopsy samples from four patients aged 6 months to 13.8 years old, with total body surface area burns between 30% and 50%.
All patients or their legal guardians, as applicable, provided written informed consent. Nontransformed primary neonatal, dermal fibroblasts (NFs) were purchased from ATCC (Manassas, VA). Fibroblasts were grown in DMEM medium containing 13% fetal bovine serum, 1% antibiotic/antimycotic containing 10,000 IU/ml penicillin, and 10,000 μg/ml streptomycin. All cells were used between passages 6 and 10. HSFs were compared with the NSFs that were excised from the same patient on the same day. For consistency, all paired fibroblasts (HSFs and NSFs) from the same patient and NFs were used at the same passage. Propranolol hydrochloride and isoproterenol were purchased from Tocris (Bristol, UK). Epinephrine was purchased from Sigma-Aldrich (St. Louis, MO).

Western blotting
After treatment, cells were lysed in buffer containing 5 mmol/L EDTA, 750 mmol/L NaCl, 250 mmol/L Tris, 5% Triton X-100, 150 mmol/L phenylmethylsulfonyl fluoride, and 0.1% phosphatase inhibitor. Protein concentration was determined using the bicinchoninic acid method (Thermo Fisher Scientific, Waltham, MA); 50 μg of protein lysate was separated by SDS-PAGE (El Ayadi et al., 2012). Antibody details are provided in Supplementary Table S1 online. Negative and positive controls were used to characterize the β1-, β2-, and β3-AR specificity in our system. Protein expression was quantified by densitometry (Xiao and Shenoy, 2011) using Image J software (available at http://imagej.nih.gov/ij/; National Institutes of Health, Bethesda, MD).

Quantitative real-time PCR
Messenger RNA was isolated using the RNasey Plus mini kit (Qiagen, Valencia, CA) and cDNA produced using a cDNA synthesis kit (Bio-Rad, Hercules, CA). PCR primer sequences are listed in Supplementary Table S2 online.

Flow cytometry
Intracellular and extracellular staining for fibroblast markers and β-ARs in HSF, NSF, and NF cells were detected using a Becton Dickinson
Accuri flow cytometer (BD Biosciences, Franklin Lakes, NJ). Data were analyzed using BD FACSDiva software (BD Biosciences, San Jose, CA). The antibodies used are listed in Supplementary Table S1.

**cAMP and PKA activity assays**

Intracellular cAMP was measured using a Catchpoint cAMP assay kit (Molecular Devices, Sunnyvale, CA) following the manufacturer’s protocol. The PKA activity was measured in 20 μg of protein using the PKA Kinase activity kit (catalog number ADI-EKS-390A, Enzo, New York, NY) following the manufacturer’s instructions.

**Cell surface biotinylation**

Cell biotinylation assays were conducted as previously described (El Ayadi et al., 2012). Control and propranolol-treated fibroblasts were incubated with 1 mmol/L cell impermeant biotinylation reagent (Sulfo-NHS-SS-Biotin; Pierce, Thermo Fisher Scientific) for 3 hours at 4°C and then quenched and lysed. Next, 500 μg protein were added to a 25-μl bed volume of NeutrAvidin agarose resin (Pierce, Thermo Fisher Scientific) and incubated overnight at 4°C. The cytosolic fraction was then collected before washing the resin with wash buffer containing protease inhibitors. Biotinylated proteins were eluted by boiling NeutrAvidin beads in SDS sample buffer and were subjected to Western blot.

**Degradation assay**

Dermal fibroblasts were treated with MG132 (Calbiochem, Millipore, MA) or leupeptin (Peptides International, Louisville, KY) (El Ayadi et al., 2012; Sarker et al., 2011). Control cells received DMSO as a vehicle. Cells were collected after 0, 3, and 6 hours. β-AR protein levels were determined by Western blot.

**Ubiquitination assays**

β-2-AR ubiquitination was determined by transfecting cells with human influenza hemagglutinin-tagged ubiquitin plasmid constructs from Addgene (Cambridge, MA): wild-type human influenza hemagglutinin–ubiquitin (plasmid #17608), Lys 48-human influenza hemagglutinin-ubiquitin (plasmid #17605), and Lys 63-human influenza hemagglutinin-ubiquitin (plasmid #17606). After 16 hours, cells were treated with MG-132 for 3 hours to induce accumulation of ubiquitinated proteins and harvested in 150 μl lysis buffer containing protease inhibitors (Roche Diagnostics, Mannheim, Germany) and 10 mmol/L deubiquitinating enzyme inhibitor N-ethylmaleimide (Sigma-Aldrich). Next, 500 μg of protein lysate was precleared before incubation with a β-2-AR antibody for 2 hours at 4°C (El Ayadi et al., 2012). Immune complexes were recovered using 50 μl protein A-Sepharose beads (Pierce, Thermo Fisher Scientific). The beads were washed with lysis buffer, boiled with SDS sample buffer, and separated by SDS-PAGE/Western blot.

**Immunohistochemistry**

β-2-AR constructs were kindly provided by Sudha Shenoy and Robert Lefkowitz. At 24 hours after transfection, fibroblasts were treated with isoproterenol with or without propranolol, GM132, or leupeptin. β-2-ARs localize to lysosomes 6 hours after activation with isoproterenol (Drake et al., 2006; Sarker et al., 2011). Thus, 6 hours after treatment, fibroblasts were fixed with 4% paraformaldehyde in phosphate buffered saline. Lysosomal localization was determined using Lysotracker (#L7528, Molecular Probes) or LAMP2 antibody with an Alexa Fluor 538 rabbit secondary antibody. Receptor trafficking was monitored using a Nikon Ti inverted microscope scope (Nikon, Tokyo, Japan) outfitted for high-resolution multicolor fluorescence microscopy and equipped with a Yokogawa CSU-X1 spinning disk, an Andor camera (iXon3 EMCCD; Oxford Instruments, Andor Technology, Belfast, UK), and a confocal set-up with 488-nm and 561-nm lasers. All images were taken at room temperature with a Nikon plan apochromatic 100X oil objective with 1.4 numerical aperture. Images were acquired using Metamorph software (Molecular Devices) and grouped in PowerPoint (Microsoft, Redmond, WA) with no manipulation. β-2-AR–GFP co-localization to lysosomes was determined using the ImageJ Colocalization Colormap plugin.

**Statistical analysis**

Statistical analysis was conducted using Prism (PRISM 5, Graph Pad Software, La Jolla, CA). One way analyses of variance or Student t tests were used as appropriate, to compare controls and treated samples. Significance was accepted at P less than or equal to 0.05.

**CONFLICT OF INTEREST**

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**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.01.037.

**REFERENCES**


