Lymphocyte-mediated regulation of β-endorphin in the myenteric plexus

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**Abstract**

Acute inflammatory cells release in a reflex manner pro- and pronociceptive mediators that inappropriately stimulate sensory afferent neurons in the gut (17). This antinociception is mediated by the gut immune system (12). We have investigated the relationship between T cells and opioid expression in the myenteric plexus. To examine this, we used in vivo and in vitro approaches. In vivo, we examined modulation of opioid peptide and receptor expression in the myenteric plexus. In vitro, we measured opioid peptide and receptor expression in the myenteric plexus. We examined opioid peptide and receptor expression in the myenteric plexus and opioid expression in the myenteric plexus. We found a positive association between T cells and opioid expression; this association was accompanied by an increase in opioid expression in vivo. In vitro, T helper (Th) type 1 (Th1) and type 2 (Th2) cytokines on the release of opioid and opioid expression in the myenteric plexus. However, exogenous β-endorphin did upregulate β-endorphin expression and both cycloheximide and naloxone methiodide inhibited peptide upregulation. Therefore, our results suggest that nonpolarized CD4+ T cells release β-endorphin, which, through an interaction with MOR, stimulates an upregulation of β-endorphin expression in the myenteric plexus. Thus, we propose that the mechanism underlying lymphocyte modulation of visceral pain involves T cell modulation of opioid expression in the myenteric nervous system.

**Materials and Methods**

**Animal housing and handling.** Male BALB/c and BALB/c SCID mice (6–8 wk of age) were purchased from Harlan (Indianapolis, IN). Mice were kept under specific pathogen-free conditions at the McMaster University Central Animal Care Facility (CAF). Upon arrival at our facility, mice were quarantined for 2 wk before the start of experiments. Cages, bedding, and food were autoclaved as per standard procedures in the McMaster University CAF. All experiments were approved by the McMaster University Animal Care Committee and the Canadian Council on Animal Care.

**Lymphocyte isolation and mouse reconstitution.** Splenocytes from male BALB/c mice were isolated in HBSS plus 10% FBS and 1% antibiotic-antimycotic. Purified cells were resuspended in PBS, and each mouse received 15 × 10⁶ cells/200 μl via an intraperitoneal injection. Cells were 95% viable as determined by Trypan blue exclusion.

**CD4+ T cell isolation.** CD4+ T cells were isolated according to the manufacturer’s instructions. Splenocytes (1 × 10⁶ cells/ml) were prepared in PBS plus 2% FBS. EasySep negative selection mouse CD4+ T cell enrichment cocktail with magnetic nanoparticles (Stem Cell Technologies, Vancouver, BC, Canada) was used to isolate CD4+ T cells. The supernatant containing CD4+ T cells was collected, and cells were resuspended in PBS. Cell purity, as determined by flow cytometry using anti-mouse CD4 (LT34) monoclonal antibody (Research Diagnostics, Flanders, NJ), was >90%.

**Immunohistochemistry.** Distal colon segments were obtained for all immunohistochemistry (IHC) measurements. Tissue was fixed in Bouin’s fixative, and paraffin cross sections were obtained for IHC staining. Sections were incubated with rabbit anti-mouse primary antibody (β-endorphin, Research Diagnostics, Flanders, NJ; μ-opioid receptor, Research Diagnostics, Flanders, NJ).
receptor (MOR), enkephalin, and δ-opioid receptor (DOR). Chemicon, Temecula, CA) for 18 h at 4°C following deparaffinization and peroxidase and protein blockade. Envision, a horseradish peroxidase-coupled anti-rabbit secondary reagent (DakoCytomation, Carpinteria, CA), was incubated with sections for 30 min at room temperature. 3,3′-Diaminobenzidine (Sigma-Aldrich, Oakville, ON, Canada) was used for color development, and modified Mayer’s hematoxylin was used to counterstain sections. The controls used included samples with omission of the primary antibody and antibody preabsorption with β-endorphin peptide (Sigma-Aldrich), MOR (3rd extracellular loop) peptide, DOR (NH2 terminal) control peptide (Chemicon), or Met-enkephalin peptide (Bachem, San Carlos, CA).

Peptide staining of the myenteric plexus was measured by immunostaining-based semiquantification. Five positions in a circular fashion of each cross section (using a 4×20 objective) were photographed by a digital camera (Olympus Q-Color). All pictures were taken under the same microscope and camera settings on the same day. Stained areas were measured by a blind observer using ImageJ software (National Institutes of Health, Bethesda, MD), and all images were quantified on the same day. Only the myenteric plexus region of each section was quantified. Results are expressed as the percent expression of naive SCID as five pictures of each colon allowed the total tissue area to be measured.

$CD^+\ T$ cell cytokine stimulation in vitro. Falcon tissue culture plates (Becton Dickinson) were precoated with purified anti-mouse CD3 antibody (10 μg/ml, BD Pharmingen) for 3 h at 37°C. $CD^+\ T$ cells were plated (1 × 10^6 cells/ml) in RPMI plus 10% FBS plus 1% antibiotic-antimycotic plus protease inhibitor cocktail (containing aprotinin, bestatin, E-64, leupeptin, and pepstatin, Sigma Aldrich) for 48 h. IL-12 (5 μg/ml) plus anti-IL-4 antibody (10 μg/ml, Sigma-Aldrich) were added to the cells for Th1-type cytokine stimulation. IL-4 (10 ng/ml) plus anti-IL-12 antibody (10 μg/ml, Sigma-Aldrich) were added to the cells for Th2-type cytokine stimulation. Unstimulated $CD^+\ T$ cells did not receive cytokine stimulation but were treated with anti-CD3. As β-endorphin could be measured from our cell culture medium, we used RPMI plus 10% FBS as our in vitro control.

Measurement of β-endorphin. Fresh supernatant was collected and assayed for β-endorphin using an enzyme immunoassay (Bachem). The minimum detectable concentration of the assay was 2–3 pg/ml.

Th1 and Th2 polarization in vivo. A single administration of recombinant adenovirus (Ad) vector expressing IL-12 (Ad5IL-12) was used for Th1 polarization. The construction and characterization of Ad5IL-12 have previously been described (4). Briefly, the vector contained an expression cassette for the p35 subunit of IL-12 in the E1 region (2). Each mouse was injected intraperitoneally with 5 × 10^8 plaque-forming units of Ad5IL-12.

Trichinella spiralis infection was used for Th2 polarization (11, 12). The T. spiralis parasites originated in the Department of Zoology at the University of Toronto. The colony was maintained through serial infections alternating between Sprague-Dawley rats and male CD1 mice. The larvae were obtained from infected rodents 60–90 days postinfection using a modification (23) of the techniques described by Castro and Fairbairn (6). Mice were infected with 400 T. spiralis larvae by oral gavage.

All mice were killed on the same day, which was day 7 after Ad5IL-12 administration and day 9 after T. spiralis infection as these time points have been described as optimal for Th1 and Th2 cell polarization (10, 14), respectively. Age-matched uninfected mice were used to obtain control, undifferentiated (Th0) $CD^+\ T$ cells. Isolated $CD^+\ T$ cells were plated and stimulated with anti-CD3 antibody (10 μg/ml) as described above for 72 h. The supernatant was collected at 24, 48, and 72 h and frozen at −70°C. IFN-γ and IL-4 concentrations in culture supernatants were measured by enzyme immunoassay techniques using commercially available kits (R&D Systems, Minneapolis, MN) at each time point. The highest ratio of IFN-γ to IL-4 was measured at 48 h, and the highest ratio of IL-4 to IFN-γ was measured at 72 h of incubation. Supernatants from these time points were then incubated with SCID LMMP tissue.

LMMP preparation and culture. Colonic tissue from BALB/c SCID mice was mounted onto a glass rod, and the mesentry was removed. The LMMP layer was carefully scraped off with a sterile cotton swab and rinsed in PBS. Tissues were placed in tissue culture plates in RPMI medium containing 10% FBS plus 1% antibiotic-antimycotic. LMMP tissues were incubated in a total volume of 1 ml consisting of either Th1 or Th2 polarized supernatant (0.5 μl supernatant + 0.5 μl medium), β-endorphin (Camel) peptide (Bachem), NLXM (Sigma-Aldrich), or cycloheximide (Sigma-Aldrich) dissolved in medium. After an 18-h incubation at 37°C, LMMP tissues were rinsed in RPMI, fixed in Bouin’s fixative, and prepared for β-endorphin IHC.

Peptide staining of the myenteric plexus was measured using the immunostaining-based semiquantification described above. Approximately 8–10 pictures of each tissue were obtained. For each picture, areas of positive staining were divided by the total tissue area to account for variability in tissue size. The average percent positive staining was then calculated per tissue sample and expressed as a percentage of the total area.

Data presentation and statistical analysis. All data are expressed means ± SD. Data were analyzed using two sample t-tests (two-tailed) between unpaired groups with $P ≤ 0.05$ considered as significant. For multiple comparisons, one-way ANOVA followed by Dunnett’s test was used to compare treatment groups with the control, and comparisons among the groups were made using Tukey’s test.

RESULTS

Our previous work demonstrated that attenuation of visceral hyperalgesia in SCID mice following reconstitution with immune cells was maximal at 12 wk postreconstitution and declined thereafter; the effect was absent by 18 wk postreconstitution, and this correlated with the absence of CD4+ T cells (22). Therefore, in this study, we compared β-endorphin expression in the myenteric plexus of the colon between naive SCID and immunocompetent Balb/c mice. We chose two time points postreconstitution (12 and 18 wk), which reflected either the presence (12 wk) or absence (18 wk) of immune cells in reconstituted SCID mice. At 12 wk postreconstitution of SCID mice, we found that β-endorphin expression in the myenteric plexus was 200% higher than that in naive SCID mice and was comparable with Balb/c mice (Fig. 1). However, at 18 wk postreconstitution, β-endorphin expression was significantly decreased compared with 12 wk postreconstitution and was ~60% of the expression of naive SCID mice.

The expression of MOR was also quantified in the myenteric plexus of colon sections. We found that when the expression of β-endorphin was increased compared with naive SCID mice at 12 wk postreconstitution, MOR expression was significantly decreased (Fig. 1). However, downregulation of MOR was no longer apparent by 18 wk postreconstitution as the expression was not significantly different from MOR expression in naive SCID mice.

We next investigated if these changes postreconstitution were applicable to other opioids in the gut. We examined the peptide enkephalin, which is a principal opioid in the gut, as well as its high-affinity receptor, DOR. We found no significant differences in the neural expression of the peptide or receptor between Balb/c and SCID mice or in SCID mice postreconstitution (Fig. 2).

Previously, we showed that lymphocytes isolated from reconstituted SCID mice with normal pain thresholds released...
increased amounts of β-endorphin. In this study, we examined whether Th1 or Th2 cytokine stimulation of lymphocytes influenced β-endorphin secretion. As shown in Fig. 3, there was a significant secretion of β-endorphin by unstimulated CD4+ T cells. However, stimulation of these cells with either IL-12 or IL-4 failed to increase β-endorphin secretion; the concentration of β-endorphin by cytokine-stimulated cells was similar to that measured in cell culture medium alone and significantly lower compared with unstimulated CD4+ T cells.

We next examined whether the Th polarity of T cells influenced the expression of β-endorphin in the LMMP. Th1 cells were isolated from mice that received a single injection of Ad5IL-12; an evaluation of cytokine production by T cells was highest at 48 h of incubation in vitro (data not shown). The supernatant from these cells was then incubated with LMMP preparations from naive SCID mice. As shown in Fig. 4, whereas lymphocytes from untreated mice induced a significant increase in β-endorphin expression, cells from Ad5IL-12-treated mice did not alter the expression of this peptide in the LMMP. A similar strategy was used for Th2-polarized lymphocytes taken from nematode-infected mice. The ratio of IL-4 to IFN-γ secretion was highest at 72 h of incubation in vitro (data not shown), but, as shown in Fig. 4, supernatant from these cells also failed to induce increased β-endorphin expression in the LMMP of SCID mice. Thus, both β-endorphin production and the induction of β-endorphin expression in the LMMP by lymphocytes are properties of nonpolarized T cells. These observations prompted an examination of β-endorphin as a lymphocyte-derived mediator involved in the regulation of endorphin expression in the myenteric plexus.

To directly examine β-endorphin as a mediator involved in neural β-endorphin upregulation, we incubated SCID LMMP tissue with increasing concentrations of exogenous β-endorphin peptide. We found that exogenous β-endorphin at...
centrations of $10^{-6}$ and $10^{-10}$ M, but not $10^{-8}$ M, significantly increased endorphin expression in the SCID LMMP compared with the LMMP incubated in medium alone (Fig. 5). The lack of statistical significance seen with $10^{-8}$ M β-endorphin is likely due to the smaller sample size of this group ($n = 3$ compared with $n = 5–6$ for $10^{-6}$ and $10^{-10}$ M). To determine whether the increased expression of β-endorphin reflected new synthesis, we coincubated tissues with $10^{-6}$ M β-endorphin and cycloheximide, a protein synthesis inhibitor. As shown in Fig. 5, LMMP tissue incubated with endorphin and cycloheximide showed significantly less expression of β-endorphin than tissue incubated with β-endorphin alone and was not different from the control. Cycloheximide alone had no significant effect (data not shown).

We next examined whether the upregulation of β-endorphin in the LMMP is mediated by MOR. Tissue was preincubated with NLXM before being exposed to β-endorphin. As shown in Fig. 6, NLXM did not alter the constitutive expression of β-endorphin but significantly prevented the induction of this peptide in the tissue by exogenous β-endorphin.

**DISCUSSION**

In this study, we examined the relationship between mucosal lymphocytes and opioid regulation in the myenteric plexus. We demonstrated a positive association between mucosal lymphocytes and β-endorphin expression in the ENS. In vitro, we determined that nonpolarized T cells secreted increased β-endorphin and that exogenous β-endorphin upregulated the expression of this peptide in the myenteric plexus through an interaction with MOR. Taken together, these results support the notion that β-endorphin, derived from CD4$^+$ T cells, is a mediator involved in the upregulation of β-endorphin in the myenteric plexus.

Previously, we showed that T cell reconstitution increased the visceral pain threshold in SCID mice to threshold values seen in immunocompetent wild-type mice. Both the presence and antinociceptive effect of T cells was marked at 12 wk postreconstitution and declined by 18 wk postreconstitution in SCID mice (22). The increase in β-endorphin expression in the myenteric plexus in reconstituted SCID mice followed a sim-ilar temporal pattern, further supporting the concept that the upregulated expression of this peptide in the ENS is a potential mechanism underlying the normalization of visceral perception in reconstituted SCID mice. It should also be noted that β-endorphin was expressed in the myenteric plexus of naive SCID mice, thus indicating a role for T cell-independent factors in the expression of this peptide in the ENS.

While several studies have implicated β-endorphin as the major opioid involved in pain regulation, other peptides can also contribute. For example, it has been shown that deep brain stimulation in patients with chronic pain stimulates the release of both β-endorphin and enkephalin into the cerebrospinal fluid (25). Our results showed that enkephalin is not subject to regulation by T cells as neither the peptide nor its receptor was different in wild-type, SCID, and reconstituted SCID mice. These data indicate that under our experimental conditions, opiate involvement in immune-mediated visceral antinociception is restricted to β-endorphin.

Our results indicated that polarization of T cells into Th1 or Th2 phenotypes does not enhance the production of β-endorphin. Neither stimulation with the Thh-polarizing cytokines IL-12 (Th1) nor IL-4 (Th2) nor the in vivo polarization of T cells resulted in increased β-endorphin release by T cells or β-endorphin expression in the myenteric plexus. Indeed, β-endorphin release was significantly lower in media from cytokine-stimulated cells compared with unstimulated cells. Interestingly, it has been shown that activation of CD4$^+$ T cells results in the degradation of β-endorphin via a peptidase secreted by T cells (17). This could account for the lower levels of β-endorphin seen in media from cytokine-stimulated cells and the inability of Th cells polarized in vivo to alter β-endorphin expression.

We propose that the mechanism underlying the lymphocyte modulation of visceral pain involves the release of β-endorphin from lymphocytes and that this induces an upregulation of the peptide in the myenteric plexus. This is supported by the following findings: 1) our previous demonstration showing that the attenuation of visceral hyperalgesia in reconstituted SCID mice is naloxone sensitive (22); 2) the demonstration of β-endorphin release by lymphocytes; and 3) the ability of exoge-
nous β-endorphin to upregulate its expression in the myenteric plexus in a protein synthesis-dependent manner. We do not know whether the induction of endogenous β-endorphin expression in the myenteric plexus is induced locally by lymphocytes present in the plexus or via a systemic effect. In that regard, it is important to point out that the lowest stimulatory concentration of β-endorphin in the present study was $10^{-10}$ M, which is physiologically relevant (20, 21).

β-Endorphin expression was mediated by MOR as it could be inhibited by NLXM. A study (3) has shown that β-endorphin induces a downregulation of MOR, and that is consistent with our finding of a reduction in MOR expression in the myenteric plexus in SCID mice in which visceral pain thresholds were normalized 12 wk postreconstitution. Interestingly, as the antinociceptive effect declined at 18 wk postreconstitution, β-endorphin expression also declined, and there was a corresponding upregulation of MOR (22). A similar relationship between β-endorphin and MOR expression has been described in the central nervous system (18, 26). This may represent a mechanism by which the persistence of β-endorphin-producing lymphocytes in the gut is not accompanied by prolonged suppression of the sensory function, which is an important component of host defense.

Our results provide the first demonstration of how immune cells regulate β-endorphin expression in the ENS. Positive regulation of opioid expression by T cell-derived opioids has significant implications for antinociception in the gut. It extends our understanding of how chronic inflammation dominated by T cells may be associated with normal pain thresholds or hypoalgesia, as seen in some subsets of inflammatory bowel disease patients (1, 7, 8, 15). Furthermore, increased CD4$^+$ T cell depletion from the gut during human immunodeficiency virus (HIV) infection (5, 16) may explain the high incidence of idiopathic abdominal pain seen in HIV/acquired immunodeficiency syndrome patients (13, 19, 24). Thus, we suggest that the neuroimmune cross-talk between mucosal T cells and the ENS via β-endorphin is involved in the homeostatic regulation of visceral pain perception.

GRANTS

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