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Methylprednisolone prevents nerve injury-induced hyperalgesia in neprilysin knockout mice

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ABSTRACT

The pathophysiology of the complex regional pain syndrome involves enhanced neurogenic inflammation mediated by neuropeptides. Neutral endopeptidase (neprilysin, NEP) is a key enzyme in neuropeptide catabolism. Our previous work revealed that NEP knock out (ko) mice develop more severe hypersensitivity to thermal and mechanical stimuli after chronic constriction injury (CCI) of the sciatic nerve than wild-type (wt) mice. Because treatment with glucocorticoids is effective in early complex regional pain syndrome, we investigated whether methylprednisolone (MP) reduces pain and sciatic nerve neuropeptide content in NEP ko and wt mice with nerve injury. After CCI, NEP ko mice developed more severe thermal and mechanical hypersensitivity and hind paw edema than wt mice, confirming previous findings. Hypersensitivity was prevented by MP treatment in NEP ko but not in wt mice. MP treatment had no effect on protein levels of calcitonin-gene related peptide, substance P, and bradykinin in sciatic nerves of NEP ko mice. Endothelin-1 (ET-1) levels were higher in naïve and nerve-injured NEP ko than in wt mice, without an effect of MP treatment. Gene expression of the ET-1 receptors ET_AR and ET_BR was not different between genotypes and was not altered after CCI, but was increased after additional MP treatment. The ET_BR agonist IRL-1620 was analgesic in NEP ko mice after CCI, and the ET_BR antagonist BQ-788 showed a trend to reduce the analgesic effect of MP. The results provide evidence that MP reduces CCI-induced hyperalgesia in NEP ko mice, and that this may be related to ET-1 via analgesic actions of ET_BR.

1. Introduction

Studies on the early changes in complex regional pain syndrome (CRPS) underscore the importance of neuropeptides. The nonapeptide bradykinin, which was found increased systemically in CRPS patients [3], is a potent algogenic mediator and is involved in edema formation [16]. In the warm and red "inflammatory phenotype" of CRPS, electrical stimulation of primary afferents results in an intense neurogenic flare and edema. The former is supposed to be mediated by calcitonin gene-related peptide (CGRP), the latter by substance P (SP) [46]. Endothelin-1 (ET-1), a potent vasoconstrictive peptide, is increased in blister fluids of patients with cold and bluish, noninflammatory CRPS extremities [18]. ET-1 is first translated as pre-pro-ET-1 and is cleaved to the active peptide ET-1 by the endothelin converting enzyme-1 (ECE-1). Exogenously administered ET-1 elicits pain in humans and experimental animals, mostly through ET_A receptors (ET_AR) [1]. ET_B receptors (ET_BR) may have an analgesic effect, depending on doses and local conditions, as demonstrated in an animal model of CRPS [34].

Because of the still-incomplete understanding of the pathophysiology of CRPS and the lack of large-scale clinical trials, treatment of CRPS remains mostly empirical [31,35]. Systemic glucocorticoids seem to be effective when administered early in the course of CRPS [4,11]. Consistent with this, continuously infused methylprednisolone (MP) reversed hind paw edema and protein extravasation in rats with tibial fracture [27] and also reversed thermal and mechanical hyperalgesia in sciatic nerve-transected rats [26].

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Human neutral endopeptidase (neprilysin, NEP), a zinc endopeptidase, degrades several peptides, including CGRP, SP, and ET-1, and regulates osteoblasts [21], which might be involved in CRPS pathogenesis [36]. We previously demonstrated that after chronic constriction injury (CCI) of the sciatic nerve, mice deficient of NEP (NEP knockout [ko] mice) were more sensitive to heat and mechanical stimuli than wild-type (wt) mice, and developed edema and changes in limb temperature resembling human CRPS II (ie, CRPS with major nerve lesion) [28]. There is no perfect animal model for CRPS, and although CCI is considered a nerve injury model, we took advantage of the CRPS-like phenotype of NEP ko mice after CCI to investigate steroid responsiveness. We hypothesized that a glucocorticosteroid such as MP, which is reportedly effective in the treatment of CRPS [4,11,23], would have analgesic effects in NEP ko mice after CCI and that the endothelin system and its receptors are the basis for this analgesic effect.

2. Materials and methods

2.1. Animals, surgery, and drug treatment

NEP ko mice on C57Bl/6 background (>10 generations backcrossed) were generated in the laboratory of B.L. at Boston Children's Hospital, Harvard Medical School [30] and were bred and maintained at the animal facilities of the University of Würzburg. C57Bl/6 wt mice were purchased from Harlan Winkelmann, Germany. The mice were age-matched (wt mice: 12 weeks ± 0.4; ko mice: 13 weeks ± 0.3) and cage-matched (ie, kept in the same cages and environment). The experiments were approved by the Bavarian State authorities.

CCI was performed according to the method described by Bennett and Xie [2] modified for mice [43]. Briefly, animals were anesthetized by isoflurane, and the right sciatic nerve was exposed at the level of the mid-thigh just proximal to its trifurcation. Three ligatures were loosely tied around the nerve at 1 mm distance using 7-0 Prolene suture material (Ethicon, Germany). MP sodium succinate (Upjohn Company, Kalamazoo, MI) was diluted in normal saline (NS) for infusions. At the time of CCI, all mice were implanted with infusion pumps (ALZET 1007D; Alza, Palo Alto, CA). NEP ko and wt mice received either NS or MP (3 mg/kg body weight/day) for 7 days. An additional group of NEP ko and wt mice received MP without CCI (n = 5 to 6 for all groups).

To investigate the role of the endothelin receptors, we applied the ET_BR agonist IRL-1620 and the ET_BR antagonist BQ-788 and assessed pain behavior. Twenty NEP ko mice each received CCI of the right sciatic nerve. Withdrawal thresholds to heat and withdrawal latencies to mechanical stimulation were recorded before CCI and at day 7 after surgery. At day 7, 5 mice received an intraplantar injection of 50 pmol of the ET_BR agonist IRL-1620 in a volume of 10 μL (Tocris Bioscience, Wiesbaden-Nordenstadt, Germany), 5 mice received an equal volume of distilled water; another 5 mice were implanted with infusion pumps (ALZET 1007D) with MP (3 mg/kg body weight/day) for 7 days and received an intraplantar injection of 60 nmol of the ET_BR antagonist BQ-788 in a volume of 10 μL (Tocris Bioscience, Wiesbaden-Nordenstadt, Germany) on day 7. Five mice received an equal volume of the solvent (ethanol). Behavioral tests were repeated 30 minutes afterward. Injection dosage and time point for testing was adjusted to published data [34]. Mice were killed in deep isoflurane anesthesia, and skin from the footpads of all mice from the agonist and antagonist experiments was obtained for gene expression analysis (see later).

2.2. Behavioral testing

For all behavioral tests, the investigator was unaware of the animal genotype.

Thermal withdrawal latencies were measured to monitor sensitivity to heat [2]. The mice were tested for paw withdrawal latencies to a thermal stimulus on 3 consecutive days before surgery to habituate animals to the testing device and to obtain baseline values. Mice were then tested at days 3 and 7 after CCI. The animals were put in Plexiglas cages on a glass plate and rested for 30 minutes before the experiment started. A radiant heat source (Plantar Tester, Ugo Basile, Comerio, Italy) was focused on the plantar surface of the hind paw, and the latency from the initiation of the radiant heat until paw withdrawal (paw withdrawal latency) was measured automatically. A maximal cutoff of 15 seconds was used to prevent tissue damage. Each paw was tested 3 times, and the mean withdrawal latency was calculated. The interval between 2 trials on the same paw was at least 5 minutes.

Withdrawal thresholds to mechanical stimuli were assessed with von Frey hairs using the up-and-down method [7] on 3 consecutive days before surgery and on days 3 and 7 after surgery. Mice were placed on a wire mesh in Plexiglas cages. The plantar surface was touched perpendicularly with a von Frey hair until the filament was slightly bent. Testing started with the 0.84 mN von Frey hair. If the mouse responded to the touch within 3 seconds by brisk withdrawal of the respective hind paw, the response was interpreted as positive. In case of a positive response, the next weaker stimulus was applied. In case of a negative response, the next stronger stimulus was used. This procedure was performed until 6 responses were recorded. The 50% threshold was calculated using the formula: 50% threshold (mN) = $(10[Xf + \kappa\delta])/10{,}000 \times 9.8$ indicating the force at which individual mice withdrew the hind paw in 50% of trials (Xf = value [log units] of the final von Frey hair used; κ = tabular value according to Dixon [15]; δ = mean of difference [log units] between stimuli). Thickness of the hind paws was assessed 1 day before and at days 1, 3, 5, and 7 after surgery using a caliper.

2.3. Protein extraction and enzyme-linked immunosorbent assay (ELISA)

Wt and NEP ko mice were killed on day 8 after surgery or after initiation of MP treatment, respectively, with an isoflurane overdose, and sciatic nerves were dissected bilaterally. Tissue was immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

Samples for ELISA studies were homogenized in protease-inhibitor-containing phosphate-buffered saline (PBS with aprotinin, leupeptin, pepstatin; Boehringer Mannheim, Germany, pH 7.4) at 39,000 rpm for 30 seconds using a Miccra D-8 power homogenizer (ART, Müllheim-Hügelheim, Germany). After centrifugation for 10 minutes at +4 °C, the supernatant was removed, aliquoted, and assayed in duplicate by a commercial CGRP (Spi-Bio, Paris, France), SP (Spi-Bio), bradykinin (Immundiagnostic AG, Bensheim, Germany), and ET-1 (Cayman, Ann Arbor, MI) enzyme immunoassay according to the manufacturer's instructions. The assay detection limits were: CGRP: 5 pg/mL; SP 4 pg/mL; bradykinin 173 pg/mL; ET-1 18 pg/mL.

2.4. Gene expression analysis

RNA extraction from sciatic nerve and from skin was performed as described earlier [42,45]. In brief, total RNA from sciatic nerve samples was extracted following the method of Chomczynski with modifications [10]. Frozen nervous tissue was incubated in TRIzol reagent (Invitrogen, Karlsruhe, Germany) and homogenized (Polytron PT 1600E, Kinematica, Luzern, Switzerland). Chloroform was added, and the samples were centrifuged (13,000 rpm, 4 °C, 15 minutes). The upper phase was mixed with glycogen and propanol, and after incubation overnight at −20 °C the samples were