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Journal

Wound Repair and Regeneration: The International Journal of Tissue Repair and Regeneration, 21(5)

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Publication Date

2013

DOI

10.1111/wrr.12083

Peer reviewed

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HHS Public Access

Author manuscript

Wound Repair Regen. Author manuscript; available in PMC 2015 July 20.

Published in final edited form as:

Wound Repair Regen. 2013 ; 21(5): 715–722. doi:10.1111/wrr.12083.

Local blockade of glucocorticoid activation reverses stress- and glucocorticoid-induced delays in cutaneous wound healing

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Abstract

Stress slows cutaneous wound healing (WH) in an endogenous glucocorticoid (GC)-dependent fashion. We investigated whether stress/GC-induced delays in WH require further intracutaneous activation of endogenous GC; and whether blockade or down-regulation of peripheral activation normalizes WH in the face of stress. Delayed WH in our motion-restricted murine model of stress could be attributed to elevated systemic GC, because blockade of GC production (using corticotropin-releasing factor inhibitor, antalarmin), or of peripheral binding to the GC receptor [GCr], with an antagonist, Ru-486, normalized WH. We next investigated whether local blockade or down-regulation of the peripheral GC-activating enzyme, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), accelerates cutaneous WH. Topical applications of nonspecific (carbenoxolone) as well as an isoform-specific 11β-HSD1 inhibitor overcame stress and exogenous GC-induced delays in WH. Moreover, two liver X receptor ligands, TO901317 and GW3695, down-regulated expression of 11β-HSD1, attenuating stress-induced delays in WH. Combined inhibitor and liver X receptor ligand applications accelerated WH in the face of stress/systemic GC. Thus: (1) intracutaneous conversion of inactive-to-active GC accounts for stress (GC)-induced delays in WH; and (2) blockade or down-regulation of 11β-HSD1 and/or GCr normalize cutaneous WH in the face of stress/GC. Local blockade or down-regulation of cutaneous GC activation could help enhance WH in various clinical settings.

Multiple types of short-term and sustained psychological stress delay mucosal wound healing (WH) in humans, including chronic stress during care of a relative with Alzheimer's disease, as well as relatively short durations of stress during examinations.[1,2] Likewise, restraint-induced stress slows WH in mice,[3] and also adversely affects barrier recovery after

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Supporting Information

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superficial injury by tape stripping of human skin.[4,5] In our work with the tape stripping model, we utilize barrier recovery as one physiological endpoint of the response, because this process includes epidermal resurfacing, with reestablishment of a competent barrier,[6] allowing survival in a terrestrial environment.[7]

A commonly proposed mechanism accounting for stress-induced delays in WH invokes stimulation of aberrant "neuro-immuno-endocrine" mechanisms, driven by a variety of biological mediators. Yet increased systemic glucocorticoids (GC) largely account for delayed permeability barrier recovery after superficial wounding in mice subjected to multiple, unrelated forms of stress (e.g., continuous sound and light exposure, crowding, restricted motion), because blockade of either GC production (by co-administration of the corticotropin-releasing factor inhibitor, antalarmin, or of GC peripheral action [by mifeprostone, Ru-486]) completely normalizes permeability barrier recovery in the face of ongoing stress.[8–10] Accordingly, the stress-induced abnormalities in permeability barrier homeostasis can be replicated in both humans and rodents with short-term administration of either systemic or topical GC.[9]

It recently became apparent that activation of endogenous GCs occurs in multiple peripheral tissues, through activity of an enzyme, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1),[11,12] which catalyzes the interconversion of inert, nonreceptor binding 11-ketosteroids to their receptor-binding, active 11-hydroxy forms (cortisone and 11-dehydrocorticosterone to cortisol and corticosterone in humans and rodents, respectively). As the predominant 11-ketoreductase in peripheral tissues, this mechanism guarantees rapid, local access of active metabolites to the GC receptor (GCr).[11,12]

Because systemic blockade of GC production/peripheral action and systemic administration of 11β-HSD1 inhibitors could risk the development of Addison's disease, we assessed the potential utility of two different strategies to overcome stress-induced delays in WH in mice; i.e., local (intracutaneous) blockade and/or down-regulation of 11β-HSD1. Within both mouse and human skin, 11β-HSD1 is expressed in epidermis/keratinocytes, dermis/fibroblasts, and hair follicle-associated cells.[13–16] To inhibit 11β-HSD1 activity, we deployed either carbenoxolone (Cbx), a nonspecific inhibitor of 11β-HSD1,[17,18] or PF-915275, a specific inhibitor of 11β-HSD1.[19] While Cbx also inhibits 11β-HSD2,[17] this isoform is expressed predominantly in mineralocorticoid-generating tissues and kidney, but only at very low levels in normal skin.[20] Hence, it can be assumed that locally administered Cbx, if not absorbed in significant quantities, would largely target intracutaneous 11β-HSD1.

While a very recent study by Terao et al.[16] demonstrated that a selective 11β-HSD1 inhibitor accelerated WH in normal skin, we explored two additional issues here: first, whether these inhibitors can overcome stress-induced delays in WH; and second, whether another approach, i.e., down-regulation of 11β-HSD1, also can overcome stress delays in WH. Both synthetic and naturally occurring activators of the liver X receptor (LXR) down-regulate 11β-HSD1 expression in both adipocytes and mouse embryonic fibroblasts.[21] As LXR ligands also inhibit GCr expression in hepatocytes in vitro and in vivo,[22] we assessed here whether one or both mechanisms could be exploited to provide an alternative (or additive)

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method to enhance WH. For this latter group of studies, we employed two chemically unrelated, but specific LXR ligands, TO901317, and GW3695. Our results indicate not only that the conversion of inactive-to-active GC accounts for stress-induced delays in WH, but also the converse, i.e., that intracutaneous blockade/down-regulation of GC activation accelerates not only normal WH, but it also overrides the negative effects of stress and systemic GC on cutaneous WH.

MATERIALS AND METHODS

Animals, stress model, and treatment protocols

Either albino (immune-competent) male hairless mice (Skh1/hr) (from Charles River Laboratories, Wilmington, MA) or previously clipped male C57BL/6 mice (from Jackson Laboratories, Bar Harbor, ME) between 9 and 12 weeks of age were used in all studies. The Skh1 strain of albino hairless mice used in these studies is immuno-competent (e.g., Benavides et al.[23]). Mice were allowed to rest in the vivarium after arrival for at least a week. Batches of 16–20 mice were used for each study, and were divided into four or five groups to treat with each compound (see below). The light cycle was 12 hours (6:00 AM–6:00 PM). Mice were restrained for 4 or 6 days during the study, between 9:00 PM and 9:00 AM. The animal experiments described in this study were conducted in accordance with accepted standards of humane animal care, under protocols approved by the local animal research committee at the San Francisco VA Medical Center.

To generate stress, animals were in motion-restricted environments once daily (12 hours) overnight. Food and water were restricted in parallel with motion restriction (control mice were allowed access to food and water only at the same time points). Plastic containers (4.0 [W] × 3.0 [H] × 11.5 [L] cm) with mesh walls on the top that minimized the ability of mice to reposition their bodies were used. Ru-486 (mifeprostone; Sigma-Aldrich, St. Louis, MO), antalarmin hydrochloride (Sigma-Aldrich) dissolved in 4% EtOH and 4% Cremophor (Sigma-Aldrich), or vehicle alone were injected intraperitoneally to stressed animals 1 hour prior to stress onset and every 24 hours thereafter for 4 days. Control mice were injected with vehicle alone at the same time points. Ru-486 and antalarmin were administered at doses of 6 and 20 mg/kg, respectively. Carbenoxolone (10 mM; Sigma-Aldrich), 1 mM PF-915275 (Tocris Bioscience, Ellisville, MO), 1 mM TO901317 (Cayman Chemical, Ann Arbor, MI), and 1 mM GW3695 (Sigma-Aldrich) in propylene glycol/ ethanol (3:7 v/v), or vehicle alone were topically applied on wound sites twice daily at 20 uL doses. In other experiments, systemic GC were administered by injecting each hairless mouse intraperitoneally with 10 μg dexamethasone (Dex) (500 μg/kg) in 0.5% DMSO 30 min prior to the initiation of stress once daily for 4 or 6 days.

Biopsy protocol for wounding and assessment of wound recovery

Full-thickness, 6 mm diameter biopsy wounds were made in Skh1 mice, or in C57BL/6 mice, after prior clipping hair off the skin ≈15 hours before wounding, and two contralateral wounds were made on the back of each mouse. Wound size was assessed in digital photographs following photo-recording and integration of resultant-involved wound areas. ImageJ software (National Institutes of Health, Bethesda, MD) was used to assess changes

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in wound area/initial wound area over time. Photos were taken daily just before drug or vehicle treatment on days 0 through 6 after wounding. A ruler is applied next to each wound, and the scale of the ruler image is set before calculating changes in wound areas in each photo. The distance from the wound to the camera was kept constant (15 cm) (Figure 1 shows wound size in mm scale as an example). Wound healing recovery kinetics represent the percent change over time from the initial size of each wound.

qRT-PCR and Western blotting

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using 50 ng of cDNA prepared from mRNA fraction of cultured human keratinocyte lysates (treated as described in the text and figure legends), the specific primer sets (200 nM), and SYBR green reagents, as we described previously.

The following primer sets were used: human 11β-HSD1, 5′-AAGCAGAGCAATGGAAGCAT-3′ & 5′-GAAGAACCCATCCAAAGCAA-3′; human GC receptor (GCr), 5′-GGAGTCAACGGATTTGGTCGTA -3′ & 5′-GCAACAATATCCACT TTACCAGAGTTAA-3′; human glyceraldehydes 3′-phosphate dehydrogenase (GAPDH), 5′-GGAGTCAACGGATTTGGTCGTA-3′ & 5′-GCAACAATATCCACTTTACCAGAGTTAA-3′. Data for 11β-HSD1 and GCr were normalized to mRNA levels of GAPDH.

Western immunoblot analysis was performed as described previously, with modification. Keratinocyte lysates (40 μg protein/lane) were prepared in RIPA buffer (Thermo Fisher Scientific, Rockford, IL) and were resolved by electrophoresis on 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA). Resultant bands were blotted onto nitrocellulose membranes, probed with anti-human β-actin (Abcam, Cambridge, MA), anti-11β-HSD1 (Abcam), and anti-GC receptor (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies, and detected using enhanced chemiluminescence (Thermo Fisher Scientific).

11β-HSD1 activity assay

For the enzyme activity assay, cortisol levels were assayed using the Cortisol EIA kit according to the instructions from the manufacturer (Cayman Chemical, Ann Arbor, MI). This assay is highly specific for cortisol with less than 2% cross-reactivity with cortisol's inactive form, cortisone. HaCaT cells (70–80% confluency), plated in a low Ca2+ (0.07 mM) keratinocyte media, were treated with 100 nM cortisone for 24 hours. Cortisols are extracted with dichloromethane from the lysates in RIPA buffer (Thermo Fisher Scientific). After evaporation of the dichloromethane under N2, the samples were immediately resuspended in EIA buffer, and either analyzed in a microplate reader (Fisher Scientific, Suwanee, GA) following the kit instructions, or frozen at −80 °C until assayed. The concentration of cortisol was quantitated and normalized to the protein content in each sample.

Measurement of serum corticosterone

Blood samples were obtained from stressed and unstressed mice immediately after a 4-day stress period, while animals were under isoflurane anesthesia. Blood was centrifuged at

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2,000×g for 10 minutes, and the serum was separated and frozen at −80 °C until assayed. Serum corticosterone level was measured with a commercially available ELISA kit (Assaypro, St. Charles, MO), according to manufacturer's protocol.

Immunohistochemistry

Immunohistochemical staining for 11β-HSD1 (primary and secondary antibodies from Abcam) was performed, as described previously.[24] Briefly, 5-μm paraffin sections were incubated with the primary antibodies overnight at 4 °C. After three washes, sections were incubated with the secondary antibody for 30 minutes. Staining was detected with the ABC-peroxidase kit from Vector Labs (Burlingame, CA). Sections were examined with a Carl Zeiss light microscope (Jena, Germany), and digital images were captured with AxioVision software (Carl Zeiss Vision, Munich, Germany).

Statistics

Data were expressed as mean ± SEM, using both data for individual wounds and averaged data when two wounds were treated similarly on the same animal. Statistical analyses were performed using paired and unpaired Student's *t*-test. A *p*-value less than 0.05 was considered significant.

RESULTS

Blockade of 11β-HSD1 accelerates normal WH and overcomes stress-induced delays in WH

As changes in the initial wound area cannot be established prior to formation of a fibrin clot, we began our wound recovery studies 15 hours following the formation of full-thickness wounds in hairless mouse skin with a 6 mm punch. We initially compared WH, quantitated as wound closure with assessments of healing rates in histologic sections. Because the former method proved to be more reproducible and accurate, we present our results here as percent wound recovery over time, supplemented by histologic images from the same wounds. Because wounds on the same animals healed at different rates, the results are shown in the figures as % change per wound. In supplementary material, we also show the data as WH/animal. The results remained significantly different, even when wounds from the same animal were averaged. We confirmed that WH is delayed in our motion-restricted stress model (Figure 1A and B). Then we determined whether localized activation of endogenous GC by the enzyme, 11β-HSD1, accounted for the stress-induced delays in WH. WH kinetics were first compared initially in stress vs. nonstressed (normal) animals ($n = 4$ mice; two sites on each mouse) treated with the nonspecific 11β-HSD inhibitor, Cbx. Despite normal (low) constitutive levels of systemic GC (see Figure 4A), Cbx significantly accelerated WH (by ≈10%) at the 2-day time-point, even in normal (nonstressed) mice (Figure 2A). More pertinent to our hypothesis, topical applications of Cbx also overcame the delayed WH in stressed mice, resulting in repair kinetics that became comparable with WH in nonstressed (control) animals (Figure 2B). Histologic images of these wounds showed that accelerated healing reflected rapid re-epithelialization of the wound bed (Supporting Information Figure S1).

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Because Cbx inhibits both 11β-HSD1 and 11β-HSD2, we next assessed whether a highly specific 11β-HSD1 inhibitor, PF-95275,[19] also improves WH in stressed mice. As shown in Figure 2C, the selective 11β-HSD1 inhibitor also completelynormalized WH in stressed mice (data shown in this and most subsequent figures are for day 4 after wounding, when maximal differences were observed). Together, these results show that 11β-HSD1/2 inhibitors accelerate healing of both normal wounds and wounds in a setting of experimental stress.

Yet intracutaneous Cbx could be absorbed, resulting in a systemic, rather than a purely local impact on cutaneous WH. Hence, we next assessed WH kinetics in stressed animals treated unilaterally with Cbx, while vehicle alone was applied to a comparable wound on the opposing flank. WH rates normalized only on the Cbx-treated sites in comparison with vehicle-treated sites on the contralateral flanks of stressed mice (Figure 2D and E). Together, these results show that local inhibition of GC activation: (1) accelerates WH in normal skin; and (2) completely overrides stress-induced delays in WH.

LXR ligands normalize, while LXR ligands plus 11β-HSD1 inhibitors further accelerate, WH in stressed/GC-treated mice

We next assessed whether two different LXR ligands normalize WH in stressed mice. While TO901317 attenuated, but did not completely normalize the stress-induced delays in WH (Figure 3A, TO/stress lane), comparable concentrations of a second, chemically unrelated, specific LXR ligand, GW3695, normalized WH in stressed mice (Figure 3B). Furthermore, in normal hairless mice, treated systemically with Dex, which slows WH, both the 11β-HSD1/2 inhibitor, Cbx, and the LXR ligand, TO901317, reversed Dex-induced delays in WH (Figure 3D). Finally, to assess whether 11β-HSD1 inhibitors and LXR ligands could exert additive or synergistic effects on WH in normal and/or stressed mice, we next applied Cbx alone, or in combination with TO901317. This combination not only accelerated WH in normal (nonstressed) mice (Figure 3C), but it also accelerated WH in the face of stress (Figure 3A). Together, these results show that (1) LXR ligands enhance WH in stressed mice; and (2) combined local applications of an 11β-HSD1 inhibitor and an LXR ligand additively accelerate WH in both normal and stressed skin.

Enzyme inhibitors and LXR ligands reverse mechanisms that account for stress-induced delays in WH

Increased systemic GC largely account for stress-induced delays in WH.[3,9,10] To ascertain whether increased levels of systemic GC account for the stress-induced delay in our WH model, we first assessed whether systemic GC levels increase in this model. As shown in Figure 4A (WH ± stress), systemic GC levels markedly increase with stress. To determine whether the increase in systemic GC levels account for the delay in WH in this model, we next assessed whether blockade of GC production or peripheral action by systemic administration of either the CRF inhibitor, antalarmin, or the GCr inhibitor, Ru-486, normalizes WH in stressed mice. While antalarmin and Ru-486 did not significantly alter the kinetics of WH in normal (nonstressed) mice (Figure 4B), GC blockade with either of these agents normalized WH in the face of ongoing stress (Figure 4C). Together, these results

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show that the stress-induced delay in cutaneous WH can be attributed to an increase in systemic GC.

To further assess whether localized activation of endogenous GC by the enzyme, 11β-HSD1, accounts for stress-induced delays in this WH model, we next assessed 11β-HSD1 expression/activity both in whole skin and in cultured keratinocytes. Immunohistochemical studies revealed that 11β-HSD1 is expressed in whole mouse skin, where it largely localizes to the suprabasal layers of the epidermis (Figure 5A, arrowheads). Western blot analyses also showed that 11β-HSD1 is expressed at high levels in preconfluent, cultured human keratinocytes, grown in 0.07 mM Ca^{++} (Figure 5).

Because cortisone, the inactive form of GC in humans, is a substrate for 11β-HSD1, which then generates cortisol, the active GC, we next assessed whether exogenous GC stimulates 11β-HSD1 activity in cultured human keratinocytes. Indeed, the conversion of cortisone to cortisol increased afterapplications of exogenous cortisone to cultured human keratinocytes in a dose-dependent manner (Figure 5B). In contrast, co-treatment of keratinocytes with either Cbx or the synthetic LXR ligand, TO901317, inhibited the conversion of cortisone to cortisol under the same conditions. Yet the basis for LXR-induced acceleration of WH could involve more than decreased 11β-HSD1 activity, because TO901317 also down-regulated not only 11β-HSD1 expression, but also GCr protein expression in cultured keratinocytes (Figure 5C). In contrast, Dex, which is not a substrate for 11β-HSD1, instead induced a dose-dependent increase in 11β-HSD1 and GCr mRNA/protein levels. Finally, co-treatment with the LXR ligand (TO901317) blocked the expected Dex-induced increase in 11β-HSD1 and GCr expression in cultured human keratinocytes (Figure 5C and D). Together, these results provide a mechanistic basis for these studies, by showing that first, 11β-HSD1 is expressed in the skin, where it largely localizes to the epidermis; second, both enzyme blockade and down-regulation inhibit the conversion of cortisone to cortisol in keratinocytes; and finally, both an enzyme inhibitor and an LXR ligand block peripheral GC activation through both blockade and down-regulation of epidermal 11β-HSD1 and the GCr, respectively.

DISCUSSION

Systematic reviews and meta-analyses show that heterogeneous forms of stress impair WH and down-regulate associated biomarkers in a wide variety of cutaneous and mucosal wounds in humans (reviewed in Walburn et al.[25]). Although such stress-induced functional abnormalities are often attributed to “psycho-neuro-endocrine-immune” abnormalities,[26,27] stress-induced stimulation of systemic GC production is well known to compromise keratinocyte proliferation, migration, and differentiation,[28] leading to impaired antimicrobial defense[29] and permeability barrier function.[10] These alterations largely account for stress-induced delays in restoration of normal barrier function after both superficial and full-thickness wounding.[3,9,10,30–32] The importance of the GC mechanism has been shown most convincingly in animals that were cotreated systemically with a GCr antagonist, mifeprostone (Ru-486), which normalizes WH rates.[3] We also showed previously that systemic administration of either antalarmin or mifeprostone normalizes healing of superficial, tape stripping-induced wounds in the face of ongoing stress.[9,10] While increased

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GC production by an intracutaneous steroidogenic system[33,34] could also contribute to stress-induced delays in WH, we confirm here that increased systemic GC accompany delayed healing of full-thickness wounds, and that systemic blockade of both GC production and peripheral action restores normal WH kinetics in this motion-restricted (frustration) model.[3]

Although either inhibition of the hypothalamic-pituitary-adrenal (HPA) axis with a CRF inhibitor or blockade of GC binding to the GCr can overcome the adverse impact of stress/GC in WH, these strategies cannot be deployed to improve WH in humans due to an attendant risk of adrenal insufficiency (Addison's disease). Systemic mifeprostone (Ru-486) can also lead to severe hypokalemia through increased cortisol level, as occurs in Cushing syndrome.[35,36] Although systemic 11β-HSD1 inhibitors are under development as alternative therapy for type 2 diabetes,[18,37] their utility could be limited by undesirable side effects (i.e., GC deficiency in peripheral tissues). Hence, based upon recent studies showing that the GC-activating enzyme, 11β-HSD1, is expressed in the skin, but primarily in the epidermis,[14–16] we assessed here the feasibility of local enzyme blockade and/or down-regulation, which should avoid the undesirable consequences of systemic GC blockade. With regard to epidermal component of WH, we confirmed that 11β-HSD1 is not only expressed in skin, but showed further that the enzyme is particularly abundant in the suprabasal layers of the epidermis.[16] In addition, we showed that both topical enzyme blockade and topical enzyme down-regulation inhibited the conversion of cortisone to cortisol in cultured human keratinocytes in a dose-dependent fashion (see Figure 5), showing that this therapeutic strategy targets the appropriate mechanism at achievable topical concentrations. Whereas Terao et al.[16] recently showed that a specific 11β-HSD1 inhibitor accelerates WH in normal and ob/ob mice, we showed for the first time that both approaches overcame stress- and GC-induced delays in WH, and further that the effect is local, and cannot be attributed to systemic absorption of the inhibitor. Although cross-sectional, morphometric studies still comprise the standard approach to compare WH kinetics, in this study, quantitation of percent WH recovery, based upon surface area changes, provided a more reproducible measure of healing in this type of punch-biopsy-initiated model. Moreover, the histologic studies provided further mechanistic insights, because they support our hypothesis that accelerated WH correlates with rapid reepithelialization at the base of treated wounds. Whether enzyme blockade also accelerates healing sequences deeper in the wound bed could not be ascertained in these studies.

11β-HSD1 is a NADP(H)-dependent enzyme that catalyzes intracellular (re)generation of active GCs (cortisol, corticosterone) from inert 11-keto forms (cortisone, 11-dehydrocorticosterone) in many peripheral tissues, thereby amplifying local GCr activation.[11,12,37] Because multiple recent lines of evidence indicate that 11β-HSD1-mediated intracellular cortisol production could have a pathogenic role in type 2 diabetes and its comorbidities,[38] 11β-HSD1 has become a recent target for anti-type 2 diabetes drug development. Although systemic 11β-HSD1 inhibitors are being developed as adjunctive therapy for diabetes (see above), their impact on cutaneous WH has not been assessed. As noted previously, systemic use of these agents carries a theoretical risk of systemic complications, because reduction in tissue-regenerated cortisol might produce signs of GC deficiency, and/or because decreased feedback to the HPA axis could instead up-regulate

adrenal cortisol production (reviewed in Harno & White[39]). Hence, a strategy that deploys topical (local), rather than systemic blockade of 11β-HSD1 to improve WH in humans would have important theoretical advantages.

To validate this possibility, we deployed two potentially complementary, and potentially additive strategies to reduce 11β-HSD1 activity[37]; i.e., either enzyme inhibitor-induced blockade or down-regulation of 11β-HSD1 expression by LXR ligands.[21] As expected, topical 11β-HSD1 inhibitors normalized WH in stressed mice, restoring recovery rates to kinetics comparable with WH in normal mice. Two chemically different, but specific LXR ligands also enhanced WH in stressed mice, but recovery rates did not always normalize at the doses and time points that we assessed. It should be noted that the mechanisms that account for LXR ligand-induced acceleration of WH likely involve more than 11β-HSD1 down-regulation, because LXR ligands also down-regulate the GCr, as reported previously.[22] We showed here that the LXR ligand, TO901317, down-regulates not only 11β-HSD1, but also GCr expression in human keratinocytes. Thus, the impact of LXR ligands on WH could also be explained, at least in part, by a localized reduction in GCr expression.

Yet we have also shown that LXR activators attenuate the adverse effects of exogenous GC on epidermal homeostasis,[24] leading to accelerated barrier recovery.[40,41] It is worth noting here that restoration of normal barrier function, though rarely assessed in WH studies, is the final step in WH, and perhaps the driving force behind the entire WH response. Hence, LXR ligands could normalize WH in stressed mice by down-regulating either 11β-HSD1 and/or the GCr. Together, our results strongly show that local blockade or down-regulation of 11β-HSD1 and/or the GCr can normalize cutaneous WH in the face of stress/GC, without the theoretical risks posed by systemic therapy, and that enzyme inhibitors even accelerate WH in normal murine skin. A similar strategy could be deployed to improve WH in normal human skin, particularly in clinical settings where there is an increased risk of secondary infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Dr. Kenneth R. Feingold for his insightful comments and suggestions regarding this work, and are grateful to Ms. Joan Wakefield for providing invaluable editorial assistance.

Source of Funding: These studies were supported by the Medical Research Service, Department of Veterans Affairs, San Francisco, CA, a grant from the U.S. Department of Defense, and the Kimjeon Foundation (South Korea).

Glossary

11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
Cbx	Carbenoxolone

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CRF	Corticotropin-releasing factor
Dex	Dexamethasone
GC	Glucocorticoid
GCr	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal
LXR	Liver X receptor
WH	Wound healing

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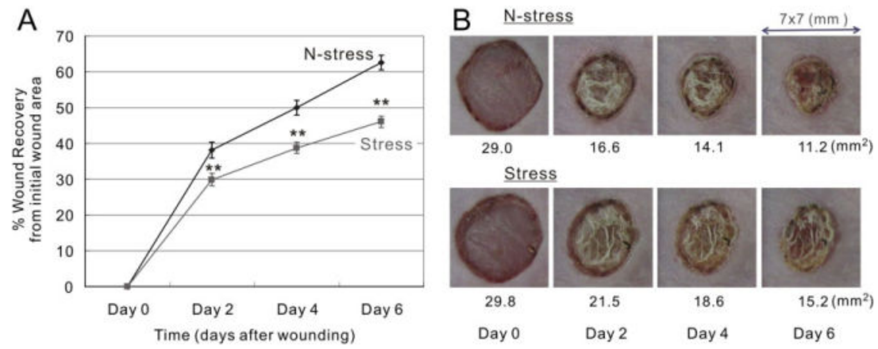

Figure 1.

Motion-restricted stress delays WH. WH, wound healing. (A–B) Motion restriction delays WH, assessed as % change in reepithelialization of wound area over time/initial wound area, in otherwise normal hairless mice ([A] $n = 16$ wounds from mice, with either stressed or nonstressed [N-Stress]; $n = 8$ mice each); (B) Digital images are from a representative experiment. Significance: $^{**}p < 0.005$.

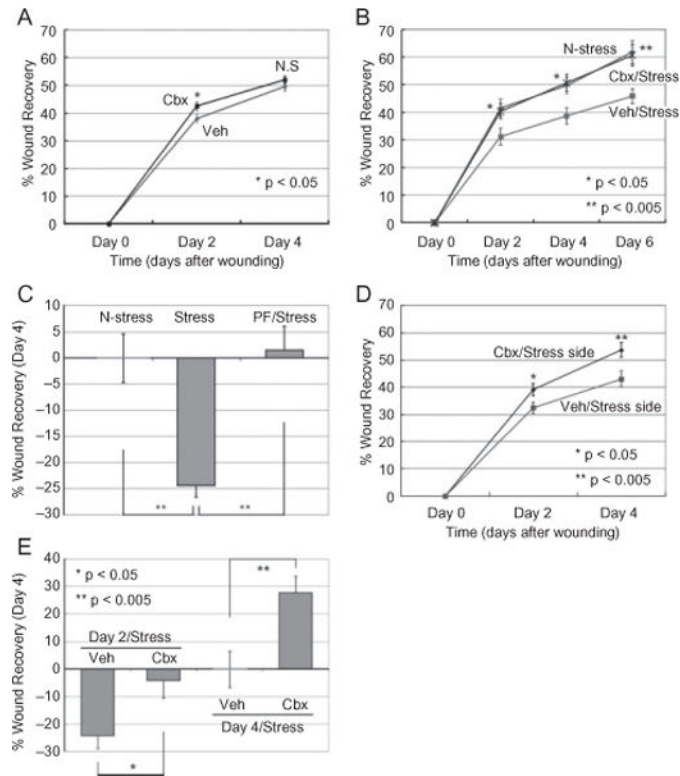

Figure 2.

Cbx accelerates WH in normal and stressed mice. WH in normal vs. stressed hairless mice, co-treated with either carbenoxolone (Cbx), or vehicle (Veh) alone, as in Methods. (A) Cbx significantly accelerated WH at day 2 in normal (N-Stress) mice ($n = 20$ from 10 mice). (B) Cbx reversed stress-induced delays in WH at each time point (Day 2, 4, 6) ($n = 8$ [two sites each on four mice]). (C) PF-915275 (PF) normalized WH in stressed mice at Day 4 after wounding (Stress and N-Stress: $n = 10$ from 5 mice, PF: $n = 8$ [two sites each on four mice]). C57BL/6 mice were utilized for these studies. (D–E) WH was compared in two comparable wounds on opposing flanks treated unilaterally with Cbx (Cbx/stress) or vehicle alone (Veh/stress) ($n = 10$ sites each from a total of 10 mice). Significance: $*p < 0.05$, $**p < 0.005$; NS, no significant difference. WH, wound healing.

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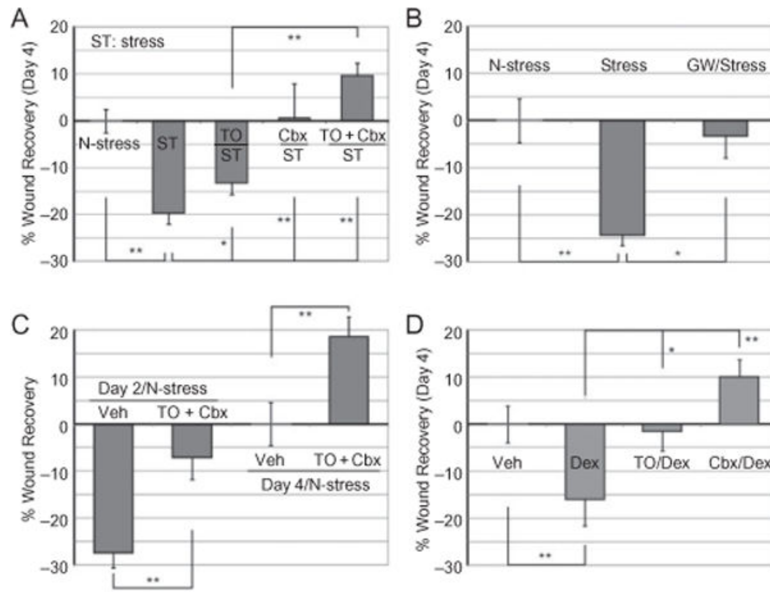

Figure 3.

LXR ligands overcome stress-induced delays in WH synergistically with 11β-HSD1 inhibitors. (A + C) Combined applications of Cbx and TO901317 (TO) accelerated WH in normal (nonstressed) and stressed mice (n = 12 from six mice in each group). (B) A chemically unrelated LXR ligand, GW3695 (GW), normalized WH in stressed mice (N-stress and Stress: n = 10 [two sites from five mice], GW: n = 8 [two sites each on four C57BL/6 mice]). C57BL/6 mice were used for this experiment. (D) Both Cbx and TO901317 reversed Dex-induced delays in WH (n = 14 [two sites each on seven mice]). N-PS, non-PS; TO+Cbx: combined applications of Cbx and TO901317. Significance: *$p < 0.05$, **$p < 0.005$. Cbx, carbenoxolone; Dex, dexamethasone; LXR, liver X receptor.

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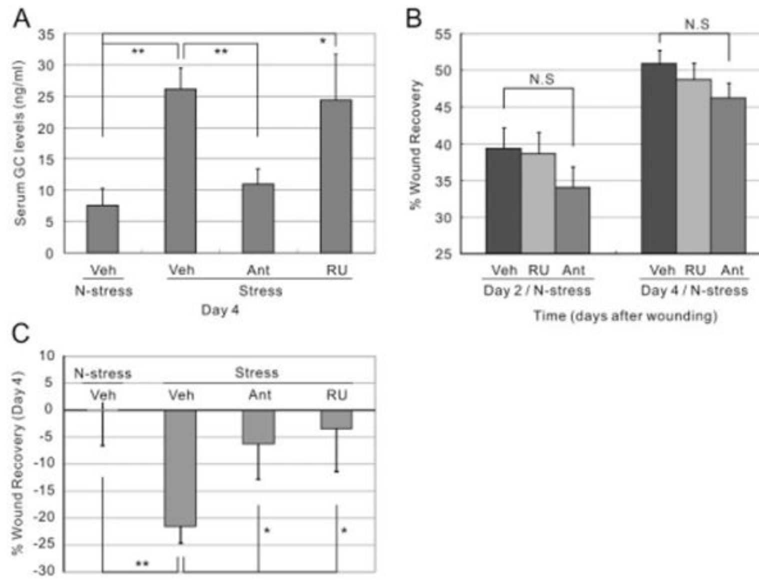

Figure 4.

Stress-induced delays in WH are due to increased systemic GC. (A–C) Hairless mice were injected with either antalarmin (Ant), Ru-486 (RU), or vehicle (Veh) once daily under stress or nonstressed conditions, as described in Methods. (A) Motion restriction-induced stress stimulated an increase in serum levels of GC ($n = 4$). (B) Neither antalarmin nor Ru-486 altered WH in normal mice ($n = 16$ wounds from eight mice each). (C) The stress-induced delay in WH was reversed by either Ant or RU administration ($n = 8$ from four mice each). Significance: $*p < 0.05$; $**p < 0.005$. GC, glucocorticoid; WH, wound healing.

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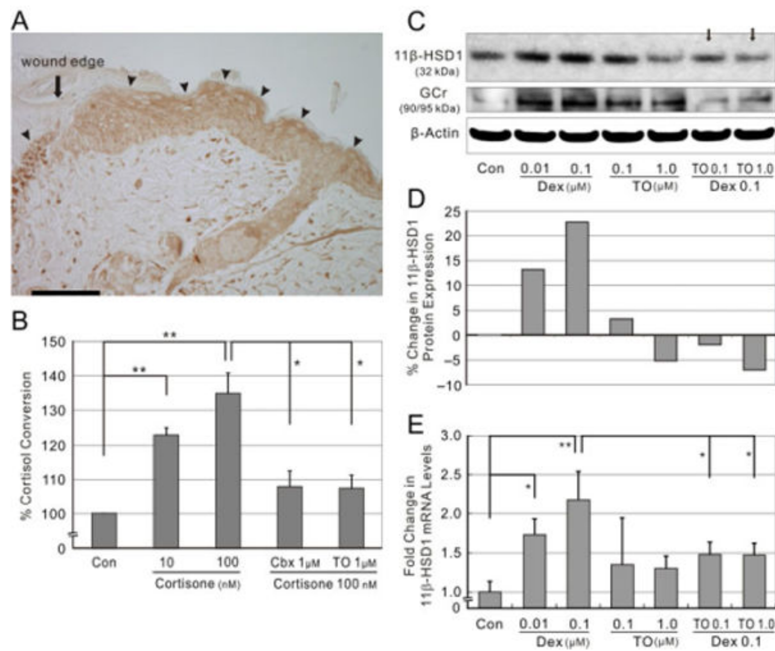

Figure 5.

11β-HSD1 blockade reduces GC-induced increase in expression and activity. (A) 11β-HSD1 was immunostained (as in Methods) in whole skin from normal mouse wounds ≈15 hours after wounding (scale bar = 50 μm). (B) Exogenous cortisone increased cortisol generation in HaCaT cells in a dose-dependent fashion, while cotreatment with either Cbx or TO901317 (TO) blocked the expected increase in cortisol generation ($n = 3$). (C) Protein levels of 11β-HSD1 and the GCr were assessed in HaCaT cells 24 hours after the Dex treatment ± cotreatment with the LXR ligand, TO901317 (TO). (D) Dose-dependent quantification of changes in 11β-HSD1 (data shown are from a representative experiment from a total of four). (E) Changes in 11β-HSD1 mRNA levels in HaCaT cells 24 hours after Dex ± TO treatment. Significance: *$p < 0.05$, **$p < 0.005$. Cbx, carbenoxolone; Dex, dexamethasone; GC, glucocorticoid; GCr, GC receptor; LXR, liver X receptor.