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

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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.

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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 [2,22,27,57]. Again, the fracture/cast CRPS model has provided results consistent with

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the clinical findings; enhanced postjunctional 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 (EIA) 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.