Perineural Clonidine Reduces Mechanical Hypersensitivity and Cytokine Production in Established Nerve Injury
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Background: Partial sciatic nerve ligation (PSNL) produces axonal damage, a local inflammatory response, and Wallerian degeneration. Cytokines secreted near the site of nerve injury are thought to play important roles in development and maintenance of central sensitization and neuropathic pain. Injection of clonidine at the site and time of nerve injury slows the development of PSNL-induced hypersensitivity and reduces local cytokine expression by actions on α2 adrenoceptors. The current study tested whether clonidine would have a similar effect in established nerve injury.

Methods: Rats underwent unilateral PSNL, and perineural saline, clonidine, or BRL44408 plus clonidine was injected 2 weeks later. Three days after perineural injection, withdrawal threshold to mechanical stimulation of the hind paw ipsilateral and contralateral to PSNL was determined, and tissues were removed for cytokine analysis.

Results: PSNL was accompanied by a proinflammatory pattern of cytokine content in neural structures and hypersensitivity ipsilateral with few changes contralaterally. Perineural clonidine, but not saline, partially reversed the hypersensitivity, accompanied by reduced concentrations of interleukin 6 and interleukin 1β in the sciatic nerve. The effect of clonidine on hypersensitivity and these cytokines was blocked by the α2-adrenoceptor antagonist, BRL44408.

Conclusions: These data suggest that perineural clonidine acts on α2 adrenoceptors to reduce hypersensitivity in established nerve injury, likely by an immunomodulatory mechanism, and may be effective in patients in the weeks after nerve injury.

INJURY to peripheral nerves provokes an inflammatory response leading to axonal regression and support cell death, a process termed wallerian degeneration. The extent and timing of this response are controlled by the balance between proinflammatory and antiinflammatory cytokine production,1,2 which orchestrates nerve demyelination by macrophages and Schwann cells.3,4 Interleukin (IL)-1α, IL-1β, IL-6, granulocyte–macrophage colony-stimulating factor, and tumor necrosis factor (TNF)-α are important signaling molecules for the early stages of wallerian degeneration,4,5 whereas IL-10 is instrumental in the later healing/regenerative phase.1

It has been suggested that mechanical hypersensitivity and pain in animals and humans after peripheral nerve injury reflect actions of these cytokines on injured nerves and their central projections.6–10 Cytokine production is critical to the initial development and early maintenance of hypersensitivity and central sensitization, including activation of microglia and/or astrocytes in the spinal cord.11–14 Central activation of glial cells often results in contralateral hypersensitivity ("mirror-image" pain) in animals,7,10,14 which occasionally also occurs in humans.15–17

Perineural injection of clonidine at the time of surgery delays the onset of hypersensitivity after partial sciatic nerve ligation (PSNL), coincident with reduced TNF-α and IL-1β concentrations in the injured nerve,9 and reduces hypersensitivity in established PSNL by actions on α2 adrenoceptors.18 Cytokine responses after nerve injury change with time, however,1,4 and whether the antihypersensitivity effect of perineural clonidine in established PSNL reflects a similar modulation of cytokine responses to that of preemptive treatment is not known.

The purpose of this study was to test the hypothesis that perineural clonidine, by actions on α2 adrenoceptors, decreases proinflammatory and/or increases antiinflammatory cytokines in the injured nerve and its central projections in established hypersensitivity after PSNL.

Materials and Methods

Partial Sciatic Nerve Ligation and Perineural Injections

After approval was obtained from the Animal Care and Use Committee of Wake Forest University Health Sciences Center (Winston-Salem, North Carolina), male Wistar rats (200 g at the time of surgery) underwent partial ligation of the left sciatic nerve (SN) during halothane anesthesia, as previously described with slight modifications.9,19 Briefly, the left SN was exposed, and one third to one half of the nerve proximal to the trifurcation of the peroneal, sural, and tibial branches was tightly ligated with silicon-treated silk suture (prolene 6–0). The incision was closed in layers, and animals recovered from anesthesia.

Two weeks after surgery, animals were briefly anesthetized and received a single perineural injection of saline or clonidine, 30 μg, a dose previously shown to reduce mechanical hypersensitivity in this model,9 alone or coadministered with BRL44408, 45 μg, an α2A-preferring adrenoceptor antagonist. Injections were administered in a 0.3-ml volume for clonidine and saline alone, or 0.4 ml for clonidine plus BRL44408 using a 25-gauge needle, which was introduced percutaneously between the greater trochanter and the ischial tuberosity and ad-
vanced in an anteromedial direction as previously described. Injections were performed in a fanning motion along the path of the SN. Control injections of this volume of ropivacaine in other animals produced temporary unilateral limb paralysis in more than 95% of cases, consistent with reliable delivery of drug near the nerve.

**Behavioral Experiments**

Animals were placed on a plastic mesh floor in individual plastic boxes for 20–30 min for acclimation. Withdrawal thresholds were assessed using calibrated von Frey filaments (Stoelting, Wood Dale, IL) and an up-down statistical method. Withdrawal thresholds were measured twice at 10-min intervals, and the average of these was used for analysis. Behavioral testing was performed before surgery, 2 weeks after PSNL (immediately before the perineural injections), and at 5 h and 1, 2, 3, and 7 days after perineural injections. Investigators were blind to treatment groups. Animals in other identical groups received perineural clonidine, saline alone, or clonidine plus BRL44408 and were killed 3 days later for analysis of cytokine concentrations.

**Tissue Collection**

For tissue collection, animals were anesthetized with halothane and killed by decapitation immediately after the last behavioral test. Four tissues were collected for analysis of cytokine concentrations: L4, L5, and L6 dorsal root ganglia (DRG), combined in each animal, and 0.5-cm sections of the SN proximal to, at, or distal to the injury site. Tissues were collected both ipsilateral and contralateral to the side of injury. We also collected the following tissues from control animals with no surgery or injections: L4, L5, and L6 dorsal root ganglia and SN. Group size was 10–12. All of the samples were collected in 1.5-ml polypropylene tubes (RNAase, DNAase, and pyrogen free) and were put in dry ice immediately after the collection. Subsequently, all of the tissues were stored at −80°C until analysis.

**Cytokine Assay**

Samples weighing less than 20 mg were combined with 150 μl regular cell culture medium, Roswell Park Memorial Institute (Buffalo, NY) medium plus 10% inactivated bovine serum (Gibco, Grand Island, NY), and samples weighing 20 mg or more were combined with this medium in a ratio of 10 μl/mg tissue. The samples were homogenized in an ice bath with an ultrasonic tissue homogenizer. The homogenates were centrifuged at 1,300g for 15 min at 4°C; the supernatants were collected and then recentrifuged at 1,500g for 10 min at 4°C. The supernatants were immediately used for multiplex cytokine assays. Assays were performed in duplicate, and the resultant data were averaged.

A 9-plex bead-based immunoassay kit for rat cytokines (Bio Rad, Hercules, CA) was used to detect the concentrations of granulocyte–macrophage colony-stimulating factor, interferon γ, IL-10, IL-1α, IL-1β, IL-2, IL-4, IL-6, and TNF-α simultaneously, according to the instructions of the manufacturer. This technique has been validated previously. For the clonidine plus BRL44408 group, only IL-1β and IL-6 were measured. Multiwavelength fluorescence was determined with a luminometer (Luminex 100 system; Luminex, Austin, TX) and analyzed using MasterPlex QT Quantitation Software (MiraiBio, Inc., Alameda, CA).

Concentrations of cytokines in samples were within the linear range of the assay for all cytokines except TNF-α, in which case most samples were below the level of detection (0.2 pg/ml). TNF-α concentrations are therefore not reported.

**Statistical Analysis**

Data are presented as mean ± SEM. The effects of PSNL surgery and drug injections on withdrawal threshold were determined using a two-way analysis of variance followed by the Dunnett test using pre-PSNL thresholds as control. Comparisons among groups for cytokine concentrations were performed using t tests or, when normality failed, Mann–Whitney U tests. A P value less than 0.05 was considered significant for withdrawal threshold. Because we performed a large number of comparisons (eight cytokines in eight tissues), we performed an experiment-wide correction for multiple analyses and considered a P value of 0.001 or less as significant for each cytokine analysis of variance.

**Results**

**Withdrawal Threshold**

Partial sciatic nerve ligation produced a significant decrease in withdrawal threshold ipsilaterally 2 weeks after surgery, and this hypersensitivity was sustained for 7 days in saline-treated animals (P < 0.05; fig. 1). Perineural clonidine partially alleviated hypersensitivity at 2, 3, and 7 days after the injection compared with the saline group (P < 0.05). The maximum effect was seen on day 3 after clonidine treatment (fig. 1). For that reason, other groups of rats were prepared to measure the cytokine levels in the collected tissue 3 days after injection. In those groups, saline did not affect withdrawal threshold (fig. 2A), whereas clonidine partially reversed the hypersensitivity at 2 and 3 days after the injection (P < 0.05; fig. 2A). The effect of clonidine was prevented by BRL44408 (P < 0.05; fig. 2A).

Partial sciatic nerve ligation produced a slight but significant decrease of withdrawal threshold contralaterally to the injury. Perineural saline or clonidine injection, ipsilateral to the injury, did not affect the withdrawal thresholds contralaterally (fig. 2B).
Effect of PSNL on Cytokine Concentration

Partial sciatic nerve ligation increased IL-1β, IL-2, and IL-6 in DRG (P ≤ 0.001; fig. 3), as well as IL-10 and IL-6 in the SN proximal to injury (P ≤ 0.001; fig. 3). Distal to injury, only IL-1β increased (P ≤ 0.001; fig. 3).

Contralaterally, we observed that IL-1β and IL-6 were increased in proximal SN, as well as the anti-inflammatory cytokine IL-10 in mid and distal SN (P ≤ 0.001; fig. 3).

Effects of Perineural Clonidine on Cytokine Concentrations

Compared with perineural saline, clonidine injection 3 days before tissue harvest was associated with significantly decreased concentrations of IL-6 in ipsilateral SN proximal to injury and IL-1β in ipsilateral SN distal to injury (P ≤ 0.001; fig. 3). Clonidine did not otherwise affect cytokine concentrations ipsilateral to PSNL, including a lack of effect of the increased IL-10 concentrations in the injured SN (fig. 3), and did not affect cytokine concentrations contralateral to PSNL (fig. 4). BRL44408 prevented clonidine-associated decreases in IL-1β in ipsilateral DRG and IL-6 in both ipsilateral DRG and SN distal to injury (P ≤ 0.001; fig. 3).

Discussion

The role of cytokine production and signaling in the pathogenesis of nerve injury and pain is of current interest. Acute administration of the proinflammatory cytokines, which are present shortly after injury, into normal peripheral nerve or tissues sensitizes afferents and results in hypersensitivity.23 Although the role of cytokines...
in pain and hypersensitivity may well diminish over time after injury, the current study suggests that they are important factors as long as 2 weeks after surgical nerve injury and can be modified at this time by perineural clonidine.

**Cytokine Expression 2 Weeks after PSNL**

Four cytokines, IL-1β, IL-2, IL-6, and IL-10, were increased in ipsilateral tissues 2 weeks after PSNL in the current study. These results confirm previous observations of increased IL-1β and IL-6 late after nerve injury.4,5 Cell types important to production of these cytokines in this setting include macrophages, mast cells, and lymphocytes. These cytokines have been described as pronociceptive8,14,23–25 and are speculated to be important determinants of hypersensitivity at this time. IL-10 is an antiinflammatory cytokine with increased expression late after nerve injury, presumably to initiate regeneration of the damaged nerves. IL-10 down-regulates expression of the major proinflammatory cytokines.1 As part of the balance of normal inflammatory processes, proinflammatory cytokines themselves increase IL-10 expression.26 Therefore, we speculate that increased IL-10 concentrations observed in the current study are secondary to previously increased proinflammatory cytokines and may be signaling regeneration 2 weeks after injury. IL-2 is a key factor in activation of the innate immune system27,28 and exerts antinociceptive effects.29–32 IL-2 concentration is increased in plasma beginning on day 11 after nerve injury in rats33 and may be important in regeneration of transected nerve.34

Others have observed changes in cytokine messenger RNA expression in neural structures contralateral to peripheral nerve transection.35 We observed a strikingly similar pattern of cytokine expression in contralateral DRG and SN as that ipsilateral to PSNL injury. Thus, there was a bilateral increase in IL-1β, IL-6, and IL-10, although the proinflammatory cytokines IL-1β and IL-6 were numerically increased less contralaterally than ipsilaterally, and the antiinflammatory cytokine IL-10 was numerically increased more. This suggests that contralateral changes in cytokine concentrations are not merely a systemic response to injury but are regionally controlled. Others have proposed a communication of activity or transport of chemicals as a mechanism to explain contralateral changes in unilateral neuropathies.36,37

Tumor necrosis factor α is markedly increased in injured peripheral nerve for 3–7 days and likely plays an important role in hypersensitivity during this period.9 Because the sensitivity of our methodology to detect TNF-α was inadequate, the current study did not address the relevance of this key cytokine 2 weeks after PSNL.

**Effects of Clonidine**

Perineural injection of clonidine reduces hypersensitivity weeks after PSNL.18 Because that study observed α2-adrenoceptor immunoreactivity in macrophages and T cells in the injured nerve, it was presumed that clonidine was altering immune cell function to produce its delayed-onset, prolonged effect. Subsequently, repeated perineural clonidine injections at the time of PSNL injury were shown to delay the development of hypersensitivity, accompanied by significant reductions in TNF-α and IL-1β concentrations in clonidine-treated animals.9 In addition, different cytokines, particularly IL-6 and IL-2, were increased in addition to IL-1β 2 weeks after PSNL, consistent with the changing cytokine expression profile accompanying wallerian degeneration.4,33 Two observations argue for an immunomodulatory effect of clonidine to reduce hypersensitivity: similar time course of behavioral and immunologic effects and presence of α2-adrenoceptors on immune cells at the site of injury.18

The blockade of the behavioral effect of clonidine confirms previous observations9,18 that clonidine is acting on α2 adrenoceptors. More importantly, parallel blockade of clonidine’s reduction of IL-1β and IL-6 as well as its antihypersensitivity effect by BRL44408 strongly supports an immunomodulatory mechanism of action of clonidine for analgesia in this setting.

The mechanisms by which perineural clonidine alters hypersensitivity and cytokine expression after nerve injury are uncertain, although they clearly involve actions on α2 adrenoceptors. The current study indicates that cytokine expression is reduced days after a single clonidine injection, and we speculate that clonidine acutely altered genetic expression which subsequently changed immune or neural cell phenotype. This speculation is supported by reduction in abnormal excitability in injured afferents days after a single perineural injection of clonidine30 and reduction in cytokine expression and response to immune challenge in leukocytes 3 days after a single perineural injection of clonidine in a model of inflammatory sciatic neuritis.40

Although others have demonstrated excitatory α2 adrenoceptors on injured nociceptors,41 there are no reports of clonidine-induced pain, and animals in the current study did not demonstrate agitation behavior after perineural clonidine. In addition, other manipulations that reduce IL-1 or IL-6 or increase IL-1044 reduce hypersensitivity in settings of nerve injury or inflammation, arguing for a causal relation between clonidine-induced reduction in IL-1β and IL-6 and its antihypersensitivity effect.

In conclusion, 2 weeks after PSNL injury, there is a different pattern of cytokine expression ipsilateral to nerve injury than closer to the time of injury, with some changes contralaterally. Perineural clonidine partially alleviates mechanical hypersensitivity at this time, and we
speculate that this is secondary to reduced cytokine expression, because both the analgesic and cytokine modulation effects of perineural clonidine are similarly blocked by an α2-adrenoceptor antagonist. These data provide the rationale for clinical trials examining the efficacy of perineural clonidine for patients with pain and hypersensitivity shortly after traumatic nerve injury, such as occurs commonly after surgery.

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References


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