Subcellular Pathways of β-Endorphin Synthesis, Processing, and Release from Immunocytes in Inflammatory Pain

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The opioid peptide β-endorphin (END) as well as mRNA for its precursor proopiomelanocortin (POMC) are found not only in the pituitary gland, but also within various types of immune cells infiltrating inflamed se tissue. During stressful stimuli END is released and interacts with peripheral opioid receptors to inhibit pain. However, the subcellular pathways of POMC processing and END release have not yet been delineated in inflammatory cells. The aim of the present study was to examine the presence of POMC, carboxypeptidase E, the prohormone convertases 1 (PC1), and 2 (PC2), PC2-binding protein 7B2, and the release of END from inflammatory cells in rats. Using immunohistochemistry we detected END and POMC alone or colocalized with PC1, PC2, carboxypeptidase E, and 7B2 in macrophages/monocytes, granulocytes, and lymphocytes of the blood and within inflamed sc paw tissue. Immunoelectron microscopy revealed that END is localized within secretory granules packed in membranous structures in macrophages, monocytes, granulocytes, and lymphocytes. Finally, END is released by noradrenaline from immune cells in vitro. Taken together, our results indicate that immune cells express the entire machinery required for POMC processing into functionally active peptides such as END and are able to release these peptides from secretory granules. (Endocrinology 145: 1331–1341, 2004)

SUBJECTS AND METHODS

Subjects

Experiments were performed in male Wistar rats (200–250 g; bred at the Charité-Universitätsmedizin Berlin, Berlin, Germany) in accordance with standard ethical guidelines and approved by the local authorities (Landesamt für Arbeitsschutz, Gesundheit und Technische Sicherheit, Landesamt für Arbeitsschutz, Gesundheit und Technische Sicherheit, ...)
Berlin, Germany). Rats were housed individually and maintained on a 12-h light, 12-h dark schedule, with food pellets and water ad libitum. Room temperature (22 ± 0.5 °C) and relative humidity (60–65%) were maintained constant.

Reagents

The following polyclonal rabbit antiserum antibodies were used: anti-END with no cross-reactivity against ACTH (supplier’s information; Peninsula Laboratories, Belmont, CA, and ProGen Biotechnik GmbH, Heidelberg, Germany), anti-DP4 against a common epitope of ACTH and POMC (provided by Dr. Y. Peng Loh, NIH, Bethesda, MD), anti-POMC antiserum directed against the N-terminal amino acid sequence 27–52 (Phoenix Pharmaceuticals, Inc., Karlsruhe, Germany), anti-PC1/3 and anti-PC2 (provided by Drs. N. G. Seidah, Clinical Research Institute of Montreal, Quebec, Canada; D. F. Steiner, University of Chicago, IL; and N. Birch, University of Auckland, Auckland, New Zealand), anti-CPE against the C or N terminal, respectively (provided by Drs. Y. Peng Loh and L. Fricker, Albert Einstein College of Medicine, Bronx, NY), and anti-7B2 (provided by Drs. N. G. Seidah, Clinical Research Institute of Montreal; P. Collini, Department of Pathology, University of Milan, Milan, Italy). Further materials included the Vectastain Elite Kit (Vector Laboratories, Inc., Burlingame, CA), paraformaldehyde and glutaraldehyde (Sigma-Aldrich Corp., St. Louis, MO), Freund’s complete adjuvant (FCA) (Calbiochem, La Jolla, CA), and halothane (Halocarbon Laboratories, Willy Rüsch GmbH, Böblingen, Germany).

Induction of inflammation

Male Wistar rats were sedated by brief halothane anesthesia and received an intraplantar injection of 0.15 ml FCA into the right hind paw. This treatment produces a localized inflammation of the inoculated paw characterized by increased susceptibility to painful stimuli (hyperalgesia) and increases in paw volume, paw temperature, and infiltration of various types of immune cells (3, 4, 19).

Western blot analysis of POMC, CPE, PC1, PC2, 7B2, and END in circulating leukocytes

The isolation of circulating leukocytes was accomplished from venous blood of animals without (control) and with paw inflammation (4 d after FCA treatment) using the dextran sedimentation method. Briefly, rats (n = 6/experiment) were anesthetized with halothane; blood samples (8 ml) were obtained by direct parasternal cardiac puncture and kept in heparin-coated tubes. Washing and centrifugation of the blood leukocytes was performed with a 12% polyacrylamide gel for detection of both groups was performed within the same blot.

Immunohistochemistry

Four days after FCA inoculation, rats were deeply anesthetized with halothane and perfused transcardially with 100 ml 0.1 M PBS (pH 7.4) and 300 ml cold PBS containing 4% paraformaldehyde and 0.2% picric acid (pH 7.4, fixative solution) for light microscopic immunohistochemistry and with 4% paraformaldehyde/0.1% glutaraldehyde/0.2% picric acid solution (pH 7.4) for electron microscopy, respectively. The skin with adjacent sc tissue was removed, postfixed for 90 min at 4°C in the fixative solution, and cryoprotected overnight at 4°C in PBS containing 10% sucrose. The tissues were then embedded in Tissue-Tek compound (OCT, Miles, Inc., Elkhart, IN) and frozen. Consecutive sections (6 µm thick) were cut from cryostat-mounted sections, and the Image-Pro Analysis package (Media Cybernetics, Analytical Imaging Solutions Group, Gießen, Germany) was used to quantify changes in immunodensities. The upper and lower threshold density ranges were adjusted to encompass and match the immunoreactivity (ECL reaction product) to provide an image with immunoreactive material appearing in color (black) pixels and nonimmunoreactive material appearing in white pixels. A standardized box was positioned over each band. In the case of PC1, the measurement was performed on the 66-kDa band. The area and density of pixels within the threshold values representing immunoreactivity were measured, and the integrated density (the product of the area and density) was calculated. Integrated densities of controls and treated groups were compared and statistically analyzed. A percent change (treated/control) was then calculated to demonstrate differences between the two groups. As Western blot ECL reactions can vary despite a standard protocol, comparison of both groups was performed within the same blot.

Single staining procedures

Immunohistochemical staining of the serial sections and circulating leukocytes was performed with a Vectastain avidin-biotin peroxidase complex (ABC; Vectastain Elite Kit, Vector Laboratories, Inc.), as described previously (22). Unless otherwise stated, all incubations were performed at room temperature, and PBS was used for washing (three times for 10 min each) after each step. The sections and circulating leukocytes were incubated in 1% H2O2/PBS, 0.3% H2O2 and 10% methanol for 45 min to block endogenous peroxidase. To prevent nonspecific binding, the sections were incubated for 60 min in PBS containing 0.3% Triton X-100, 1% BSA, 4% goat serum, and 4% horse serum (block solution). The sections were then incubated overnight at 4°C with rabbit polyclonal antibodies against END, PC1/3, PC2, CPE, POMC, or 7B2 (1:1000 dilution). Thereafter, the sections were incubated for 1 h with a goat antirabbit biotinylated secondary antibody (Vector Laboratories, Inc.)
and then with ABC for 45 min. Finally, the sections were washed and stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) containing 0.01% H₂O₂, in 0.05 M Tris-buffered saline (pH 7.6) for 3–5 min. After the enzymatic reaction, the sections and circulating leukocytes were washed in tap water, counterstained with thionin, then dehydrated in alcohol, cleared in xylene, and mounted in DPX (Merck & Co., Darmstadt, Germany).

Immunoreactive cells were identified by the following morphological criteria: 1) macrophages/monocytes in inflamed sc tissue by large cell bodies, vacuolated cytoplasm, and irregular-shaped nuclei; 2) monocytes in circulation by large cell bodies and kidney-shaped nuclei; 3) lymphocytes by small cell bodies, large nuclei, and small amounts of cytoplasm; and 4) polymorphonuclear leukocytes by large cell bodies and multisegmented nuclei.

**Double staining procedures**

Immunohistochemical double staining of tissue sections and circulating leukocytes was performed as described previously (23–25). Briefly, sections and circulating leukocytes stained in the first sequence with antibody against END, POMC, or PC2 (as described above) were treated with 0.3% H₂O₂ for 30 min to inactivate peroxidase in ABC, washed in several changes of PBS, incubated with blocking solution, and then processed as follows in a second sequence: 1) sections and circulating leukocytes stained for END were incubated with a second primary antibody against POMC or PC2; 2) sections and circulating leukocytes stained for POMC were incubated with a second primary antibody against CPE, PC1, or PC2; and 3) sections and circulating leukocytes stained for PC2 were incubated with a second primary antibody against 7B2. All incubations were performed overnight at 4°C, and slides were then washed in PBS and exposed to the biotinylated secondary antibody (Vector Laboratories) for 1 h and to ABC for 45 min. Finally, the sections were washed and stained using a HistoGreen Peroxidase-Substrate Kit (Linaris, Wertheim-Bettingen, Germany). The chromogen DAB used for the first primary antiserum appeared brown, whereas Histogreen used for the second primary antiserum appeared green. After the enzymatic reaction, the sections and circulating leukocytes were washed in distilled water, dehydrated in alcohol, cleared in xylene, and mounted in DPX.

**Specificity controls**

To demonstrate specificity of staining, the following controls were included. 1) Preabsorption of diluted antibody against END, POMC, CPE, PC1, PC2, or 7B2 with 5 µg/ml purified END (Peninsula Laboratories, Belmont, CA). POMC (Phoenix Pharmaceuticals), CPE (provided by Dr. Y. Peng Loh), recombinant PC1 or PC2 antigens (provided by Drs. N. G. Seidah and D. F. Steiner, respectively), or 7B2 (provided by Dr. N. G. Seidah), respectively, was performed for 24 h at 4°C. The control incubation of diluted POMC, CPE, PC1, PC2, and 7B2 antisera with END did not block their immunoreactivity, indicating that these antibodies have no cross-reactivity with END. Additionally, preincubation of POMC and PC2 antisera with END had no effect on POMC or PC2 immunoreactivity in either circulating immune cells or inflamed sc paw tissue. 2) Omission of the primary antiserum, the secondary antibodies, or ABC did not show any staining. 3) Either the first or second secondary antibody and either the first or second secondary antibody were omitted. 4) The primary antiserum was reversed between the first and second sequences of the immunostaining procedure. 5) The percentages of immunoreactive cells in the single immunostaining experiments were compared with those in the second sequence of double immunostaining.

**Quantification of immunostaining**

In sc paw tissue and circulating leukocytes, immunoreactive cells were counted by a blinded experimenter in three tissue sections or three cytospin slides per animal. Five squares (38.4 mm² each) per section were analyzed using a Zeiss microscope (objective, ×40 ×10; Carl Zeiss, Oberkochen, Germany). The percentage of immunostained cells was determined by the formula: immunostained cells/total number of immune cells × 100. In double labeling experiments, the percentage of POMC- or PC2-positive cells expressing END was calculated as the number of POMC- or PC2-immunoreactive cells double-stained against END, divided by the number of all POMC- or PC2-immunoreactive cells, respectively. The percentage of CPE- or PC1-positive cells expressing PC2 and the percentage of 7B2-positive cells expressing PC2 were calculated analogously. Five rats per group were used for analysis. Values are the mean ± SEM.

**Immunoelectron microscopy**

Free-floating sc paw sections (40 µm) were incubated with antibody against END. The immunostaining was performed in the same way as for light microscopy. The immunoreaction was then visualized by incubation with nickel chloride-enhanced DAB (DAB containing 0.01% H₂O₂ and 0.08% nickel chloride in 0.05 M Tris-buffered saline, pH 7.6) for 3–5 min (26). The sections were postfixed in 1% tannic acid (in 0.1 m phosphate buffer) and 1% osmium tetroxide solution (in 0.1 m PBS), dehydrated in ethanol, and embedded in Epon. Semithin and ultrathin sections were cut on a Reichert Ultracut (Leica, Nussloch, Germany), followed by contrast staining with toluidine blue. The ultrathin sections were examined under a transmission electron microscope (TEM 10, Zeiss).

**Tissue preparation and release experiments**

Four days after FCA treatment, rats were euthanized with halothane, and popliteal lymph nodes were collected from inflamed hind limbs (n = 20 rats). Lymph nodes were minced, homogenized, and filtered through a 70-µm pore size sieve (BD Biosciences, Franklin Lakes, NJ). Cells were washed in Hanks’ balanced salt solution (HBSS; Sigma-Aldrich) and centrifuged at 300 × g for 10 min at 20°C using a swinging bucket rotor of a Heraeus centrifuge (Heraeus-Christ GmbH, Osterode, Germany). After three washing procedures, cells were reconstituted in HBSS containing 5 µg/ml bestatin (Sigma-Aldrich) and 40 µg/ml aprotinin (Sigma-Aldrich), aiming at a concentration of 1.3 × 10⁶ cells/ml. Cell viability determined by the trypan blue exclusion method was greater than 97%. Cell suspension (300 µl) was preincubated with either HBSS or with a combination of phenolamine (50–250 ng; Sigma-Aldrich) and...
propranolol (50–250 ng; Sigma) at 37°C in a shaking water bath. After 5 min, either HBSS or noradrenaline (100 ng; Sigma) was added. Five minutes later, the suspension (total volume, 400 μl) was centrifuged at 300 × g at 4°C for 10 min. Three-hundred-microliter aliquots of the supernatants and the cell pellets were stored at −20°C until further processing.

**RIA**

Cell pellets were thawed and reconstituted in 0.3 ml RIA buffer (0.1 M sodium phosphate, 0.05 M NaCl, 0.01% NaN3, 0.1% BSA, 0.1% Triton X-100, 5 μg/ml bestatin, and 40 μg/ml aprotinin) using an RIA kit (Peninsula Laboratories). Afterward, cell pellets were lysed by a freezing/thawing procedure and sonicated for 5 min using an ultrasonic water bath (RK 52H, Sonorex, Hamburg, Germany). Lysates were centrifuged for 10 min at 14,000 × g at 4°C. Using the RIA kit (Peninsula Laboratories), the END content was determined in the supernatants of the centrifuged lysates and in the thawed supernatants of the release experiments supplemented with serum albumin (0.1%; Sigma-Aldrich) and Triton X-100 (0.1%; Sigma-Aldrich), respectively. The content of END in the samples was calculated by extrapolation from the standard curve.

**Statistical analysis**

Data are represented as the mean ± SEM and were analyzed by t test for parametric data and by Mann-Whitney U test for nonparametric data. Differences were considered significant at P < 0.05. All tests were performed using SigmaStat 2.03 statistical software (Jandel Corp., San Ramon, CA).

**Results**

**Western blot analysis of POMC, CPE, PC1, PC2, 7B2, and END in circulating leukocytes**

Circulating leukocytes isolated from control and FCA-treated animals displayed CPE-, POMC-, and END-immunoreactive bands with apparent molecular masses of approximately 53, 31, and 3.5 kDa, respectively (Fig. 1). The anti-PC1 antibody detected a double band of 66 and 60 kDa. The anti-PC2 and anti-7B2 antibodies recognized only 65- and 23-kDa bands for PC2 and 7B2, respectively (Fig. 1). There were no differences in CPE and 7B2, but there was a significant increase in the density of POMC- (36.7 ± 3.1%), PC1- (63.3 ± 5.9%), PC2- (38.5 ± 4.7%), and END- (29.9 ± 3.9%)-immunoreactive bands of leukocytes from treated vs. untreated rats (P < 0.05; Fig. 1).

**Single immunolabeling in circulating leukocytes**

In control animals, circulating leukocytes were immunoreactive for END (37.0 ± 3.5%), POMC (40.0 ± 2.0%), CPE (47.6 ± 4.9%), PC1 (33.8 ± 3.6%), PC2 (37.6 ± 2.7%), and 7B2 (49.6 ± 3.8%; Fig. 2, A–F). In FCA-treated animals, a higher proportion of leukocytes was immunoreactive for END (51.0 ± 4.6%), POMC (55.3 ± 2.6%), CPE (50.3 ± 6.8%), PC1 (49.7 ± 2.2%), PC2 (46.9 ± 2.7%), and 7B2 (52.6 ± 3.7%; Fig. 2, G–K).
These differences were significant for POMC, PC1, PC2, and END (P < 0.05), but not for CPE or 7B2. The immunoreactivity could be detected in cells morphologically identified as macrophages/monocytes, polymorphonuclear leukocytes (granulocytes), and lymphocytes from FCA-treated animals (Fig. 3, A–F) and as macrophages/monocytes and granulocytes from nontreated animals (Fig. 2, A–F). Preabsorption of all antibodies with 5 μg/ml of their respective antigen completely abolished immunostaining (data not shown).

**Single immunolabeling in sc paw tissue**

Staining of serial sections from inflamed sc paw tissue using END, POMC, CPE, PC1, PC2, or 7B2 antibody revealed positive staining in various immune cells (Fig. 4). These cells occurred in the inflammatory foci within the plantar sc tissue and had morphological appearances consistent with macrophages/monocytes, granulocytes, and lymphocytes (Fig. 4, A, D, F, H, J, and L). END−, POMC−, CPE−, PC1−, PC2−, and 7B2−immunoreactive immune cells were undetectable in noninflamed sc paw tissue (Fig. 2L). When immunostained serial sections for END, POMC, CPE, PC1, PC2, or 7B2 were compared and matched, an almost identical distribution pattern around the inflammatory foci was observed. Preabsorption of all antibodies with 5 μg/ml of their respective antigen completely abolished immunostaining (data not shown).

**Double immunohistochemistry in circulating leukocytes**

Circulating leukocytes of control animals showed colocalization of END with POMC (75.2 ± 6.3%) and PC2 (90.4 ± 4.4%; Fig. 2, G and H). Also, these cells showed a colocalization of POMC with CPE (86.4 ± 3.9%), PC1 (94.8 ± 3.8%), and PC2 (83.6 ± 7.3%; Fig. 2, I–K). Circulating leukocytes of FCA-treated animals revealed colocalization of END with both POMC (77.0 ± 6.9%) and PC2 (93.1 ± 4.3%; Fig. 3, G and H). POMC was colocalized in CPE− (89.8 ± 4.3%), PC1− (96.7 ± 3.3%), and PC2− (86.5 ± 6.3%) immunoreactive immune cells. Few cells were immunolabeled for CPE (10.1 ± 4.3%), PC1 (3.6 ± 0.5%), or PC2 (13.5 ± 6.3%) alone (Fig. 3, I–K). Almost all cells positive for CPE, PC1, or PC2 were also immunoreactive for POMC. PC2 was colocalized in 7B2− (78.3 ± 2.6%)-immunoreactive immune cells. Some cells (21.7 ± 2.7%) showed immunoreactivity of 7B2 alone (Fig. 3L). There was no statistical difference (P > 0.05) in these percentages between control and FCA-treated animals.

Omission of either the first or second primary antibody and omission of either the first or second secondary antibody did not produce the first or second (double) color, respectively. Reversing the primary antisera between the first and second sequences of the immunostaining procedure yielded the same results. There was no significant difference in the percentages of POMC− (55.3 ± 2.6), CPE− (50.3 ± 6.8), PC1− (49.7 ± 2.2), PC2− (46.9 ± 2.7), and 7B2− (52.6 ± 3.7)-immunoreactive immune cells (data not shown).
noreactive circulating leukocytes obtained by single immunostaining compared with those of POMC- (56.2 ± 3.2), CPE- (49.2 ± 4.3), PC1- (46.8 ± 2.4), PC2- (42.7 ± 2.9), and 7B2- (54.9 ± 3.8)-immunoreactive circulating leukocytes obtained by the second sequence of double immunostaining (P < 0.05 for each comparison).

**Double immunohistochemistry in inflamed sc paw tissue**

POMC was colocalized in CPE- (88.6 ± 1.0%), PC1- (94.4 ± 0.5%), and PC2- (97.0 ± 0.4%)-immunoreactive immune cells. Few cells were immunolabeled for CPE (11.4 ± 1.0%), PC1 (3.6 ± 0.5%), or PC2 (3.0 ± 0.4%) alone (Fig. 5). Almost all cells positive for CPE, PC1, or PC2 were also immunoreactive for POMC (Fig. 5). END was colocalized in POMC- (91.0 ± 1.2%) and PC2 (98.4 ± 0.5%)-immunoreactive immune cells. Few cells were labeled for POMC (7.0 ± 1.5%) or PC2 (1.6 ± 1.1%) alone (Fig. 6). Almost all cells immunoreactive for PC2 were also immunostained for 7B2 (90.2 ± 1.6%). Some cells showed immunoreactivity of 7B2 (9.8 ± 1.6%) alone (Fig. 6, G–I).

Omission of either the first or second primary antibody and omission of either the first or second secondary antibody did not produce the first or second (double) color, respectively. Reversing the primary antisera between the first and second sequences of the immunostaining procedure yielded the same results. There was no significant difference in the percentages of POMC- (73.2 ± 2.5), CPE- (73.0 ± 4.6), PC1- (65.44 ± 4.8), PC2- (68.7 ± 4.7), and 7B2 (68.5 ± 5.8)-immunoreactive inflammatory cells obtained by single immunostaining compared with those of POMC- (75.1 ± 3.0), CPE- (70.0 ± 4.5), PC1- (69.8 ± 5.9), PC2- (70.0 ± 5.2), and 7B2 (65.7 ± 5.5)-immunoreactive inflammatory cells obtained by the second sequence of double immunostaining (P > 0.05 for each comparison).

**Immunoelectron microscopy of END in inflamed sc tissue**

END-immunoreactive cells were morphologically identified as macrophages, monocytes, granulocytes, and lymphocytes (Fig. 7). These cells showed a highly developed rough ER and an extensive Golgi apparatus (Fig. 8, A and B). Immunoreactive cells contained numerous secretory granules packed in small or
large membranous structures. Immunostaining was confined to secretory granules, which were grouped in small or large membranous vesicular structures (Fig. 8, E and F). The smaller membranous structures containing END-immunoreactive granules were found within the deep cytoplasm, and the larger ones were arranged at the cell periphery and in extended processes (Fig. 8, E and F). Some secretory granules located at the trans-side of Golgi stacks showed immunostaining (Fig. 8B). Some secretory granules were packed in membranous compartments extending from Golgi cysternae (Fig. 8, C and D). No immunoreactivity was found in association with the plasma membrane or the nucleus. Preabsorption of antibody against END with 5 µg/ml purified END completely abolished immunostaining (Fig. 8A).
Noradrenaline-induced END release

In immune cell suspensions obtained from popliteal lymph nodes of inflamed hind paws, noradrenaline (100 ng) induced a significant release of immunoreactive END into the supernatant \( (P < 0.05) \). END release resulted in a concomitant reduction of END cell content \( (P < 0.05) \). Noradrenaline-induced release was reversed by two different doses of the combined administration of the adrenergic antagonists phentolamine plus propranolol \( (P < 0.05) \), indicating the specificity of the release (Table 1).

Discussion

Our study demonstrates the essential components of the regulated secretory pathway for POMC and END in a model of painful inflammation by 1) the identification of POMC, CPE, PC1, PC1, 7B2, and END proteins in circulating leukocytes by Western blotting; 2) the expression of POMC and END alone or colocalized with the proteolytic enzymes CPE, PC1, PC2, and 7B2 in circulating leukocytes as well as in resident inflammatory cells by immunohistochemistry; 3) the ultrastructural identification of END in secretory granules in macrophages, monocytes, granulocytes, and lymphocytes within inflamed sc tissue; and 4) the induction of END release from immune cells by noradrenaline in vitro.

The POMC gene has three exons and two introns. Exon 2 encodes a signal peptide essential for classical protein processing and secretion (27), and exon 3 encodes various bioactive peptides, such as ACTH, βLPH, MSH, and END. There have been numerous reports of POMC mRNA in immune cells, but some studies report a lack of full-length transcripts \( (28–30) \). A lack of exon 2 encoding the signal sequence necessary for ER membrane translocation suggests that the propeptide products are neither processed nor secreted and therefore are nonfunctional (31). Recently, Lyons and Blalock (32) detected the full-length POMC mRNA in immune cells by rapid amplification of cDNA ends-PCR. The researchers suggested that the apparent differences from previous results may be due to differences in PCR techniques, primer extension, and/or ribonuclease (18). Furthermore, they reported that in nonstimulated mononuclear cells the amount of full-length POMC mRNA is low, but that mitogenic stimulation enhanced the abundance of this mRNA (32).
model involves a strong inflammatory stimulus in vivo, which is conceivably similar to the in vitro stimulation used in the aforementioned studies. Thus, it was our objective to extend those studies and to examine the enzymatic machinery required for POMC sorting, processing, storage, and vesicular release of END in inflammatory cells.

In the present studies we provide evidence for the expression of the POMC precursor, POMC-derived peptides (END), the sorting receptor (CPE), proteolytic enzymes (PC1 and PC2), and the binding protein 7B2 in circulating and resident leukocytes. Our Western blot analysis of CPE, POMC, and END in circulating leukocytes shows bands of approximately 53, 31, and 3.5 kDa, respectively, comparable to those previously reported in neuroendocrine cells (13, 33). Our anti-PC1 antibody detected a double band of 66 and 60 kDa. The 66-kDa band corresponds to the form previously observed in rat alveolar macrophages and spleen mononuclear cells (20) and in a human monocytic leukemia cell line (34). The 60-kDa form may represent degradation products of PC1, as the activated isoform has been found to be unstable (35). The anti-PC2 and anti-7B2 antibodies recognized bands of approximately 65 and 23 kDa, respectively. These values correspond to those previously estimated for PC2 and 7B2 in pituitary cell lines and pancreatic islets (36, 37). Thus, all of the antibodies we used are characterized by their respective recognition of specific bands with different molecular masses similar to those detected in neuroendocrine cells (12, 13, 33, 36, 38). Importantly, these results demonstrate that cross-reactivity is excluded. We also found an increase in POMC, PC2, and END in circulating leukocytes from treated vs. untreated rats, indicating an up-regulation of these proteins under conditions of inflammatory pain. Since circulating cells of both treated and untreated animals displayed immunoreactive POMC, PC1, PC2, and END, painful inflammation appears to increase, but not induce, gene expression of these proteins.

Our immunohistochemistry shows that END, POMC, CPE, PC1, PC2, and 7B2 are expressed within immune cells in blood and in inflamed, but not in noninflamed, sc tissue. In inflamed tissue, immune cells expressing these compounds are found mainly in the periphery of inflammatory foci. Their morphological appearances are consistent with

![Fig. 8. Electron micrographs showing END immunoreactivity within immune cells in inflamed sc paw tissue. A, Pre-absorption of END antibody with END completely abolished END immunoreactivity within secretory granules in membranous structures (arrows) in cytoplasmic areas of immune cells. B, END-immunoreactive secretory granules located at the trans-side of Golgi stacks (G). C–E, END-immunoreactive secretory granules packed in membranous dilatations of Golgi cisternae (*). F and G, END-immunoreactive secretory granules packed in membranous structures in close contact with Golgi apparatus (G) and rough ER (small arrow) or arranged at the cell periphery and extended processes (large arrows). Magnification: A, ×5,000; and B–F, ×10,000.](https://academic.oup.com/endo/article-abstract/145/3/1331/2878207)
cells. Immunostaining of END was confined to secretory granules, which were grouped in small or large membranous vesicular structures. The smaller END-immunoreactive secretory granules were localized within cytoplasm, and the larger ones were arranged at the cell periphery and within extended processes ready for the exocytosis process, similar to the pituitary (43). Intense stimulation of such cells, which can be provided by persistent inflammatory pain and by environmental stress in our model (3, 4, 7), conceivably leads to END secretion.

As stress is typically associated with catecholamine release, we studied the liberation of END from an immune cell suspension by noradrenaline in vitro. We found that noradrenaline induced the release of immunoreactive END into the supernatant. This release was reversed by the adrenergic antagonists phentolamine and propranolol, indicating that it was mediated by the activation of adrenergic receptors on the cells, consistent with earlier indirect evidence (5). These results support and extend our previous studies showing receptor-specific END release from immune cells by CRH and IL-1β (6).

**Conclusions**

Immune cells are a source of opioid peptides, which can inhibit pain by a local interaction with peripheral opioid receptors within inflamed tissue (6, 7, 22). Our present study demonstrates the presence of POMC protein and POMC-derived peptides as well as crucial components (PC1, PC2, CPE, and 7B2) required for POMC sorting to the regulated secretory pathway and for posttranslational processing into biologically active peptides in inflammatory cells. The POMC end product, END, is apparently stored and released from secretory granules, similar to the classical pathway described in the pituitary gland.

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**References**

2. Sharp B, Litner K 1993 What do we know about the expression of proopiomelanocortin transcripts and related peptides in lymphoid tissue? Endocrinology 133:1921A–1921B
3. Stein C, Hassan AH, Przewlocki R, Gramsch C, Peter K, Herz A 1990 Opioids from immunocytes interact with receptors on sensory nerves to inhibit noci-
ception in inflammation. Proc Natl Acad Sci USA 87:5935–5939

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**TABLE 1. END release from immune cell suspensions from popliteal lymph nodes of inflamed hind paws**

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Pellet (pg/10^7 cells)</th>
<th>Supernatant (pg/10^7 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (not incubated)</td>
<td>33.1 ± 2.6</td>
<td>0</td>
</tr>
<tr>
<td>Control incubated with HBSS</td>
<td>33.7 ± 2.4</td>
<td>2.9 ± 0.4</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>25.9 ± 1.4^a</td>
<td>5.4 ± 0.9^a</td>
</tr>
<tr>
<td>Noradrenaline + 50 ng phen</td>
<td>28.6 ± 1.9</td>
<td>3.3 ± 1.0^b</td>
</tr>
<tr>
<td>Noradrenaline + 250 ng phen</td>
<td>29.3 ± 2.3^b</td>
<td>2.9 ± 0.4^b</td>
</tr>
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</table>

Values are the means ± SEM obtained from five rats per experiment and are expressed as END immunoreactivity (picograms per 10^7 cells). Each experiment was performed three times. phen, Phentolamine; propra, propranolol.

^a Significant difference vs. incubated control.

^b Significant difference vs. noradrenaline alone.

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Macrophages/monocytes, granulocytes, and lymphocytes. In the blood of untreated animals, immunoreactive cells can be differentiated into macrophages/monocytes and granulocytes. Consistent with our findings in Western blots, there was a significant increase in POMC-, PC1-, PC2-, and END-immunoreactive leukocytes within the circulation after induction of inflammation. Thus, our single staining results in this model of inflammatory pain agree with previous studies showing PC2 in peripheral and liver-infiltrating granulocytes, PC1 in alveolar macrophages and spleen mononuclear cells from lipopolysaccharide-treated rats (20), as well as PC1, PC2, and POMC in spleen macrophages/monocytes and lymphocytes from diabetic (39) and untreated (40) rats.

To date there has been no evidence for colocalization of POMC or END together with these processing enzymes in immune cells. Our double-staining experiments show that 7B2 and PC2 are clearly coexpressed within immune cells. Our double-staining experiments show that 7B2 and PC2 are clearly coexpressed within immune cells. This model of inflammatory pain agree with previous studies showing PC2 in peripheral and liver-infiltrating granulocytes, PC1 in alveolar macrophages and spleen mononuclear cells from lipopolysaccharide-treated rats (20), as well as PC1, PC2, and POMC in spleen macrophages/monocytes and lymphocytes from diabetic (39) and untreated (40) rats.

**TABLE 1. END release from immune cell suspensions from popliteal lymph nodes of inflamed hind paws**

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Pellet (pg/10^7 cells)</th>
<th>Supernatant (pg/10^7 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (not incubated)</td>
<td>33.1 ± 2.6</td>
<td>0</td>
</tr>
<tr>
<td>Control incubated with HBSS</td>
<td>33.7 ± 2.4</td>
<td>2.9 ± 0.4</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>25.9 ± 1.4^a</td>
<td>5.4 ± 0.9^a</td>
</tr>
<tr>
<td>Noradrenaline + 50 ng phen</td>
<td>28.6 ± 1.9</td>
<td>3.3 ± 1.0^b</td>
</tr>
<tr>
<td>Noradrenaline + 250 ng phen</td>
<td>29.3 ± 2.3^b</td>
<td>2.9 ± 0.4^b</td>
</tr>
</tbody>
</table>

Values are the means ± SEM obtained from five rats per experiment and are expressed as END immunoreactivity (picograms per 10^7 cells). Each experiment was performed three times. phen, Phentolamine; propra, propranolol.

^a Significant difference vs. incubated control.

^b Significant difference vs. noradrenaline alone.


38. Shen FS, Loh YP 1997 Intracellular misrouting and abnormal secretion of adrenocorticotropic hormone and growth hormone in cpefat mice associated with a carboxypeptidase E mutation. Proc Natl Acad Sci USA 94:5319–5324


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