Epidermal adrenergic signaling contributes to inflammation and pain sensitization in a rat model of complex regional pain syndrome

Wenwu Li a,b,c,1, Xiaoyou Shi a,b,c,1, Liping Wang a, Tianzhi Guo a, Tzuping Wei a, Kejun Cheng d,e, Kenner C. Rice d,e, Wade S. Kingery a, J. David Clark b,c,*

a Physical Medicine and Rehabilitation Service, Veterans Affairs Palo Alto Health Care System, Palo Alto, CA, USA
b Anesthesiology Service, Veterans Affairs Palo Alto Health Care System, Palo Alto, CA, USA
c Department of Anesthesia, Stanford University School of Medicine, Stanford, CA, USA
d National Institutes of Health/National Institute on Drug Abuse, Bethesda, MD, USA
e National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD, USA

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ABSTRACT

In many patients, the sympathetic nervous system supports pain and other features of complex regional pain syndrome (CRPS). Accumulating evidence suggests that interleukin (IL)-6 also plays a role in CRPS, and that catecholamines stimulate production of IL-6 in several tissues. We hypothesized that norepinephrine acting through specific adrenergic receptors expressed on keratinocytes stimulates the production of IL-6 and leads to nociceptive sensitization in a rat tibial fracture/cast model of CRPS. Our approach involved catecholamine depletion using 6-hydroxydopamine or, alternatively, guanethidine, to explore sympathetic contributions. Both agents substantially reduced nociceptive sensitization and selectively reduced the production of IL-6 in skin. Antagonism of IL-6 signaling using TB-2-081 also reduced sensitization in this model. Experiments using a rat keratinocyte cell line demonstrated relatively high levels of β2-adrenergic receptor (β2-AR) expression. Stimulation of this receptor greatly enhanced IL-6 expression when compared to the expression of IL-1β, tumor necrosis factor (TNF)-α, or nerve growth factor. Stimulation of the cells also promoted phosphorylation of the mitogen-activated protein kinases P38, extracellular signal-regulated kinase, and c-Jun amino-terminal kinase. Based on these in vitro results, we returned to animal testing and observed that the selective β2-AR antagonist butoxamine reduced nociceptive sensitization in the CRPS model, and that local injection of the selective β2-AR agonist terbutaline resulted in mechanical allodynia and the production of IL-6 in the cells of the skin. No increases in IL-1β, TNF-α, or nerve growth factor levels were seen, however. These data suggest that in CRPS, norepinephrine released from sympathetic nerve terminals stimulates β2-ARs expressed on epidermal keratinocytes, resulting in local IL-6 production, and ultimately, pain sensitization.

1. Introduction

The participation of the sympathetic nervous system (SNS) in complex regional pain syndrome (CRPS), once referred to as reflex sympathetic dystrophy, has long been appreciated. In 1916, Leriche proposed that sympathetic hyperactivity caused the development of posttraumatic CRPS, and he advocated surgical sympathectomy and sympathetic anesthetic nerve blocks for the treatment of this condition [28]. Sympathetic blocks are still a widely utilized treatment option for CRPS, but there is considerable controversy regarding the mechanisms by which SNS activity supports pain and the other components of CRPS [5]. Distal limb fracture is the most common cause of CRPS [7,44,56], and we have developed a rat tibia fracture/cast immobilization model that at 4 weeks postfracture closely mimics the vascular, boney, nociceptive, and inflammatory changes observed in early CRPS [10,59]. This model provides an opportunity to investigate the role of sympathetic signaling in persistent CRPS-like postfracture pain.

A large body of clinical evidence points to facilitated peripheral neurogenic inflammation, involving neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP), as contributing to some of the signs and symptoms of CRPS, including pain sensitization, warmth, and edema [22,27,57]. Again, the fracture/cast CRPS model has provided results consistent with
the clinical findings; enhanced postjunctural facilitation of SP signaling was observed, as was upregulation of SP and CGRP in peripheral nerves, and expression of NK1 receptors [17,58]. Furthermore, transgenic mice lacking functional SP or CGRP signaling had reduced postfracture allodynia, unweighting, and vascular changes. These mice also failed to develop increased interleukin (IL)-1β, tumor necrosis factor (TNF), and nerve growth factor (NGF) in the hind paw skin. Curiously, the transgenic mice did have increased IL-6 levels after fracture similar to the responses measured in control animals [12]. The upregulation of IL-6 in CRPS may, therefore, occur via a distinct mechanism.

An increase in the abundance of IL-6 may be important in supporting CRPS. Levels of IL-6 are upregulated in experimental skin blisters in CRPS limbs [14,15] and we have observed that IL-6, along with TNF-α, IL-1β, and NGF are upregulated in hind paw skin at 4 weeks after tibia fracture in rats [41,42,59]. While effective, previous studies using anti-IL-1β, TNF, and NGF agents provided only partial reversal of CRPS-like changes in the fracture/cast model [31,41,42]. On the other hand, it has been observed that intraplantar injection of IL-6 into normal hind paw skin rapidly induces nociceptive sensitization [29]. Furthermore, it is known that keratinocytes express adrenoceptors and are capable of responding to norepinephrine, though the consequences of such stimulation have not been fully explored [39,45,50]. Beta-2-adrenergic receptors (β2-ARs) appear to be the most abundantly expressed subtype in vivo [49]. We therefore hypothesized that SNS activity partially supports the inflammatory and nociceptive changes observed in the fracture/cast model of CRPS, and that norepinephrine released from sympathetic fibers acts through the stimulation of IL-6 production to support these CRPS-like changes.

2. Materials and methods

These experiments were approved by the Veterans Affairs Palo Alto Health Care System Institutional Animal Care and Use Committee (Palo Alto, CA, USA) and followed the animal subjects guidelines of the International Association for the Study of Pain. One hundred forty-five adult (9-month-old) male Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA, USA) were used in these experiments. The animals were housed individually in isolator cages with solid floors covered with 3 cm of soft bedding and were given food and water ad libitum. During the experimental period, the animals were fed Lab Diet 5012 (PMI Nutrition Institute, Richmond, IN, USA), which contains 1.0% calcium, 0.5% phosphorus, and 3.3 IU/g vitamin D3, and were kept under standard conditions with a 12-hour light-dark cycle.

2.1. Surgery

Tibia fracture was performed under 2%–4% isoflurane to maintain surgical anesthesia as we have previously described [10]. The right hind limb was wrapped in stockinet (2.5 cm wide) and the distal tibia was fractured using pliers with an adjustable stop (Visegrip, Petersen Manufacturing, Dewitt, NE, USA) that had been modified with a 3-point jaw. The hind limb was wrapped in casting tape (Delta-Lite, Johnson & Johnson, New Brunswick, NJ, USA) so the hip, knee, and ankle were flexed. The cast extended from the metatarsals of the hind paw up to a spica formed around the abdomen. The cast over the paw was applied only to the plantar surface; a window was left open over the dorsum of the paw and ankle to prevent constriction when postfracture edema developed. To prevent the animals from chewing at their casts, the cast material was wrapped in galvanized wire mesh. The rats were given subcutaneous saline and buprenorphine immediately after the procedure (0.03 mg/kg) and on the first day after fracture for postoperative hydration and analgesia. At 4 weeks the rats were anesthetized with isoflurane and the cast removed with a vibrating cast saw. All rats used in this study had union at the fracture site after 4 weeks of cast immobilization.

2.2. Drug treatment protocols

To test the hypothesis that sympathetic signaling can regulate cutaneous inflammation and nociceptive thresholds after tibia fracture, chemical sympathectomy was performed in fracture rats with either 6-hydroxydopamine (6-OHDA; Sigma-Aldrich, St. Louis, MO, USA) or guanethidine (Sigma-Aldrich). The 6-OHDA treatment was started at 7 days postfracture using a progressive dosing schedule (50 mg/kg/day 1, 50 mg/kg/day 2, 100 mg/kg/day 3, 100 mg/kg/day 4, 100 mg/kg/day 5, intraperitoneal injection [i.p.]) that we had previously utilized to reduce cutaneous norepinephrine (NE) levels by 90% [19]. The guanethidine treatment was started at 14 days postfracture and continued for 2 weeks (a total of 14 daily treatments, 50 mg/kg, subcutaneous injection [s.c.] daily). This treatment protocol severely depletes NE levels in the peripheral sympathetic fibers [4,37]. There were 2 sets of controls for these experiments, control rats that had no fracture or injections, and fracture control rats that were fractured/casted and received saline injections. Hind paw nociceptive testing and assessment of warmth and edema were performed prior to fracture and at 4 weeks postfracture (the day after cast removal). The next day the rats were euthanized and hind paw skin was collected for enzyme immunoassay (ELIA) assays.

To test the hypothesis that β2-ARs mediate nociceptive and vascular changes in the CRPS model, rats underwent tibia fracture and were casted for 4 weeks, then the cast removed and the next day they were treated with a β2-AR antagonist, butoxamine (Sigma-Aldrich; 2 mg/kg i.p.). Hind paw nociceptive testing and assessment of warmth and edema were performed before and 30 minutes after butoxamine injection. There were 2 sets of controls for these experiments, control rats that had no fracture or injections, and fracture control rats that were fractured/casted and received a saline injection. To further examine the pronociceptive effects of β2-AR activation in normal skin, the β2-AR agonist terbutaline was injected intradermally into the plantar hind paw skin of normal control rats (Sigma-Aldrich; 5 μg/30 μL saline, intraplantar injection). Nociceptive testing was performed at baseline and at 0.5, 1, and 3 hours after intraplantar terbutaline injection. In another group of rats, the hind paw skin was harvested 1 hour after terbutaline injection to determine if β2-AR activation upregulated inflammatory cytokine and NGF production. Immunohistochemistry was used to identify the cellular origin of the upregulated inflammatory mediators. The dosages of butoxamine and terbutaline used in the current study were based on previous reports of effective doses in another pain model [32].

To assess if IL-6 supported allodynia, unweighting, warmth, and edema in the CRPS model, rats underwent tibia fracture and were casted for 4 weeks, then the cast removed and the next day they were treated with TB-2-081 (2 mg/kg, s.c., a generous gift from Dr Kenner Rice, National Institute on Drug Abuse) [20]. TB-2-081 is an orally active small-molecule IL-6 receptor antagonist originally isolated from the skin of a toad. The TB-2-081 dosage used in the current study was based on a previous report of alleviation of chronic pancreatitis pain in rats with 1 mg/kg s.c. [55]. Hind paw nociceptive testing and assessment of warmth and edema were performed before and 15 minutes after TB-2-081 injection. There were 2 sets of controls for these experiments, control rats that had no fracture or injections, and fracture control rats that were fractured/casted and received a saline injection.
2.3. Hind paw nociception testing

To measure mechanical allodynia in the rats, an up-down von Frey testing paradigm was used, as we have previously described [10,11,18]. Hind paw mechanical nociceptive thresholds were analyzed as the difference between the treatment side and the contralateral untreated side.

An incapacitation device (IITC Inc. Life Science, Woodland Hills, CA, USA) was used to measure hind paw unweighting. The rats were manually held in a vertical position over the apparatus with the hind paws resting on separate metal scale plates, and the entire weight of the rat was supported on the hind paws. The duration of each measurement was 6 seconds, and 10 consecutive measurements were taken at 60-second intervals. Eight readings (excluding the highest and lowest) were averaged to calculate the bilateral hind paw weight-bearing values [10,11,18]. Right hind paw weight-bearing data were analyzed as a ratio between the right hind paw and the sum of right and left hind paws values ([2R/(R+L)] × 100%).

2.4. Hind paw volume testing

A laser sensor technique was used to determine the dorsal-ventral thickness of the hind paw, as we have previously described [10,11,18]. Hind paw volume data were analyzed as the difference between the treatment side and the contralateral untreated side.

2.5. Hind paw temperature testing

For these experiments, room temperature was maintained at 23°C and humidity ranged between 25% and 45%. The temperature of the hind paw was measured using a fine wire thermocouple (Omega, Stamford, CT, USA) applied to the paw skin, as previously described [38]. Hind paw temperature data were analyzed as the difference between the treatment side and the contralateral untreated side.

2.6. Cell culture

The rat keratinocyte cell line was generously provided by Dr. Howard Baden (Massachusetts General Hospital, Boston, MA, USA) and grown as previously described [38]. In brief, cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 1% penicillin-streptomycin, and 0.4 μg/mL hydrocortisone (Sigma-Aldrich), 0.75 mM aminoguanidine (Sigma-Aldrich), and passed weekly. Rat keratinocytes were plated at 1 × 10⁴ cells per 60-mm dish. Cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C and culture medium was changed every other day. On reaching approximately 80% confluence, the cells were treated with the above culture medium supplemented with only 1% fetal bovine serum for overnight before addition of SP, NE, cirazoline, dobutamine, and NGF proteins were calculated from the standard curve prepared (Oakridge, CA, USA). Real-time polymerase chain reactions (PCRs) were conducted using the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA). Real-time PCR amplification of ARs (α1A-AR, α1B-AR, α1D-AR, α2A-AR, α2B-AR, α2C-AR, β1-AR, β2-AR, and β3-AR), IL-1β, IL-6, TFN-α, and NGF was performed on an ABI 7900HT sequencing detection system (Applied Biosystems). To validate the primer sets used, we performed dissociation curves to document single product formation, and agarose gel analysis was conducted to confirm the size (Table 1). The data from real-time PCR experiments were analyzed by the comparative connecticut method as described in the manual for the ABI prism 700 real-time systems. All results were confirmed by repeating the experiment 3 times.

2.9. Enzyme immunoassay for TNF-α, IL-1β, IL-6, and NGF

Hind paw dorsal skin was collected after behavioral testing or at time points as indicated and frozen immediately on dry ice. Skin tissue was cut into fine pieces in ice-cold PBS, pH 7.4, containing protease inhibitors (aprotinin [2 μg/mL], leupeptin [5 μg/mL], pepstatin [0.7 μg/mL], and PMSF [100 μg/mL]; Sigma-Aldrich) followed by homogenization using a rotor/stator homogenizer. Homogenates were centrifuged for 5 minutes at 14,000 g, 4°C. Supernatants were transferred to fresh precooled microcentrifuge tubes. Triton X-100 (Boehringer, Mannheim, Germany) was added at a final concentration of 0.01%. The samples were centrifuged again for 5 minutes at 14,000 g at 4°C. The supernatants were aliquoted and stored at −80°C. TNF-α, IL-1β, and IL-6 protein levels were determined by using EIA kits (R&D Systems, Minneapolis, MN, USA). The NGF concentrations were determined by using the NGF Emax ImmunoAssay System kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The optical density of the reaction product was read on a microplate reader at 450 nm, and values were normalized per gram of tissue assayed. The concentrations of TNF-α, IL-1β, IL-6, and NGF proteins were calculated from the standard curve prepared using reagents provided with each kit. Positive and negative controls were included in each assay as well. Total protein contents in all tissue extracts were measured by the Coomassie Blue Protein Assay Kit (Pierce, Rockford, IL, USA). Each protein concentration was expressed as pg/mg total protein.

2.10. Western blot analysis

For Western blot analysis of nuclear factor (NF)-κB p65, nuclear extract was prepared as previously described [51]. Briefly, keratinocytes were washed with ice-cold PBS, scraped, and briefly centrifuged. The cell pellets were then resuspended in a hypotonic lysis buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acids, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 μg/mL goat α2C (1:50, Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4°C. Cells were then treated with Texas red (1:200, Santa Cruz Biotechnology) or cy3-conjugated secondary antibodies (1:400, Jackson Immunoresearch Laboratories, Inc. West Grove, PA, USA) for 1 hour at room temperature. Control slides were stained with just the secondary antibody, which yields low-intensity nonspecific staining patterns. Immunostained cells were visualized with a Zeiss LSM 510 META laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) and the confocal software was used for acquisition of the data and merging of the digital images.
leupeptin, and 6.4% Nonidet P-40 (Fisher Scientific, Pittsburgh, PA, USA) and incubated for 15 minutes on ice. After another brief centrifugation, the nuclear pellet was collected and suspended in nuclear extraction buffer containing 20 mM ethylenediaminetetraacetic acid, 25% glycerol, 0.5 mM dithiothreitol, 0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 5 mg/mL pepstatin A, and 5 mg/mL leupeptin. After incubation on ice for 30 minutes, the nuclear extract was collected; the concentration of protein was measured using a DC Protein Assay Kit (Bio-Rad Laboratories). Equal amounts of protein were boilded with 3× sodium dodecyl sulfate (SDS) sample buffer, and then subjected to SDS electrophoresis. The results of this assay were confirmed by repeating the experiment 3 times.

For Western blot analyses of p38, extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), phosphorylated p38, phosphorylated ERK, and phosphorylated JNK, cultured keratinocytes were harvested and homogenized in Tris buffer pH 6.8 with freshly added protease inhibitors, β-mercaptoethanol, and glycerol. The homogenate was centrifuged at 13,000 g for 15 minutes at 4°C. The concentration of protein was measured by using a DC Protein Assay Kit (Bio-Rad Laboratories). Equal amounts of protein were size fractionated by 10% SDS-polyacrylamide gel electrophoresis and then subjected to SDS electrophoresis. The results of this assay were confirmed by repeating the experiment 3 times.

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2.11. Immunohistochemistry and confocal microscopy

Animals were euthanized and immediately perfused with 4% paraformaldehyde in PBS, pH 7.4, via the ascending aorta; the hind paw skin, including subdermal layers, was removed and postfixed in 4% paraformaldehyde for 2 hours, and then the tissues were treated with 30% sucrose in PBS at 4°C before embedding in optimal cutting temperature (Sakura Finetek, Torrance, CA, USA). Following embedding, 10-μm-thick slices were made using a cryo-stat, mounted onto Superfrost microscope slides (Fisher Scientific, Pittsburgh, PA, USA), and stored at −80°C. To examine the effects of intraplantar terbutaline on IL-6 expression in epidermal keratinocytes, double immunolabeling was performed as previously described [29]. Briefly, frozen skin sections were permeabilized and blocked with PBS containing 10% donkey serum and 0.3% Triton X-100, followed by exposure to a rabbit anti-IL-6 primary antibody (1:400; LifeSpan Biosciences, Inc., Seattle, WA, USA) overnight at 4°C in PBS containing 2% serum. Sections were then rinsed in PBS and incubated with donkey antigoat immunoglobulin (IgG) (1:500) conjugated with fluorescein secondary antibody (Jackson ImmunoResearch Laboratories). Upon detection of the IL-6, the sections were exposed to monoclonal antirat keratin (clone AE1/AE3, diluted 1:50; Thermo Fisher Scientific, Waltham, MA, USA) overnight at 4°C in PBS containing 2% serum, and then the keratin was detected using donkey antimouse IgG (1:500) conjugated with cyanine dye 3 (Jackson ImmunoResearch Laboratories, diluted 1:500). Images were obtained using confocal microscopy (Zeiss LSM/510 upright 2 photon; Carl Zeiss) and stored on digital media. Control experiments included incubation of slices in primary and secondary antibody-free solutions, which led to low-intensity non-specific staining patterns in preliminary experiments (data not shown). Preabsorption controls were also completed. For these experiments, 250–500 ng of the primary antibody was incubated with 2.5–6 μg of the corresponding immunizing antigen in 500 μL of PBS at 4°C overnight, before incubation with slices. These experiments also showed only low-level staining.

2.12. Statistical analysis

Statistical analysis was performed using a 1-way analysis of variance (ANOVA) followed by post hoc Newman-Keuls multiple-comparison testing to compare effects of each drug (ie, 6-OHDA, butoxamine, TB-2-081, and terbutaline) on nociceptive, vascular, or inflammatory mediator changes after tibia fracture with vehicle treatment and naive control group. Behavioral data collected over time after intraplantar injections of terbutaline were analyzed by a 2-way ANOVA on data for each test time point, comparing with vehicle treatments. For simple comparisons of 2 means, unpaired Student’s t-test was performed. For in vitro (keratinocyte culture) experiments, 1-way ANOVA followed by post hoc Newman-Keuls test was performed to compare dose responses of each drug (ie, norepinephrine, cirazoline, dexmedetomidine, dobutamine, and...
terbutaline). All data are presented as the mean ± SEM, and differences are considered significant at a P value < 0.05 (Prism 5, Graph-Pad Software, San Diego, CA, USA).

3. Results

3.1. Blockade of sympathetic signaling reduced inflammation, nociceptive sensitization, and IL-6 production

To test the hypothesis that sympathetic signaling supports hind paw pain and inflammation in the CRPS tibia fracture model, fracture rats with cast immobilization were treated with either 6-OHDA to abolish sympathetic nerve terminals starting 7 days after fracture and continuing for 5 days, guanethidine to deplete NE from sympathetic nerve terminals once daily starting 14 days after fracture and continuing for 5 days, or saline vehicle. We observed that blockade of sympathetic signaling by 6-OHDA reduced mechanical allodynia, hind limb unweighting, warmth, and edema observed 4 weeks postfracture (Fig. 1A–D). As determined by EIA, 6-OHDA significantly decreased hind paw skin interleukin (IL)-6 levels (E), but had no effect on IL-1β, TNF-α, and NGF (Fig. 1E–H). Similar patterns were seen after guanethidine treatment. Guanethidine treatment reduced allodynia by 44% (P < 0.01), unweighting by 26% (P < 0.05), and decreased IL-6 by 47% (P < 0.001). These results suggest that sympathetic signaling contributes to inflammation, nociceptive sensitization, and the postfracture increase in IL-6.

3.2. Keratinocytes express adrenergic receptors (ARs) and the expression of those receptors was increased by nor epinephrine

Because keratinocytes are the primary cellular source for the inflammatory mediators supporting cutaneous nociceptive sensitization in early CRPS [29], we were interested to know if sympathetic signals can support keratinocyte activation and inflammatory mediator production, thus maintaining nociceptive sensitization, allodynia, and unweighting. To test this hypothesis, we first studied AR expression in cultured rat keratinocytes. The expression of ARs was determined by real-time PCR and later confirmed by immunofluorescence staining. Fig. 2A demonstrates that mRNAs of all AR subtypes were detectable in cultured rat epidermal keratinocytes, but the β2-AR subtype was expressed at much higher levels than any other subtypes. Immunofluorescence staining demonstrated the expression of ARs α1 (Fig. 2B), α2A (Fig. 2C), α2B (Fig. 2D), α2C (Fig. 2E), β1 (Fig. 2F), β2 (Fig. 2G), and β3 (Fig. 2H) in the cultured cells.

We then examined the effects of NE on AR expression in cultured rat epidermal keratinocytes by real-time PCR. NE increased the expression of all AR subtypes, except α2A-AR and β1-AR (Fig. 3). The stimulation effects of NE were maximal at 10⁻⁷ M for β2-AR, 10⁻⁶ M for α2B-AR, and 10⁻⁵ M for α1A-AR, α1B-AR, α1D-AR, α2C-AR, and β3-AR measured at 3 hours after NE addition (Fig. 3).

3.3. Activation of ARs by NE and various AR subtype agonists stimulated inflammatory mediator expression in keratinocytes

Next we looked at the effectiveness of NE and various AR subtype selective agonists in stimulating rat epidermal keratinocyte expression of IL-1, IL-6, TNF, and NGF by real-time PCR. As shown in Fig. 4, for all the agents we tested, effects were generally dose dependent. The endogenous sympathetic neurotransmitter NE stimulated expression of all 4 inflammatory mediators, but the most robust effect was on IL-6 (Fig. 4A, 3-fold increase). Cirazoline (α1-AR agonist) increased expression of all 4 inflammatory media-
Adrenergic receptor (AR) expression in cultured rat epidermal keratinocytes. For all ARs, expression was detectable at the mRNA level, though the β2-AR subtype was expressed at much higher levels than the others (A). Expression of each AR subtype was detectable at the protein level as well. Panels B–H are representative fluorescence photomicrographs of α1-AR (B), α2A (C), α2B (D), α2C (E), β1 (F), β2 (G), and β3 (H).

Fig. 2. Adrenergic receptor (AR) expression in cultured rat epidermal keratinocytes. For all ARs, expression was detectable at the mRNA level, though the β2-AR subtype was expressed at much higher levels than the others (A). Expression of each AR subtype was detectable at the protein level as well. Panels B–H are representative fluorescence photomicrographs of α1-AR (B), α2A (C), α2B (D), α2C (E), β1 (F), β2 (G), and β3 (H).

tors, up to 2-fold (Fig. 4E–H). Dexmedetomidine (α2-AR agonist) increased expression of IL-1, up to 2-fold (Fig. 4J). Dobutamine (β1-AR agonist) increased expression of IL-6, IL-1, and NGF by up to 50% (Fig. 4M, N, and P). Terbutaline (β2-AR agonist) increased expression of IL-6 by 18-fold, IL-1 by 2-fold, and NGF by 50% (Fig. 4Q, R, and T). Taken together, these results suggest that activation of ARs by NE and various AR subtype agonists stimulated inflammatory mediator expression in keratinocytes. However, enhanced IL-6 production via the stimulation of β2-ARs is by far the most robust response.

3.4. Norepinephrine (NE) stimulates phosphorylation of MAP kinases P38, ERK, and JNK

We sought to investigate the effectiveness of NE in stimulating phosphorylation of mitogen-activated protein (MAP) kinases p38, ERK, and JNK, as well as nuclear translocation of NF-kB in keratinocytes by Western blot. Fig. 5 demonstrated that the application of NE (10^{-3} M) caused peak phosphorylation of all 3 MAPKs at 5 minutes. However, NE did not affect nuclear translocation of NF-kB in rat keratinocytes.

3.5. Blockade of β2-AR by butoxamine in fracture rats reduced allodynia, unweighting, and warmth, but had no effect on edema

Given that the β2-AR subtype is predominant and functional in keratinocytes and that sympathetic signaling supports inflammation and pain sensitization, we hypothesized that β2-AR receptors mediate nociceptive changes observed in the CRPS model. To test this hypothesis, fracture rats and noninjured animals were treated with systemic administered β2-AR antagonist, butoxamine (2 mg/kg i.p. 30 minutes prior to testing). As shown in Fig. 6, butoxamine treatment reduced hind paw allodynia and hind limb unweighting that developed after fracture. However, it didn’t change hind paw warmth or edema. In noninjured animals, administration of butoxamine at the same dose did not change hind paw nociceptive responses, edema, or warmth (data not shown).

3.6. Blockade of the IL-6 receptor by TB-2-081 in fracture rats reduced allodynia, unweighting, and warmth, but had no effect on edema

IL-6 is an inflammatory mediator well known to support nociception, including mechanical allodynia in skin [1,29,53]. The
blockade of sympathetic signaling (Fig. 1) reduced inflammation, nociceptive responses, and IL-6 production. This led us to hypothesize that sympathetic signaling may regulate nociceptive responses via IL-6 in CRPS, that is, NE may promote IL-6 production and secretion from keratinocytes, which, in turn, acts directly or indirectly on sensory nerve terminals in the skin to support allodynia and unweighting seen in the CRPS model.

To examine the hypothesis of IL-6 supporting nociceptive response in the CRPS model, tibia fracture rats and noninjured animals were treated with an IL-6 receptor antagonist, TB-2-081 (2 mg/kg s.c. 15 minutes prior to testing or vehicle). We observed that treatment with TB-2-081 reduced hind paw allodynia and unweighting that developed after fracture. However, this agent did not significantly alter hind paw warmth and edema (Fig. 7).

In noninjured animals, administration of TB-2-081 at the same dose did not change hind paw nociceptive responses, edema, or warmth (data not shown).

3.7. Activation of β2-AR by terbutaline in control rats induced local mechanical allodynia and IL-6 production in keratinocytes

Because the systemic administration of butoxamine does not allow us to conclude that local hind paw ARs are the population of ARs supporting peripheral inflammatory changes, we determined if terbutaline (a selective β2-AR agonist) applied locally could induce mechanical allodynia and IL-6 production in skin in normal control rats. Our behavioral studies indicated that intraplantar application of terbutaline (5 μg/30 μL) caused hind paw mecha-
cal allodynia and hind limb unweighting at 1 hour after injection (Fig. 8A, B). When terbutaline was given systemically at the same dose used in the plantar hind paw injection experiment (5 μg/30 μl saline, i.p.), there was no effect on nociceptive thresholds (data not shown). On the other hand, using EIA, we detected that the injection of terbutaline induced a significant increase in IL-6.
cytokine level (Fig. 8C) but did not change IL-1β, TNF-α, or NGF levels (Fig. 8D, E, and F, respectively) in skin. Fig. 8G shows representative confocal images of IL-6 (green) and keratin (a keratinocyte marker, red) in hind paw skin sections from control rats, and from the ipsilateral hind paw of control rats treated with terbutaline. Only low levels of IL-6 were detected in control skin (Fig. 8G, upper row of panels), but skin sections from terbutaline-injected rats demonstrated large amounts of IL-6 protein in cells in the epidermis co-labeled for keratin (Fig. 8G, middle and bottom rows of panels).

4. Discussion

In this study we used 2 well-characterized strategies to reduce SNS activity in fracture rats, including administration of 6-OHDA, a catechol synthesis inhibitor, and guanethidine, an agent that depletes NE from sympathetic nerve terminals. Using these agents, nociceptive changes were reduced and the vascular changes were partially normalized (Fig. 1). Because TNF-α and IL-6 levels are elevated in the skin blister fluid of CRPS-affected limbs, and TNF-α, IL-1β, IL-6, and NGF levels are elevated at 4 weeks postfracture in the
Fig. 8. Activation of β2-AR by terbutaline in control rats induced local mechanical allodynia and interleukin (IL)-6 production in keratinocytes. Top panels show intraplantar administration of terbutaline (5 μg/30 μL) caused hind paw mechanical allodynia (A), and slight hind limb unweighting (B) at 1 hour after injection in control rats. *P < 0.05, and **P < 0.01 for terbutaline treatment vs saline group or baseline values, #P < 0.05 and ##P < 0.01 for terbutaline treatment vs saline-treated group (n = 8 for each group). Middle panels are changes in hind paw skin cytokine and nerve growth factor (NGF) protein levels in the plantar skin of control rats with and without terbutaline treatment. Cytokine production was measured by enzyme immunoassay 1 hour after intraplantar injection of vehicle or terbutaline. Intraplantar injection of terbutaline induced a significant increase in IL-6 (C) cytokine level but did not change IL-1β (D), tumor necrosis factor (TNF)-α (E), or NGF (F) levels (n = 7-8 per cohort). **P < 0.01 for terbutaline vs. vehicle controls. Bottom panels (G) are representative fluorescence photomicrographs of co-staining of IL-6 and keratinocytes (keratin label) in hind paw skin sections from control rats, and from the ipsilateral hind paw of control rats treated with intraplantar-injected terbutaline. This study demonstrated that keratinocytes are the main source of IL-6 in responding to terbutaline in skin (Panel G, n = 3–4 per cohort). Scale bar = 20 μm.
tibia fracture/cast model of CRPS, we went on to examine the effects of sympathectomy on inflammatory mediator levels in the hind paw skin after fracture. Curiously, we found that 6-OHDA or guanethidine treatment inhibited the expected increase in IL-6 levels in the fracture hind paw skin, while levels of the other mediators were elevated at levels similar to those observed in untreated control animals after fracture (Fig. 1).

The effects of sympathectomy on postfracture pain behavior and mediator expression correlates with our recent study examining the effects of fracture in transgenic mice lacking either SP or the CGRP receptor RAMP1 [12]. At 3 weeks postfracture, these mice had attenuated nociceptive sensitization compared to wild-type fracture mice, and while there were no increases in IL-1β in the skin, IL-6 levels were increased, suggesting that this cytokine is regulated by a distinct mechanism. We postulated that the residual sensitization observed in the transgenic fracture mice was attributable to the increased IL-6 levels. In fact, the IL-6 receptor antagonist TB-2-081 partially reversed allodynia and unweighting. Previously, we demonstrated that fracture rats treated with the IL-1 receptor antagonist anakinra, the TNF inhibitor etanercept, or the NGF antibody tanezumab had attenuated allodynia and hind limb unweighting at 4 weeks postfracture [31,41,42]. Because these cytokine and NGF inhibitors are large molecules that don’t cross the blood–brain barrier, we suspect that the pronociceptive effects of these mediators occur at the nociceptor level in the skin. Furthermore, we identified keratinocytes as the primary cellular source of IL-1β, IL-6, TNF-α, and NGF in the fracture hind paw [29]. Collectively, these results suggest that exaggerated neuropeptide signaling induces the upregulation of IL-1β, TNF-α, and NGF in cutaneous keratinocytes of the fracture limb, thus contributing to nociceptive sensitization. Similarly, the sympathectomy experiments indicate that SNS signaling contributes to postfracture nociceptive sensitization and to the upregulation of IL-6 in keratinocytes.

A rat keratinocyte cell line was then employed to identify the adrenergic receptor populations responsible for the sympathetic regulation of inflammatory mediator expression and to study potential mechanisms for those effects [48]. While the expression of many adrenergic receptor subtypes was identified in these cells, β2-AR expression was remarkably high (Fig. 2). Expression of this receptor, like several other types of adrenergic receptors, was enhanced after exposing keratinocytes to NE (Fig. 3). These results were complemented by expression studies looking at IL-1β, IL-6, TNF-β, and NGF, all mediators found in elevated levels in the skin of fracture/cast animals [29]. Stimulation of the keratinocytes with NE increased the levels of several cytokines and NGF, though IL-6 was by far the most robustly stimulated (Fig. 4). Importantly, the highly selective β2-AR agonist terbutaline caused an 18-fold increase in IL-6 expression, more than an order of magnitude greater increase than for any of the other mediators studied. It should be noted that selective stimulation of other classes of adrenergic receptors did, in fact, lead to measurable increases in cytokine levels, though the increases were typically less dramatic than the terbutaline-mediated effects on IL-6. Specifically, the 4 mediators followed in these experiments were all increased after exposure of the cultures to the selective α1-AR agonist cirazoline.

In addition, stimulation of keratinocytes with terbutaline activated all 3 of the principal families of the MAPK signaling system, but not the NF-κB transcriptional signaling pathway (Fig. 5). The MAPK signaling system has been implicated in promoting cytokine production (IL-1β, IL-6, TNF-α) in many tissues, including skin [25,35,47]. Particularly strong evidence relates p38 MAPK activation to IL-6 production in keratinocytes, which is consistent with our observations [26]. In our experiments, phosphorylation of MAPK-related enzymes was observed within 5 minutes of exposure of keratinocyte cultures to NE. Strong evidence also links activation of the NF-κB system to cytokine production in various types of cells [3,24]. This, however, was not observed in our system. We did not attempt to determine whether these signaling systems were activated under conditions in vivo.

These in vitro observations were then translated to the animal model. As predicted from the cell culture results, systemic administration of the selective β2-AR antagonist butoxamine reduced nociceptive sensitization in the fracture/cast animals (Fig. 6). These results closely parallel those obtained when chemical sympathectomy was performed. Furthermore, the systemic administration of the selective IL-6 receptor antagonist TB-2-081 also reduced hind paw nociceptive sensitization in the fracture rats (Fig. 7).
Complementing those observations, the intradermal injection of terbutaline in the hind paw caused mechanical allodynia, hind paw unweighting, and upregulated the production of IL-6 (but not other mediators) in the keratinocyte cell layer of hind paw skin (Fig. 8). Previous studies involving the local administration of SP showed a more generalized increase in cytokine and NGF levels in paw skin [43], suggesting that specific transmitters generate specific patterns of mediator production. Thus, the keratinocyte β2-AR/IL-6 regulatory axis could be reproduced in whole animals, adding credence to the notion that this axis is functional in causing some of the changes characterizing CRPS.

Our observation that β2-ARs are the predominant adrenergic receptor population expressed in cultured keratinocytes is consistent with existing literature. Though many subtypes of adrenergic receptors exist, keratinocytes predominantly express β2-ARs in vivo [49], especially those in the basal layer of the epidermis, where we observed the majority of the IL-6 expression in our experiments [46]. We observed apparent upregulation of β2-AR expression in response to NE in cell culture experiments, though the dose-response curve was U-shaped. This may be due to receptor desensitization, internalization, uncoupling, or depletion of second messengers [9]. Actual changes in functional expression were not assessed and require formal binding studies. These receptors have well-established roles in controlling proliferation and differentiation of keratinocytes through control of cAMP and calcium ion levels [13,33]. The mechanism for β2-AR stimulation on IL-6 production in epithelial cells is less well understood, though β2-AR stimulation causes the enhanced production of IL-6 in airway epithelial cells [8,52]. The evoked release of norepinephrine in skin is similar between CRPS and control subjects, suggesting that target tissue changes in adrenergic signaling may be important [54]. It is also noteworthy that catecholamines released from sympathetic nerve terminals may not be the only source of these transmitters in skin. Pollar et al. have reported that keratinocytes synthesize and release epinephrine, and that β2-ARs expressed on these keratinocytes may regulate cell proliferation in response to this epinephrine release [40]. We did not, in these studies, examine the question of increased adrenergic sensitivity of afferent neurons, which may support pain in conditions such as Varicella zoster infection [23].

These data, along with others previously reported, can be assembled into an integrative model of neurocutaneous signaling in CRPS. Fig. 9 updates our previously proposed model to accommodate sympathetic signaling [30]. Any such model needs to take into account the demonstrated functions of primary afferent fibers, as well as sympathetic input. The main demonstrated sources of cutaneous cytokines and NGF in this model are the keratinocytes [29]. As diagrammed in Fig. 9, the neuropeptides SP and CGRP stimulate keratinocyte production of several different inflammatory mediators, including IL-1β, TNF-α, and NGF. In parallel, the SNS likely has a predominant, though perhaps not exclusive, role in stimulating IL-6 production via β2-ARs activation on epidermal keratinocytes. All these inflammatory mediators can cause rapid cutaneous nociceptive sensitization after intraplantar injection [29] and can immediately evoke spontaneous firing and sensitization in primary sensory neurons at the nociceptors level [6,16,36]. We suggest that partially overlapping neurocutaneous mechanisms support the production of nociceptive mediators in CRPS, and that the relative balance of these systems may explain in part the heterogeneous clinical presentation and differences in treatment responses in the CRPS patient population.

In conclusion, the results from the keratinocyte in vitro studies and the rat fracture CRPS model support our hypothesis that the release of NE from the sympathetic terminals in the skin can activate β2-ARs on epidermal keratinocytes, resulting in the production and secretion of the proinflammatory cytokine IL-6, subsequently causing sensitization of cutaneous sensory neuron terminals with allodynia and unweighting in the fracture limb. We postulate that adrenergic-keratinocyte-sensory neuron neuro-inflammatory signaling also plays a role in the modulation of nociceptive thresholds in CRPS patients. Previous reports that CRPS patients frequently express β2-AR antibodies that can act as β2-AR agonists [21] and that IL-6 levels are upregulated in experimental skin blister fluid in the CRPS affected limb [14,34] further support this hypothesis. Collectively, these data support mechanism-based pharmacologic trials examining the role of β2-AR signaling and IL-6 immune responses in early CRPS.

Conflict of interest statement

The authors do not have financial or other relationships that might lead to conflict of interest.

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