European Journal of Pharmacology 654 (2011) 33–41

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Neuropharmacology and Analgesia

Antagonism of 5-HT_{2A} receptors inhibits the expression of pronociceptive mediator and enhances endogenous opioid mechanism in carrageenan-induced inflammation in rats

Jian Huang [1], Yanmei Fan [1], Yue Jia, Yanguo Hong *

College of Life Sciences, Fujian Normal University, Key Provincial Laboratory of Developmental and Neurological Biology, Fuzhou, 350108, People's Republic of China

ARTICLE INFO

Article history:
Received 11 July 2010
Received in revised form 17 November 2010
Accepted 11 December 2010
Available online 24 December 2010

Keywords:
Inflammatory pain
5-HT_{2A} receptor
Endogenous opioid mechanism
CGRP (Calcitonin gene-related peptide)
DRG (Dorsal root ganglia)
Spinal dorsal horn

ABSTRACT

We have recently reported that treatment with the 5-HT_{2A} receptor antagonist ketanserin in the inflamed paw raises the nociceptive threshold above normal level (hypoalgesia) and this response is naloxone-reversible. The present study aimed to investigate neurochemical changes at the site of inflammation and in dorsal root ganglia (DRG) and the spinal cord following the blockade of 5-HT_{2A} receptors. Intraplantar injection of ketanserin (20 μg) inhibited carrageenan-induced increase in CGRP immunoreactivity-positive neurons in DRG. On the other hand, administration of ketanserin (20 μg) and 5-HT (10 μg), but not vehicle, enhanced and inhibited recruitment of β-endorphin-expressing immune cells, respectively, in subcutaneous loci of inflamed hindpaw. Moreover, the treatment with ketanserin increased the number of endomorphine-containing cells in the inflamed paw and μ-opioid receptor-expressing neurons in DRG at L4–5 but reduced the expression of endomorphine in superficial layers of the lumbar spinal cord. The present study provided evidence at the cellular level showing that the blockade of 5-HT_{2A} receptors inhibited inflammation-associated increase in pronociceptive mediator, and that the pronociceptive property of 5-HT is mediated by the suppression of inflammation-activated opioid mechanism. Therefore, targeting the 5-HT_{2A} receptors in the site of inflammation may be a promising approach to inhibit inflammatory pain.

1. Introduction

Tissue destruction produces and releases pronociceptive chemicals or mediators as a repairing response to injury. These mediators produce inflammation and hyperalgesia (exacerbated nociception) or allodynia (pain perception by innocuous stimuli). 5-hydroxytryptamine (5-HT) is one of important pronociceptive mediators (Schaible et al., 2002; Bolay and Moskowitz, 2002) and is released from platelets, mast cells and endothelial cells into an injured site during inflammation (Parada et al., 2001; Rowley and Benditt, 1956). The involvement of 5-HT in the induction of pathological pain has been documented in several animal pain models, such as formalin- (Abbott et al., 1997), carrageenan- (Di Rosa et al., 1971) as well as complete Freund's adjuvant-induced inflammation (Okamoto et al., 2002; Pertsch et al., 1993) and nerve injury (Theodosiou et al., 1999; Wang et al., 2009). Previous studies have demonstrated that 5-HT activates or sensitizes nociceptors by exciting C-fiber afferents (Beck and Handwerker, 1974; Herbert and Schmidt, 1992), increasing the excitability of small-diameter neurons in dorsal root ganglia (DRG (Cardenas et al., 2001)), releasing calcitonin gene-related peptide (CGRP (Tramontana et al., 1993)) and activating tetrodotoxin-resistant sodium current (Gold et al., 1996) or PKC and PKA signaling pathways (Ohta et al., 2006). However, it has never been found that pronociceptive property of 5-HT is attributed to the inhibition of endogenous opioid mechanism.

The endogenous opioid mechanism is activated during inflammation as a self-protection mechanism to inhibit pain perception (Stein et al., 2001). It includes migration of opioid-containing leukocytes into the inflamed tissue (Cabot et al., 1997, 2001), the release of opioid peptides (Cabot et al., 1997, 2001) and synthesis as well as transportation of opioid receptors (Mousa et al., 2001). However, it is not clear how these activities are regulated. We observed that a removal of 5-HT activity by the blockade of 5-HT_{2A} receptors using ketanserin in the inflamed, but not naïve, hindpaw raised nociceptive threshold above the normal level (Huang et al., 2009). This type of pain perception is referred to as "hypoalgesia", a raised threshold for nociception, which was reported previously (Franca et al., 2006; Francischi et al., 2002). It is noticed that the ketanserin-induced hypoalgesia was seen at 21 h after the injection (Huang et al., 2009). Obviously, this response was not attributed to ketanserin itself since this drug is relatively short-lasting and fast in the onset (Heykants et al., 1986; Nitanda et al., 2005). Instead, the hypoalgesia must be due

* Corresponding author. Department of Anatomy and Physiology, College of Life Sciences, Fujian Normal University, Minhou County, Fuzhou, Fujian 350108, People's Republic of China. Tel.: +86 591 22868211; fax: +86 591 83465091.
E-mail address: yanguo_hong@hotmail.com (Y. Hong).
[1] These authors contributed equally to this work.

0014-2999/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2010.12.007

to the events that were secondary to the blockade of 5-HT_{2A} receptors. This assumption was supported by the result showing that the ketanserin-induced response was abolished by the opioid receptor antagonist naloxone (Huang et al., 2009), suggesting the involvement of endogenous opioid mechanism. The present study was designed to investigate the effect of ketanserin on opioid peptides and receptors at the site of inflammation and in DRG or the spinal cord. To explore a cellular mechanism underlying the low sensitivity status in the inflamed paw following the treatment with ketanserin, the current study also examined the inflammation-associated expression of the pronociceptive mediator CGRP.

2. Materials and methods

2.1. Animals

This study was carried out using male Sprague–Dawley rats weighing 250–330 g. Care and treatment of animals were according to the guidelines for investigations of experimental pain in conscious animals and were approved by the animal care committee at the Fujian Normal University.

2.2. Experimental procedures

Carrageenan (2%, 100 μl) and ketanserin (20 μg) or vehicle (0.8% DMSO) were injected intraplantarly (i.pl.) at 0 and 1 h, respectively, in the hindpaw in rats. In addition, two groups of animals received i.pl. injections of carrageenan and 5-HT (10 μg) or vehicle (saline) at 0 and 18 h, respectively. Rats were sacrificed at 24 h following the injection of carrageenan. Hindpaw skin, DRG at L3–L5 or the lumbar spinal cord on the both sides were harvested.

2.3. Immunohistochemistry

Rats were deeply anesthetized with sodium pentobarbital (65 mg/kg, i.p., Shenggong Chemicals, Shanghai, China). The animals were perfused through the heart with 200 ml of phosphate-buffered saline (PBS; 0.05 M; pH 7.4) followed by 500 ml of fresh cold fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Paw skin, the spinal cord and DRG at L3–L5 were harvested. The tissues were post-fixed for 4–6 h at 4 °C in the fixative solution and cryoprotected overnight at 4 °C in PBS containing 30% sucrose. The tissues was frozen and cut with a cryostat (paw skin: 7 μm; spinal cord: 40 μm; DRG: 10 μm). Tissue sections were then processed on the slides or by a free-floating slice as described previously (Cai et al., 2007). Staining of the sections was performed with a vectastain avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA). Unless otherwise stated, all incubations were done at room temperature and PBS was used for washing (10 min for three times) after each step. The sections were incubated with PBS containing 0.3% H_2O_2 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, 3% bovine serum, 4% goat serum and 4% horse serum. The sections were then incubated overnight at 4 °C with rabbit anti-β-endorphin (1:500, Chemicon international Inc. USA), rabbit anti-endomorphine (1:1000), rabbit anti-μ-opioid receptor (1:1000) or chicken anti-CGRP (1:6000) diluted with 3% bovine serum albumin (Sigma, St. Louis. MO), and then with secondary antibody (1:2000 in 10% goat serum in PBS) for 2 h. The sections were incubated with avidin–biotin-conjugated peroxidase (1:100, Vector Laboratories, Burlingame, CA, USA) for 1 h. Finally, the sections were stained with 3',3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) containing 0.01% H_2O_2 in 0.05 M Tris-buffered saline (pH 7.6) for 3–5 min. Sections were then mounted on gelatin-coated slides, air dried, dehydrated in a series of graded alcohols, cleared in xylene and coverslipped.

To verify the specificity of immunostaining, a few sections from three rats were treated with the identical protocol except being incubated in 2% normal rabbit serum instead of primary antibody against β-endorphin or endomorphine. In this case, no immunolabelling was observed. The specificity of staining was also tested with preabsorption procedures. Antibodies against β-endorphin and endomorphine were preabsorbed with β-endorphin peptide (10^{-6}, 10^{-5} and 10^{-4} M, Huadatianyuan Biological Co, Shanghai, China) and endomorphine peptide (10^{-5}, 10^{-4} and 10^{-3} M, Huadatianyuan), respectively. After being rotated for 2 h at room temperature, the antiserum/peptide solutions were agitated for 24 h at 4 °C. Then, the solution was microcentrifuged at 13,000 *g* for 30 min. The supernatant was collected as the preabsorbed serum. The sections were immunostained with the identical procedure described above (n = 3 each).

2.4. Double labeling

Sections of subcutaneous tissue of the hindpaw were also counterstained with Harris haematoxylin (haematoxylin 2.5 g; ethanol 25 ml; $KAl(SO_4)_2 \cdot 12H_2O$ 50 g; HgO 1.25 g; CH_3COOH 20 ml in 500 ml of distilled water) in β-endorphin- and endomorphine-reacted sections to show nucleus in immune cells. The reaction for immunocytochemical study was detected by standard DAB with brown color while nucleus staining with Harris haematoxylin was shown by blue color.

2.5. Immunofluorescence

Sections were incubated with a rabbit anti-μ-opioid receptor (1:1000) or chicken anti-CGRP antiserum (1:1000; Chemicon International, Inc. USA) in 0.1 M PBS with 5% normal goat serum overnight at 4 °C, followed by secondary incubation with FITC-conjugated donkey polyclonal antibody against rabbit IgG (1: 200, Abcam, Cambridge Science Park, Cambridge, UK) or donkey polyclonal antibody against chicken Rhodamine conjugated affinity purified secondary antibody (1:200, Chemicon) for 2 h. The sections were rinsed and mounted in Vectashield. Fluorescence digital images were captured using Olympus fluorescence microscope (Olympus Optical CO., LTD, BX51 and DP70, Tokyo, Japan) outfitted with a Hamamatsu (Bridgewater, NJ) C5810 color CCD camera that output to a Pentium microcomputer.

2.6. Enzyme immunoassay (ELIZA)

Paw skin was minced and homogenized using a sonicator in ice-cold PBS containing Tris–HCl (40 mM, pH 7.5), SDS (2%), aprotinin (2 mg/ml), leupeptin (2 mg/ml), pepstatin-A (2 mg/ml), phenylmethylsulfonyl fluoride (1 mM), dithiothreitol (1 mM) and EDTA (1 mM). Homogenates were shaken at 4 °C for 30 min followed by centrifuging at 15,000 ×*g* for 30 min. The supernatant was then aliquoted and stored at −80 °C. Quantitative determination of β-endorphin (Phoenix Pharmaceuticals, Inc., Belmont, CA, USA) was performed using a commercially available ELIZA kit according to manufacturer's instruction. Microplates were read using a microplate reader (Synergy HT, BioTek Instruments Inc, Vermont, USA). The value (pg/mg tissue protein) averaged from 3 rats was taken to express the level of the peptide in each group. Concentration of protein in tissue samples was determined in the crude supernatants using the BCA protein assay kit (Bio-Rad).

2.7. Drugs

Carrageenan (lambda type IV), ketanserin tartrate and 5-hydroxytryptamine hydrochloride (5-HT) were purchased from Sigma (St. Louis, MO, U.S.A.). Carrageenan and 5-HT were dissolved in sterile

saline. Stock solution of ketanserin was prepared in 20% dimethylsulfoxide (DMSO, Shenggong Inc, Shanghai, China). The stocks were stored at 4 °C and diluted to the working concentration with sterile saline before the experiment. The concentration of DMSO in the vehicle for 20 μg of ketanserin was 0.8%. For i.pl. injection, the chemicals were administered in a volume of 100 (carrageenan) or 50 (ketanserin or 5-HT) μl with a 30-gauge needle.

2.8. Statistical analysis

2.8.1. Quantification of β-endorphin- or endomorphine-containing cells in subcutaneous loci of inflamed hindpaw

Sections were visually scanned and photographed via bright field microscopy (Vanox; Olympus Corp., Tokyo, Japan). The individual sections were captured with CCD camera. Three sections from each animal were randomly selected for the quantification of β-endorphin- or endomorphine-containing cells. All positive cells in a section were counted manually by an observer blind to the treatment conditions. The number of β-endorphin- or endomorphine-containing cells was determined by averaging the counts made using 10 squares per section (6250 μm^2) and four sections per animal.

2.8.2. Quantitation of μ-opioid receptor- or CGRP-positive DRG neurons

Four rats per group were used for quantification of μ-opioid receptor- or CGRP-positive neurons in L3–5 DRG following various treatments. One field (850 μm × 640 μm) was randomly selected from each of 15 DRG sections per animal. For neuron-size measurement, the neuronal soma area was calculated by the imaging software Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA). Small neurons were <600 μm^2, medium neurons were 600–1200 μm^2, and large neurons were >1200 μm^2 (Gendron et al., 2006).

2.8.3. Quantification of endomorphine-immunoreactivity in the spinal cord

Ten sections were randomly selected from L3–L5 segment of the spinal cord from each rat and the density of endomorphine immunoreactivity in these sections was measured. Measurement was made for positive staining which was in superficial layers (laminae I and II). The density of endomorphine staining was measured with a computer-assisted imaging analysis system (Image-Pro Plus). The number of pixels in white matter was taken as background level. The number of brown pixels in the superficial laminae was subtracted from the background to yield a corrected optical density for each section. Ten readings were averaged to obtain the number of pixels for each animal.

Quantification of the positive neurons in immunohistochemical studies was counted by an experimenter who was blind to the treatment conditions using image-analysis software. Data were expressed as the mean ± standard error of mean (S.E.M.). Statistical significance was analyzed using Student's *t*-test for two groups or one-way ANOVA for multiple groups. A *P* value less than 0.05 denoted the presence of a statistically significant difference.

3. Results

3.1. Effect of ketanserin on inflammation-induced expression of CGRP in DRG

To investigate neurochemical change at 23 h following injection of ketanserin, CGRP immunoreactivity in DRG was examined. As our

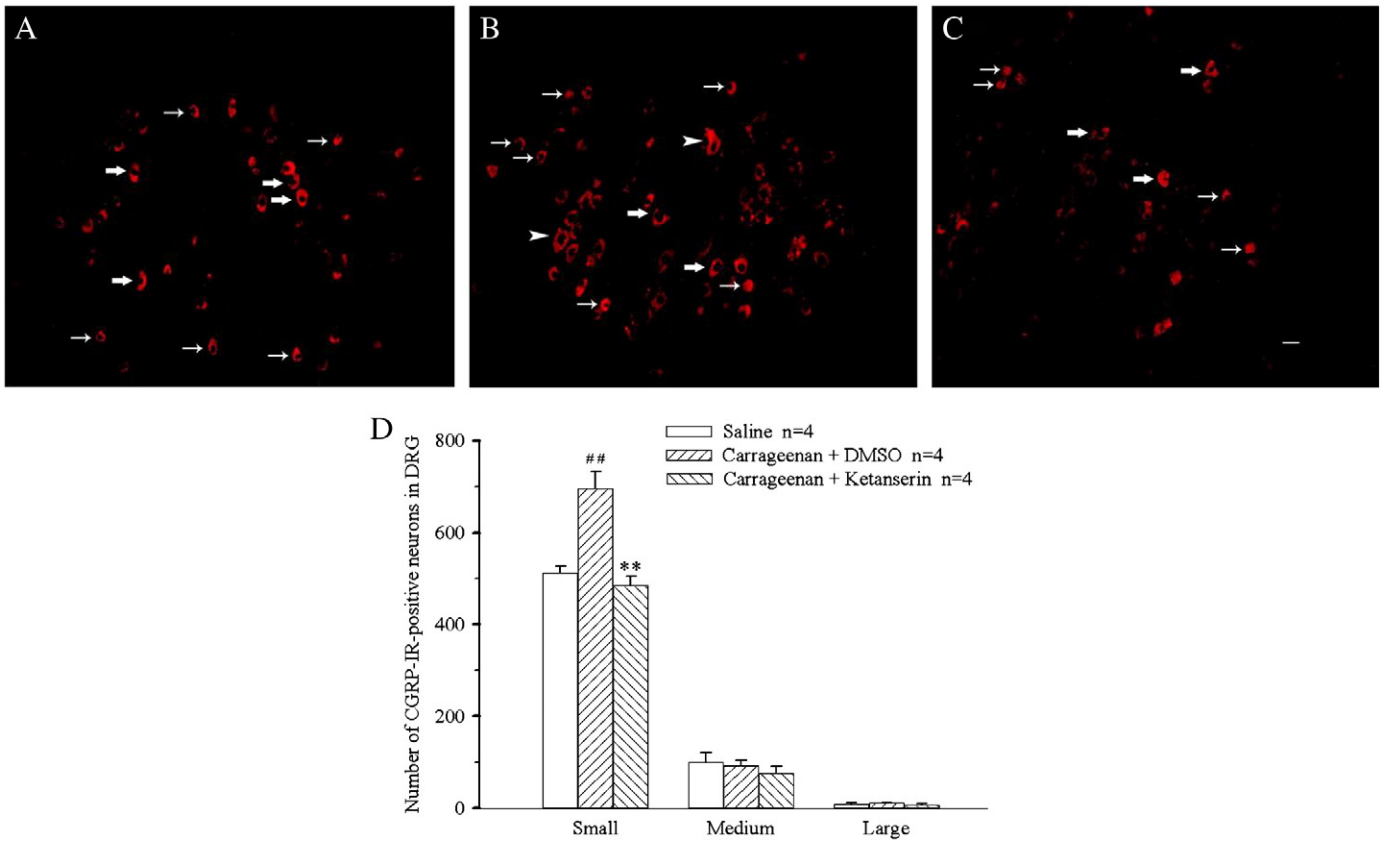

Fig. 1. Effect of post-treatment with ketanserin on carrageenan-induced expression of CGRP in DRG. Saline and 0.8% DMSO (A), carrageenan (2%, 100 μl) and 0.8% DMSO (B) or carrageenan and ketanserin (20 μg, C) were injected i.pl at 0 and 1 h, respectively. DRGs at L3–L5 were harvested at 24 h. Photomicrographs of transverse sections of DRG demonstrate that CGRP-like immunoreactivity is mainly seen in small (small arrows) DRG neurons. Large arrows and arrow heads show medium and large DRG neurons, respectively. Histograms illustrate the numbers of CGRP-positive neurons in small-, medium- and large-diameter subpopulations (D). ** represent $P<0.01$ compared with both saline and carrageenan/DMSO groups. N = 4 each. Scale bar = 100 μm.

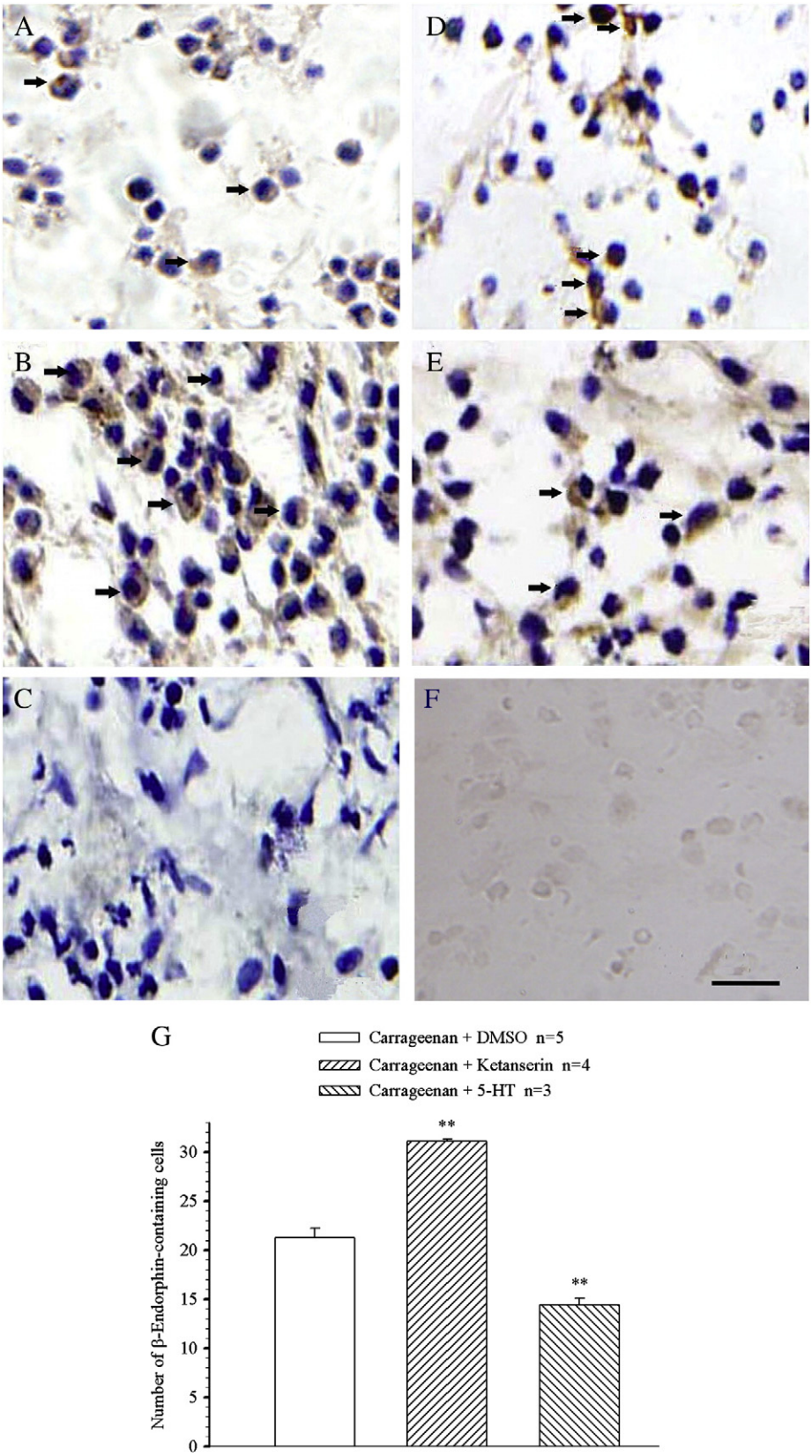

Fig. 2. Immunohistochemical localization of β-endorphin in subcutaneous loci of inflamed paw. Carrageenan and 0.8% DMSO (A) or ketanserin (B) were injected i.pl at 0 and 1 h, respectively. Carrageenan and saline (D) or 5-HT (E) were injected i.pl. at 0 and 18 h, respectively. Paw skin tissue was harvested at 24 h. Harris haematoxylin stained nucleus with blue color while immunohistochemical procedure displayed β-endorphin in cytoplasm with brown color. β-Endorphin positive cells are in leukocytes in inflamed paw tissue while in the absence of primary antibody, no appreciable staining is observed (C). β-Endorphin-like immunoreactivity positive cells are increased (B) and decreased (E) compared with vehicle (A or D). F shows paw skin section that was incubated with anti-β-endorphin antiserum preabsorbed with 10^{-5} β-endorphin peptide. Histograms (G) represent the mean ± S.E.M. of number of β-endorphin-containing cells. ** represent $P<0.01$ compared with group of carrageenan/vehicle. Scale bar = 40 μm.

previous study has demonstrated that 20 μg of ketanserin is effective to increase nociceptive threshold in the carrageenan-induced inflammation (Huang et al., 2009), this dose was used in this and following studies.

Intense staining of CGRP immunoreactivity was exhibited in cytoplasm of DRG neurons in animals that received i.pl. saline. These neurons were mainly of small and medium diameter subpopulations. Injection of carrageenan (2%, i.pl.) only altered the number of small, but not medium and large, neurons. On the side ipsilateral to saline injection, 512.8 ± 13.9 small neurons were CGRP-immunostaining positive (saline group, n = 4, Fig. 1). I.pl carrageenan increased the number of CGRP neurons in the small-sized subpopulation to 695.8 ± 37.2 (carrageenan group, n = 4). This number was significantly different from that in saline group ($P<0.01$). Following i.pl. administration of ketanserin (20 μg), carrageenan injection only induced 485.0 ± 19.6 CGRP-immunoreactivity positive neurons (n = 4), which was significantly lower than carrageenan group ($P<0.01$) but very similar to saline group ($P>0.05$, Fig. 1D).

3.2. Expression of β-endorphin in subcutaneous loci of inflamed paw following injection of ketanserin or 5-HT

Carrageenan (i.pl.) and 0.8% DMSO or ketanserin (20 μg) or 5-HT (10 μg) were injected into the right hindpaw at 0 and 1 (18 h for 5-HT) hour, respectively. Inflamed paw skin was harvested at 24 h.

Sections of paw skin were processed with double immunostaining. Histological procedure with Harris haematoxylin revealed dense nucleus of various immune cells with blue-color. Immunolabelling for β-endorphin (Fig. 2A, B, D and E) showed brown staining diffused throughout the cytoplasm. The cells had morphological appearances consistent with macrophages, lymphocytes and polymorphonuclear leucocytes. Cell counts of β-endorphin immunoreactivity revealed that carrageenan plus 0.8% DMSO produced 21.3 ± 1.0 cells in the area of 6250 μm^2 of subcutaneous loci (n = 5). However, the treatment with carrageenan plus ketanserin induces 31.1 ± 0.2 β-endorphin immunoreactivity positive cells in the same size area (n = 4). There was significant difference for the cell number between these two groups ($P<0.01$, Fig. 2G). As hypoalgesia response started at 21 h after administration of ketanserin (Huang et al., 2009), carrageenan and 5-HT (10 μg) was injected i.p. at 0 and 18 h, respectively, to determine whether 5-HT could inhibit the expression of β-endorphin. Fig. 2G illustrates that the number of β-endorphin immunoreactivity positive cells reduced to 14.4 ± 0.7 in the site of inflammation following the injection of 5-HT (n = 3) which was significantly less than that in vehicle group ($P<0.01$).

To further confirm the alteration of β-endorphin, the content of this peptide in the flamed paw skin was measured using ELIZA kit. The assay revealed that carrageenan plus 0.8% DMSO produced 25.9 ± 3.1 pg/mg of β-endorphin (Fig. 3, n = 3). However, the content of the peptide increased to 52. 6 ± 5.7 pg/mg in the group of carrageenan plus ketanserin (n = 3). There was a significant difference between these two groups ($P<0.05$, Fig. 3).

3.3. Effect of ketanserin on the expression of endomorphine in subcutaneous loci of inflamed paw

Double staining study using Harris haematoxylin and an antibody against endorphin-1 and 2 (Fig. 4A–B) also produced positive staining in immune cells. These cells displayed dense nucleus stained with haematoxylin (blue color) and diffused endomorphine staining throughout the cytoplasm (brown color). The treatment with carrageenan plus 0.8% DMSO induced 12.5 ± 0.8 endomorphine cells in the area of 6250 μm^2 of subcutaneous loci (n = 4). The number of endomorphine immunoreactivity-stained cells was increased to 18.2 ± 0.7 in the same size area following the treatment with carrageenan plus ketanserin (n = 4). This number was significantly higher than vehicle group ($P<0.01$, Fig. 4E).

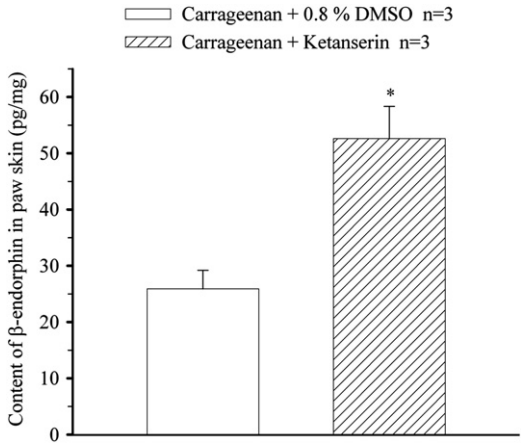

Fig. 3. Content of β-endorphin in the inflamed paw skin. Carrageenan and 0.8% DMSO or ketanserin were injected i.pl at 0 and 1 h, respectively. Paw skin tissue was harvested at 24 h. Content of β-endorphin was assayed with ELISA kit. * represents $P<0.05$ compared with group of carrageenan/0.8% DMSO. N = 3 each.

3.4. Effect of ketanserin on expression of μ-opioid receptors in DRG

μ-Opioid receptor immunoreactivity was detected in DRG in rats that received i.pl. injection of saline. The positive staining was mainly located in the cytoplasm of neurons. These neurons were mainly a small diameter subpopulation (Fig. 5A, 270.3 ± 15.0) although a few medium sized neurons also displayed μ-opioid receptor immunoreactivity (59.3 ± 6.7, n = 4). Carrageenan injection produced no changes in the expression of μ-opioid receptors compared to naïve rats. The numbers of positive immunoreactivity neuron were 247.0 ± 10.3 and 53.0 ± 8.4 in small and medium subpopulation, respectively (Fig. 5B, n = 4). However, the treatment with ketanserin in the inflamed paw induced a significant increase in the number of μ-opioid receptor-immunoreactivity neurons in the small-sized subpopulation (Fig. 5C, 358.0 ± 26.1, n = 4, $P<0.01$ compared to carrageenan group). Although ketanserin also increased the number of μ-opioid receptor-expressing neurons in medium-sized subpopulation, the increase did not reach significant level (84.3 ± 16.9, $P>0.05$).

3.5. Effect of ketanserin on expression of endomorphine in the spinal dorsal horn

Staining process with endomorphine antiserum displayed moderate labeling in laminae I and II of the lumbar spinal cord (Fig. 6B, n = 4) following i.pl. unilateral injection of carrageenan plus 0.8% DMSO. There was no difference in the intensity of endomorphine immunoreactivity between the ipsilateral and contralateral (data not shown) sides with pixels of 66.0 ± 1.3 and 67.2 ± 1.9, respectively. The treatment with carrageenan plus ketanserin produced reduction of endomorphine immunoreactivity in both distribution area and density (Fig. 6C and D, 57.8 ± 2.1, n = 4, $P<0.05$) which was significantly different from the other side (Fig. 6A) or ipsilateral side in the group of carrageenan/0.8% DMSO (Fig. 6B).

4. Discussion

We observed in previous study that i.pl. injection of the 5-HT_{2A} receptor antagonist ketanserin (Hoyer et al., 2002) in the inflamed hindpaw dose-dependently (2–20 μg) elevated pain threshold to the level that was above normal baseline, called hypoalgesia, in the late phase (post-20 h) of carrageenan-induced inflammation. This response developed slowly starting at 21 h after injection, peaked at 24 h and lasted for at least 10 h. Interestingly, the hypoalgesia response was abolished following s.c. or i.pl injection of naloxone methiodide (Huang et al., 2009). Therefore, the present study aimed

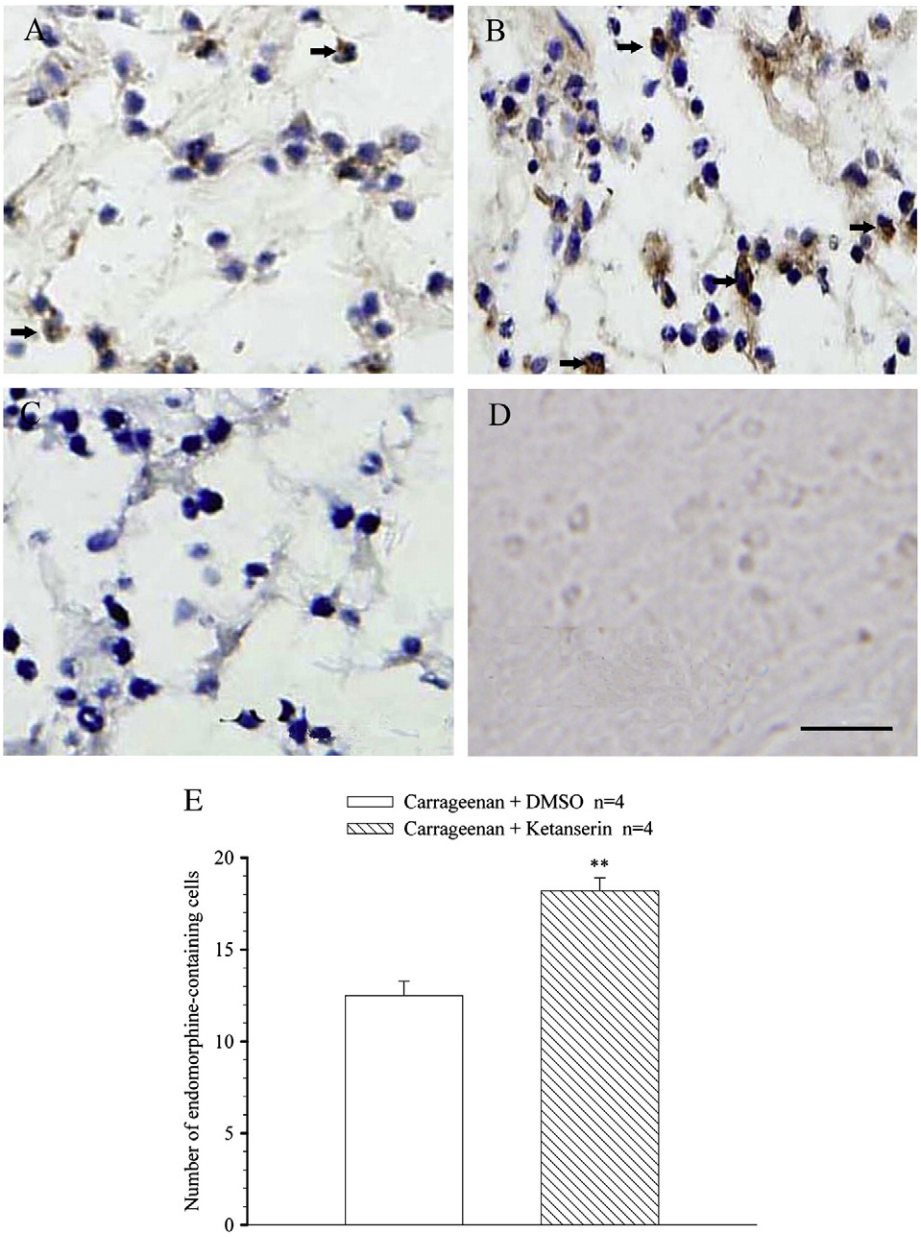

Fig. 4. Immunohistochemical localization of endomorphine in subcutaneous loci of inflamed paw. Carrageenan and 0.8% DMSO (A) or ketanserin (B) were injected i.pl at 0 and 1 h, respectively. Paw skin tissue was harvested at 24 h. Harris haematoxylin stained nucleus with blue color while immunohistochemical procedure displayed endomorphine in cytoplasm with brown color. Endomorphine-like immunoreactivity positive cells are in leukocytes in inflamed paw tissue while in the absence of primary antibody, no appreciable staining is observed (C). D shows paw skin section that was incubated with anti-endomorphine antiserum preabsorbed with 10^{-4} endomorphine peptide. Histograms (E) represent the mean ± S.E.M. of number of endomorphine-containing cells. ** represent $P<0.01$ compared with group of carrageenan/vehicle. Scale bar = 40 μm. N = 4 each.

to investigate neurochemical changes underlying the ketanserin-induced response. It was found that ketanserin completely inhibited carrageenan-induced increase in CGRP in DRG. Furthermore, the injection of ketanserin increased the number of β-endorphin-expressing leukocytes in subcutaneous loci of inflamed paw while similar treatment with 5-HT reduced it. ELISA assay also demonstrated the increase in β-endorphin content in the inflamed skin following the injection of ketanserin. Moreover, the treatment with ketanserin, but not vehicle, induced the increase in the numbers of endomorphine immunoreactivity positive cells in the inflamed paw and μ-opioid receptor-expressing neurons in the small-sized subpopulation in DRG. Taken together, these data suggest that the injection of ketanserin in the inflamed paw suppresses the expression of pronociceptive mediator and enhances the endogenous opioid mechanism.

To confirm the low sensitivity status in the inflamed paw following the treatment with ketanserin, the present study examined the level of the pronociceptive mediator CGRP in DRG since inhibition of hyperalgesia should be associated with suppression of its increase. CGRP is released from both peripheral (Ferreira et al., 2000) and central terminals (Garry and Hargreaves, 1992; Sluka and Westlund, 1993) of primary afferents during inflammation, leading to the sensitization of nociceptors (Li et al., 2008) and nociceptive neurons in the spinal cord (Miletic and Tan, 1988; Oku et al., 1988; Ryu et al., 1988a) as well as DRG (Ryu et al., 1988b). Especially, the blockade of spinal CGRP receptors inhibits inflammation-associated activation of spinal dorsal horn neurons (Neugebauer et al., 1996) and hyperalgesia (Yu et al., 1998; Kawamura et al., 1989; Sun et al., 2003). In agreement with previous reports (Stanfa et al., 1997; Beland and Fitzgerald,

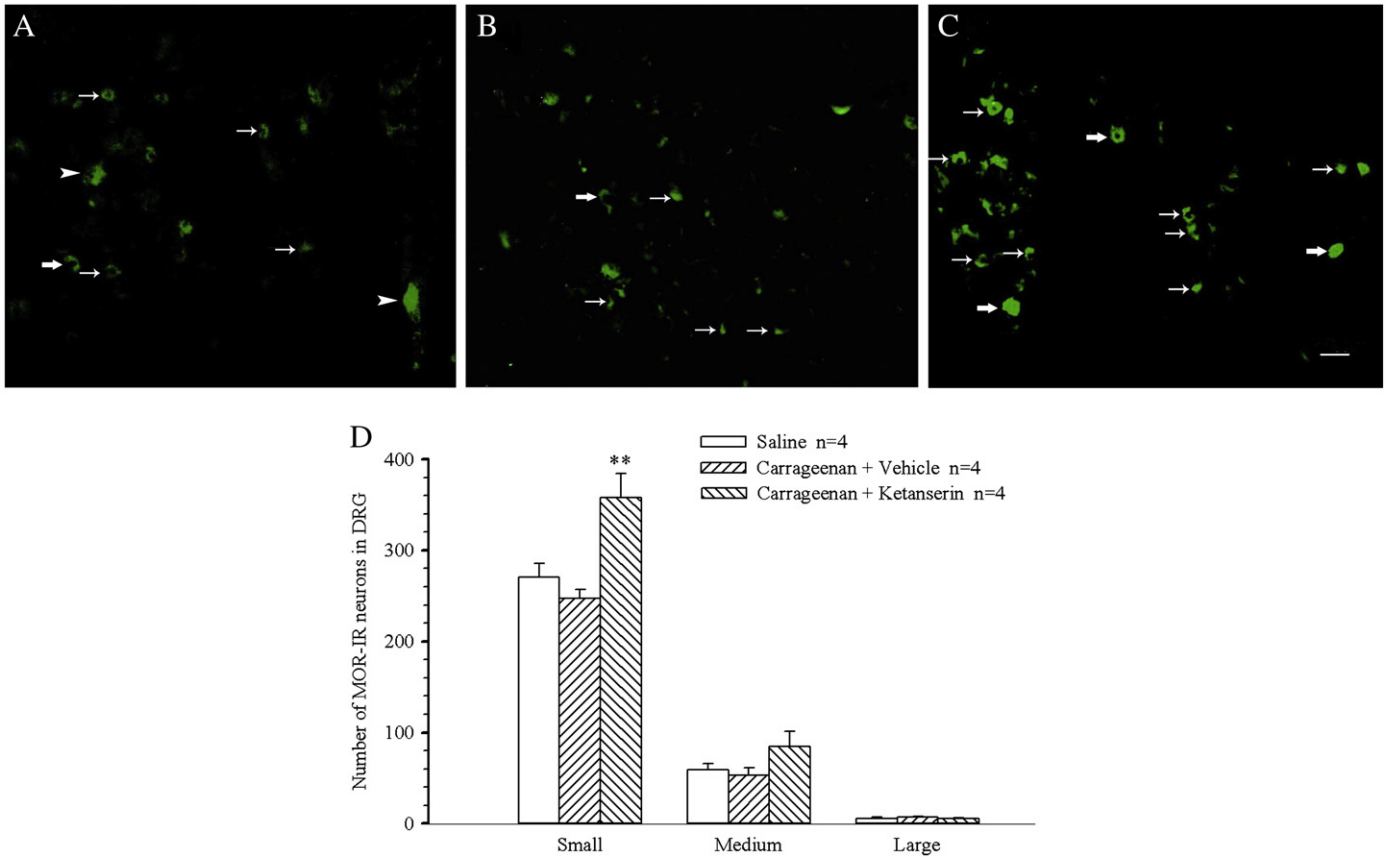

Fig. 5. Expression of μ-opioid receptors in DRG. Carrageenan and 0.8% DMSO (B) or ketanserin (C) were injected i.pl at 0 and 1 h, respectively. Control animals received only saline injections in the hindpaw at 0 and 1 h (A). DRGs at L3–L5 were harvested at 24 h. Photomicrographs of transverse sections of DRG demonstrate that μ-opioid receptors-like immunoreactivity is mainly seen in small (small arrows) and medium (large arrows) DRG neurons. Arrow heads show large DRG neurons. Histograms (D) illustrate the numbers of μ-opioid receptors-positive neurons in small-, medium- and large-diameter subpopulations. ** Represent $P<0.01$ compared with both saline and carrageenan/DMSO groups. Scale bar = 100 μm. N = 4 each.

2001), the present study showed that CGRP was mainly expressed in small-sized DRG neurons and its expression was increased in carrageenan-induced inflammation. However, this increase was completely abolished following the treatment with ketanserin. The reversal of increase in CGRP expression in DRG was consistent with the inhibition of hyperalgesia following the injection of ketanserin, suggesting that pronociceptive activity of 5-HT is associated with the enhancement of CGRP activity. The release of CGRP by 5-HT was reported previously (Tramontana et al., 1993). Our result also suggests that the suppression of CGRP underlies the inhibition of carrageenan-induced hyperalgesia. The present study, together with our previous observation showing that i.pl injection of 1% formalin induced less defensive behaviors and expression of c-Fos in the spinal dorsal horn (Huang et al., 2009), confirmed the low sensitivity status in the inflamed site following the treatment of ketanserin.

The inhibition of CGRP expression in DRG following the injection of ketanserin can be attributed to the opioid mechanism as our previous study showed that the ketanserin-induced hypoalgesia was abolished by naloxone (Huang et al., 2009). We therefore determined the effect of ketanserin on the expression of opioid peptides and receptors. Opioid-containing leukocytes are known to be absent in naïve peripheral tissue (Machelska et al., 1998). After complete Freund's adjuvant (CFA) is injected in the hindpaw, opioid-containing immune cells are recruited to the site of inflammation (Cabot et al., 1997, 2001). These cells release β-endorphin, met-enkephalin and dynorphin A inhibiting nociception during inflammation (Machelska et al., 2003). The present study showed that similar to CFA, i.pl. carrageenan recruited β-endorphin- and endomorphine-containing cells in subcutaneous loci of the inflamed hindpaw at 24 h following the injection. Importantly, post-treatment with ketanserin further increased the numbers of both cells at the site of inflammation. Another quantitative assay by ELISA also revealed an increase in the content of β-endorphin in the subcutaneous tissue of inflamed paw following the blockade of 5-HT_{2A} receptors. The facts that ketanserin increased the number of β-endorphin-containing cells by 33.9% but the content of β-endorphin by 90.8% imply that the release of β-endorphin in the site of inflammation was also increased. The finding that the removal of 5-HT activity by the blockade of 5-HT_{2A} receptors increased the number of β-endorphin-containing cells suggests that 5-HT suppressed migration of inflammation-activated β-endorphin-containing cells toward the site of inflammation. This notion was supported by the result that injection of 5-HT in inflamed paw reduced the accumulation of β-endorphin-containing cells in the site of inflammation.

It has been demonstrated that peripheral sensory neurons express opioid receptors. Previous studies have shown that inflammation upregulates the expression of μ-, but not δ- and *k*-, opioid receptors, in DRG (Ji et al., 1995; Mousa et al., 2001). Unexpectedly, we failed to observe the increase in μ-opioid receptor immunoreactivity positive neurons in DRG following the induction of inflammation. The discrepancy may be due to the difference in the models (carrageenan vs CFA (Mousa et al., 2001)) or stimulation intensity (100 μl of 2% carrageenan in the present study vs 200 μl of 4% carrageenan (Ji et al., 1995)). Importantly, we found that ketanserin increased the number of μ-opioid receptor-expressing neurons in DRG and this increase was restricted to the small-sized subpopulation of cells. In accordance with a previous study using CFA model (Mousa et al., 2002), carrageenan did not increase the expression of endomorphine in the spinal dorsal horn.

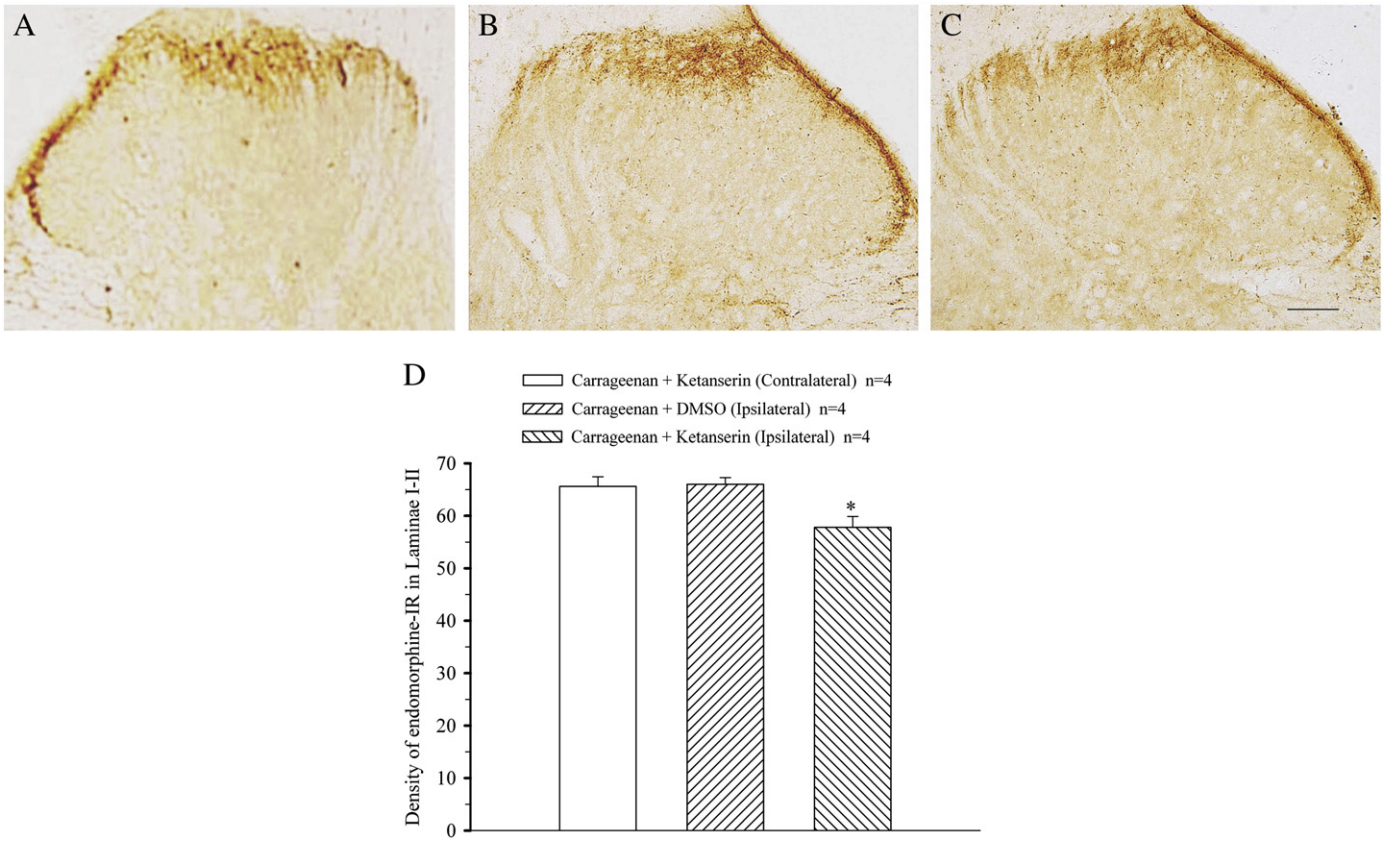

Fig. 6. Immunohistochemistry of endomorphine fibers in spinal dorsal horn. Carrageenan and 0.8% DMSO (B) or ketanserin (C) were injected i.pl at 0 and 1 h, respectively. The lumbar spinal cord was harvested at 24 h. The contralateral side (A) of the spinal dorsal horn following the treatment with carrageenan/ketanserin is also presented for comparison. Histograms (D) show mean pixels (±S.E.M.) of endomorphine-like immunoreactivity in laminae I–II of the spinal cord. N = 4 each. Scale bar = 500 μm.

However, the treatment with ketanserin significantly decreased the expression of endomorphine in superficial layers of the spinal cord compared to vehicle injection. Thus, this result suggests that the removal of biological activity of 5-HT facilitated the release of endomorphine from primary afferents to decrease the content of this peptide remaining in the afferent terminals in dorsal horn. Our observation was in agreement with previous studies showing that i.t. injection of cholera toxin and clonidine suppressed hypersensitivity and the expression of prodynorphin (Caudle et al., 2001) and met-enkephalin (Vazquez et al., 1998), respectively, in spinal dorsal horn. The release of opioid peptides following the injection of carrageenan in the periphery (inflamed paw) has been also observed (Kayser and Guilbaud, 1991). Taken together, the present study provided evidence at cellular level showing that the treatment with ketanserin enhanced endogenous opioid mechanisms at the site of inflammation, DRG and the spinal cord. The present study proposes that 5-HT inhibits inflammation-activated opioid mechanism, and that removing activity of 5-HT by the blockade of 5-HT_{2A} receptors disinhibits the endogenous opioid mechanism.

In summary, the treatment with the 5-HT_{2A} receptor antagonist ketanserin, but not vehicle, in inflamed paw increases the levels of β-endorphin and endomorphine in the local loci and the expression of μ-opioid receptors in DRG. This treatment also enhanced the release of endomorphine in spinal dorsal horn. The enhanced opioid mechanism following the blockade of 5-HT_{2A} receptors can be ascribed for the inhibition of increased expression of the pronociceptive mediator CGRP. Therefore, the present study suggests that the pronociceptive activity of 5-HT is mediated not only by enhancement of pronociceptive mediator but also by inhibition of intrinsic opioid mechanism during inflammation. As the treatment of ketanserin in the inflamed site reduced hyperalgesia (Huang et al., 2009) by both enhanced endogenous opioid mechanisms and reduced expression of pro-inflammatory mediator (this study), one would expect this antagonist to be very useful to inhibit pain in inflammation.

Acknowledgements

This work was supported by grants from Science and Technology Ministry of Fujian Province (2006F6001, 2008J0323) and the National Natural Science Foundation of China (30570600).

References

Abbott, F.V., Hong, Y., Blier, P., 1997. Persisting sensitization of the behavioural response to formalin-induced injury in the rat through activation of serotonin2A receptors. Neuroscience 77, 575–584.
Beck, P.W., Handwerker, H.O., 1974. Bradykinin and serotonin effects on various types of cutaneous nerve fibers. Pfluegers Arch. 347, 209–222.
Beland, B., Fitzgerald, M., 2001. Influence of peripheral inflammation on the postnatal maturation of primary sensory neuron phenotype in rats. J. Pain 2, 36–45.
Bolay, H., Moskowitz, M.A., 2002. Mechanisms of pain modulation in chronic syndromes. Neurology 59, S2–S7.
Cabot, P.J., Carter, L., Gaiddon, C., Zhang, Q., Schafer, M., Loeffler, J.P., Stein, C., 1997. Immune cell-derived beta-endorphin. Production, release, and control of inflammatory pain in rats. J. Clin. Invest. 100, 142–148.
Cabot, P.J., Carter, L., Schafer, M., Stein, C., 2001. Methionine-enkephalin-and dynorphin A-release from immune cells and control of inflammatory pain. Pain 93, 207–212.
Cai, M., Chen, T., Quirion, R., Hong, Y., 2007. The involvement of spinal bovine adrenal medulla 22-like peptide, the proenkephalin derivative, in modulation of nociceptive processing. Eur. J. Neurosci. 26, 1128–1138.
Cardenas, L.M., Cardenas, C.G., Scroggs, R.S., 2001. 5HT increases excitability of nociceptor-like rat dorsal root ganglion neurons via cAMP-coupled TTX-resistant Na(+) channels. J. Neurophysiol. 86, 241–248.
Caudle, R.M., Mannes, A.J., Benoliel, R., Eliav, E., Iadarola, M.J., 2001. Intrathecally administered cholera toxin blocks allodynia and hyperalgesia in persistent pain models. J. Pain 2, 118–127.

Di Rosa, M., Giroud, J.P., Willoughby, D.A., 1971. Studies on the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine. J. Pathol. 104, 15–29.
Ferreira, P.K., Campos, M.M., Calixto, J.B., 2000. The role of sensorial neuropeptides in the edematogenic responses mediated by B(1) agonist des-Arg(9)-BK in rats pre-treated with LPS. Regul. Pept. 89, 29–35.
Franca, D.S., Ferreira-Alves, D.L., Duarte, I.D., Ribeiro, M.C., Rezende, R.M., Bakhle, Y.S., Francischi, J.N., 2006. Endogenous opioids mediate the hypoalgesia induced by selective inhibitors of cyclo-oxygenase 2 in rat paws treated with carrageenan. Neuropharmacology 51, 37–43.
Francischi, J.N., Chaves, C.T., Moura, A.C., Lima, A.S., Rocha, O.A., Ferreira-Alves, D.L., Bakhle, Y.S., 2002. Selective inhibitors of cyclo-oxygenase-2 (COX-2) induce hypoalgesia in a rat paw model of inflammation. Br. J. Pharmacol. 137, 837–844.
Garry, M.G., Hargreaves, K.M., 1992. Enhanced release of immunoreactive CGRP and substance P from spinal dorsal horn slices occurs during carrageenan inflammation. Brain Res. 582, 139–142.
Gendron, L., Lucido, A.L., Mennicken, F., O'Donnell, D., Vincent, J.P., Stroh, T., Beaudet, A., 2006. Morphine and pain-related stimuli enhance cell surface availability of somatic delta-opioid receptors in rat dorsal root ganglia. J. Neurosci. 26, 953–962.
Gold, M.S., Reichling, D.B., Shuster, M.J., Levine, J.D., 1996. Hyperalgesic agents increase a tetrodotoxin-resistant Na + current in nociceptors. Proc. Natl Acad. Sci. USA 93, 1108–1112.
Herbert, M.K., Schmidt, R.F., 1992. Activation of normal and inflamed fine articular afferent units by serotonin. Pain 50, 79–88.
Heykants, J., Van, P.A., Woestenborghs, R., Gould, S., Mills, J., 1986. Pharmacokinetics of ketanserin and its metabolite ketanserin-ol in man after intravenous, intramuscular and oral administration. Eur. J. Clin. Pharmacol. 31, 343–350.
Hoyer, D., Hannon, J.P., Martin, G.R., 2002. Molecular, pharmacological and functional diversity of 5-HT receptors. Pharmacol. Biochem. Behav. 71, 533–554.
Huang, J., Cai, Q., Chen, Y., Hong, Y., 2009. Treatment with ketanserin produces opioid-mediated hypoalgesia in the late phase of carrageenan-induced inflammatory hyperalgesia in rats. Brain Res. 1303, 39–47.
Ji, R.R., Zhang, Q., Law, P.Y., Low, H.H., Elde, R., Hokfelt, T., 1995. Expression of mu-, delta-, and kappa-opioid receptor-like immunoreactivities in rat dorsal root ganglia after carrageenan-induced inflammation. J. Neurosci. 15, 8156–8166.
Kawamura, M., Kuraishi, Y., Minami, M., Satoh, M., 1989. Antinociceptive effect of intrathecally administered antiserum against calcitonin gene-related peptide on thermal and mechanical noxious stimuli in experimental hyperalgesic rats. Brain Res. 497, 199–203.
Kayser, V., Guilbaud, G., 1991. Physiological relevance and time course of a tonic endogenous opioid modulation of nociceptive messages, based on the effects of naloxone in a rat model of localized hyperalgesic inflammation. Brain Res. 567, 197–203.
Li, D., Ren, Y., Xu, X., Zou, X., Fang, L., Lin, Q., 2008. Sensitization of primary afferent nociceptors induced by intradermal capsaicin involves the peripheral release of calcitonin gene-related peptide driven by dorsal root reflexes. J. Pain 9, 1155–1168.
Machelska, H., Cabot, P.J., Mousa, S.A., Zhang, Q., Stein, C., 1998. Pain control in inflammation governed by selectins. Nat. Med. 4, 1425–1428.
Machelska, H., Schopohl, J.K., Mousa, S.A., Labuz, D., Schafer, M., Stein, C., 2003. Different mechanisms of intrinsic pain inhibition in early and late inflammation. J. Neuroimmunol. 141, 30–39.
Miletic, V., Tan, H., 1988. Iontophoretic application of calcitonin gene-related peptide produces a slow and prolonged excitation of neurons in the cat lumbar dorsal horn. Brain Res. 446, 169–172.
Mousa, S.A., Zhang, Q., Sitte, N., Ji, R., Stein, C., 2001. Beta-endorphin-containing memory-cells and mu-opioid receptors undergo transport to peripheral inflamed tissue. J. Neuroimmunol. 115, 71–78.
Mousa, S.A., Machelska, H., Schafer, M., Stein, C., 2002. Immunohistochemical localization of endomorphin-1 and endomorphin-2 in immune cells and spinal cord in a model of inflammatory pain. J. Neuroimmunol. 126, 5–15.
Neugebauer, V., Rumenapp, P., Schaible, H.G., 1996. Calcitonin gene-related peptide is involved in the spinal processing of mechanosensory input from the rat's knee joint and in the generation and maintenance of hyperexcitability of dorsal horn-neurons during development of acute inflammation. Neuroscience 71, 1095–1109.
Nitanda, A., Yasunami, N., Tokumo, K., Fujii, H., Hirai, T., Nishio, H., 2005. Contribution of the peripheral 5-HT 2A receptor to mechanical hyperalgesia in a rat model of neuropathic pain. Neurochem. Int. 47, 394–400.
Ohta, T., Ikemi, Y., Murakami, M., Imagawa, T., Otsuguro, K., Ito, S., 2006. Potentiation of transient receptor potential V1 functions by the activation of metabotropic 5-HT receptors in rat primary sensory neurons. J. Physiol. 576, 809–822.
Okamoto, K., Imbe, H., Morikawa, Y., Itoh, M., Sekimoto, M., Nemoto, K., Senba, E., 2002. 5-HT2A receptor subtype in the peripheral branch of sensory fibers is involved in the potentiation of inflammatory pain in rats. Pain 99, 133–143.
Oku, R., Nanayama, T., Satoh, M., 1988. Calcitonin gene-related peptide modulates calcium mobilization in synaptosomes of rat spinal dorsal horn. Brain Res. 475, 356–360.
Parada, C.A., Tambeli, C.H., Cunha, F.Q., Ferreira, S.H., 2001. The major role of peripheral release of histamine and 5-hydroxytryptamine in formalin-induced nociception. Neuroscience 102, 937–944.
Pertsch, M., Krause, E., Hirschelmann, R., 1993. A comparison of serotonin (5-HT) blood levels and activity of 5-HT2 antagonists in adjuvant arthritic Lewis and Wistar rats. Agents Actions 38 Spec No, C98-101.
Rowley, D.A., Benditt, E.P., 1956. 5-Hydroxytryptamine and histamine as mediators of the vascular injury produced by agents which damage mast cells in rats. J. Exp. Med. 103, 399–412.
Ryu, P.D., Gerber, G., Murase, K., Randic, M., 1988a. Actions of calcitonin gene-related peptide on rat spinal dorsal horn neurons. Brain Res. 441, 357–361.
Ryu, P.D., Gerber, G., Murase, K., Randic, M., 1988b. Calcitonin gene-related peptide enhances calcium current of rat dorsal root ganglion neurons and spinal excitatory synaptic transmission. Neurosci. Lett. 89, 305–312.
Schaible, H.G., Ebersberger, A., Von Banchet, G.S., 2002. Mechanisms of pain in arthritis. Ann. N.Y. Acad. Sci. 966, 343–354.
Sluka, K.A., Westlund, K.N., 1993. Behavioral and immunohistochemical changes in an experimental arthritis model in rats. Pain 55, 367–377.
Stanfa, L.C., Singh, L., Williams, R.G., Dickenson, A.H., 1997. Gabapentin, ineffective in normal rats, markedly reduces C-fibre evoked responses after inflammation. NeuroReport 8, 587–590.
Stein, C., Machelska, H., Schafer, M., 2001. Peripheral analgesic and antiinflammatory effects of opioids. Z. Rheumatol. 60, 416–424.
Sun, R.Q., Lawand, N.B., Willis, W.D., 2003. The role of calcitonin gene-related peptide (CGRP) in the generation and maintenance of mechanical allodynia and hyperalgesia in rats after intradermal injection of capsaicin. Pain 104, 201–208.
Theodosiou, M., Rush, R.A., Zhou, X.F., Hu, D., Walker, J.S., Tracey, D.J., 1999. Hyperalgesia due to nerve damage: role of nerve growth factor. Pain 81, 245–255.
Tramontana, M., Giuliani, S., Del, B.E., Lecci, A., Maggi, C.A., Evangelista, S., Geppetti, P., 1993. Effects of capsaicin and 5-HT3 antagonists on 5-hydroxytryptamine-evoked release of calcitonin gene-related peptide in the guinea-pig heart. Br. J. Pharmacol. 108, 431–435.
Vazquez, J., Covenas, R., Munoz, M., Garcia, J.A., Victoria, A., 1998. Modifications in the distribution of met-enkephalin in the cat spinal cord after administration of clonidine. An immunocytochemical study. Histol. Histopathol. 13, 955–959.
Wang, D., Gao, Y., Ji, H., Hong, Y., 2009. Topical and systemic administrations of ketanserin attenuate hypersensitivity and expression of CGRP in rats with spinal nerve ligation. Eur. J. Pharmacol. 627, 124–130.
Yu, L.C., Hansson, P., Lundeberg, S., Lundeberg, T., 1998. Effects of calcitonin gene-related peptide-(8–37) on withdrawal responses in rats with inflammation. Eur. J. Pharmacol. 347, 275–282.