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Original Article

Increased chromogranin A and neuron-specific enolase in rats with chronic nonbacterial prostatitis induced by 17-beta estradiol combined with castration

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Abstract: Although chronic nonbacterial prostatitis (CNBP) is a common diagnosis in middle-aged men, the etiology of this disease remains poorly understood. Neuroendocrine cells play an important role in the neuroendocrine regulation of the prostate, and chromogranin A (CgA) and neuron-specific enolase (NSE) are regarded as classic markers of neuroendocrine cells. This study aimed to determine CgA and NSE levels in a CNBP rat model to evaluate the role of neuroendocrine cells in the pathogenesis of CNBP. For developing a CNBP rat model, we examined the ability of 17-beta estradiol and surgical castration alone or in combination to induce CNBP. Histologic inflammation of the prostate was assessed in CNBP-induced rats by hematoxylin-eosin staining, whereas CgA and NSE protein levels were assessed by immunohistochemistry, Western blot analysis, and enzyme-linked immunosorbent assays. Our results showed that 17-beta estradiol combined with castration successfully induced CNBP and that CgA and NSE levels were increased in the prostate of CNBP rats as compared to those without CNBP. These findings indicate that the neuroendocrine regulation mediated by neuroendocrine cells may be involved in the pathogenesis of CNBP.

Keywords: Chromogranin A, neuron-specific enolase, chronic nonbacterial prostatitis, neuroendocrine

Introduction

Chronic prostatitis is a common disease that occurs primarily in middle-aged men and accounts for 25% of urology outpatients [1, 2]. As a commonly diagnosed type of chronic prostatitis, chronic nonbacterial prostatitis (CNBP) has a high recurrence rate and low cure rate, and can lead to male infertility and sexual dysfunction, thus affecting the patient's physical and psychological health and quality of life [3-6]. However, despite the importance and prevalence of CNBP, its etiology remains poorly understood. Hence, determining the pathogenesis of this condition could lead to treatments for improving the cure rate and reducing the recurrence rate.

Several studies have found that a neuroendocrine mechanism might be associated with the pathogenesis of chronic prostatitis [7-9]. In particular, the findings have suggested that neuroendocrine cells in the prostate might play a role in this process. Neuroendocrine cells (NECs) are distributed throughout normal prostate tissue, secrete a variety of biologically active substances, and play important roles in the growth and development of the prostate [10]. Protein chromogranin A (CgA) and neuron-specific enolase (NSE) are classic markers of neuroendocrine cells [11]. Previous studies on CgA and NSE have shown that CgA and NSE are increased in many diseases and have indicated both proteins function as important mediators of inflammation [12, 13]. However, no previous studies have examined the expression of CgA and NSE in CNBP. Thus, the present study aimed to investigate the potential role of CgA and NSE in CNBP by examining their expression in a CNBP rat model.

Materials and methods

Experimental induction of a CNBP rat model

Sixty male Sprague-Dawley rats weighing 250 ± 20 g were purchased from the Experimental Animal Center of Anhui Medical University. The

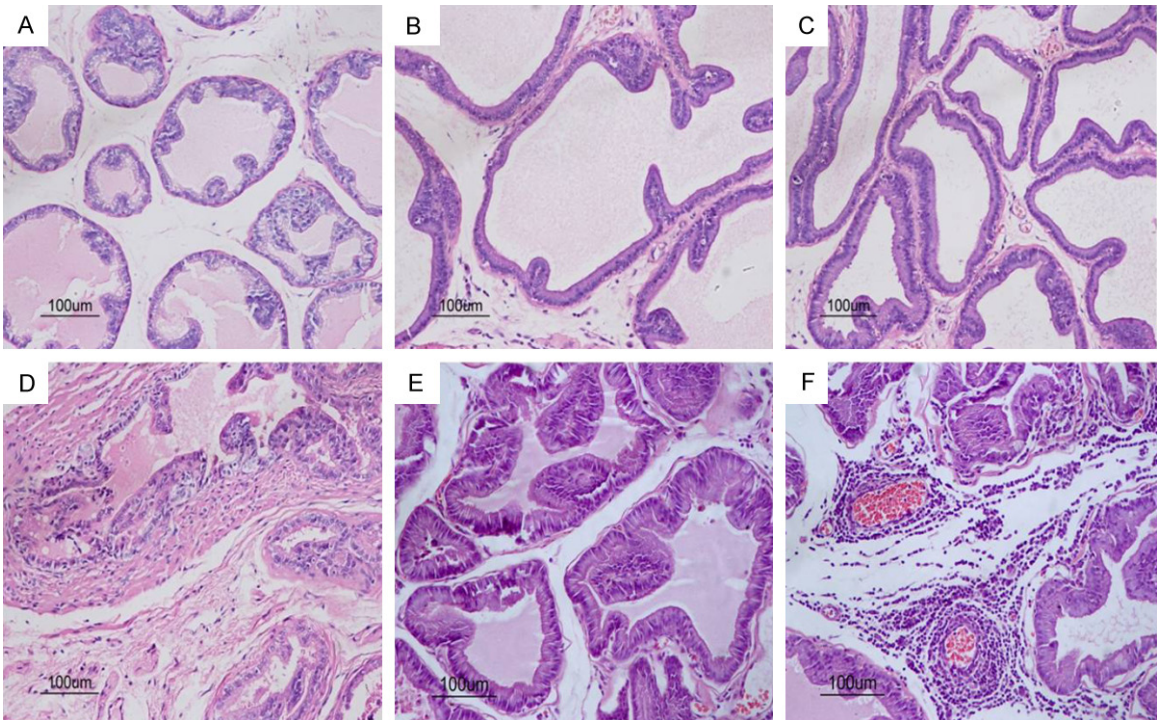

Figure 1. Histologic findings of the prostate in the six study groups. A. Vehicle control group. B. Normal saline group. C. Sham group. D. Castration group. E. 17-beta estradiol group. F. 17-Beta estradiol and castration group. The histopathology of the prostate was characterized by marked inflammatory cell infiltration. The inflammation changes were similar to those in clinical pathologic lesions.

animals were housed in a specific pathogen-free room with a controlled ambient temperature (22 ± 2°C) and humidity (40% ± 70%). The ethics committee of Anhui Medical University approved this study, and all the experiments were performed in accordance with the guidelines of Anhui Medical University. The rats were randomly assigned to the following groups based on the experimental treatment: Group 1: vehicle control; Group 2: the rats were injected subcutaneously with normal saline; Group 3: the rats received a sham operation; Group 4: the rats underwent surgical castration; Group 5: the rats were injected with 17-beta estradiol (0.25 mg/kg, Sigma, USA) for 30 consecutive days; Group 6: the rats underwent surgical castration and were injected 7 days later with 17-beta estradiol at the same dose and duration as Group 5. On day 38, all rats were anesthetized with chloral hydrate and sacrificed for further analysis (n = 10 per group).

Histological examination of prostate inflammation

The prostate was extirpated after fixation in 10% neutral buffered formalin and cut into coronal blocks. The tissue samples were dehydrated, embedded in paraffin, and sectioned at a thickness of 3 ± 4 mm. The sections were stained with hematoxylin-eosin and examined microscopically.

Western blot analysis of CgA and NSE

Isolated rat prostates were washed twice with ice-cold phosphate-buffered saline (PBS) and subsequently lysed in RIPA lysis buffer for 30 min on ice. The concentration of the extracted protein was determined by using the Lowry assay. A mixture of the lysates with 5 × loading buffer (1:4) was heated for 10 min at 95°C. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on precise 10% polyacrylamide gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Millipore, USA). Membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for 1 h. Then membranes with incubated with rabbit polyclonal antibody against CGA (1:1000 diluted in TBST, Abcam, USA), rabbit polyclonal antibody against NSE (1:800 diluted in TBST, Abcam, USA), and

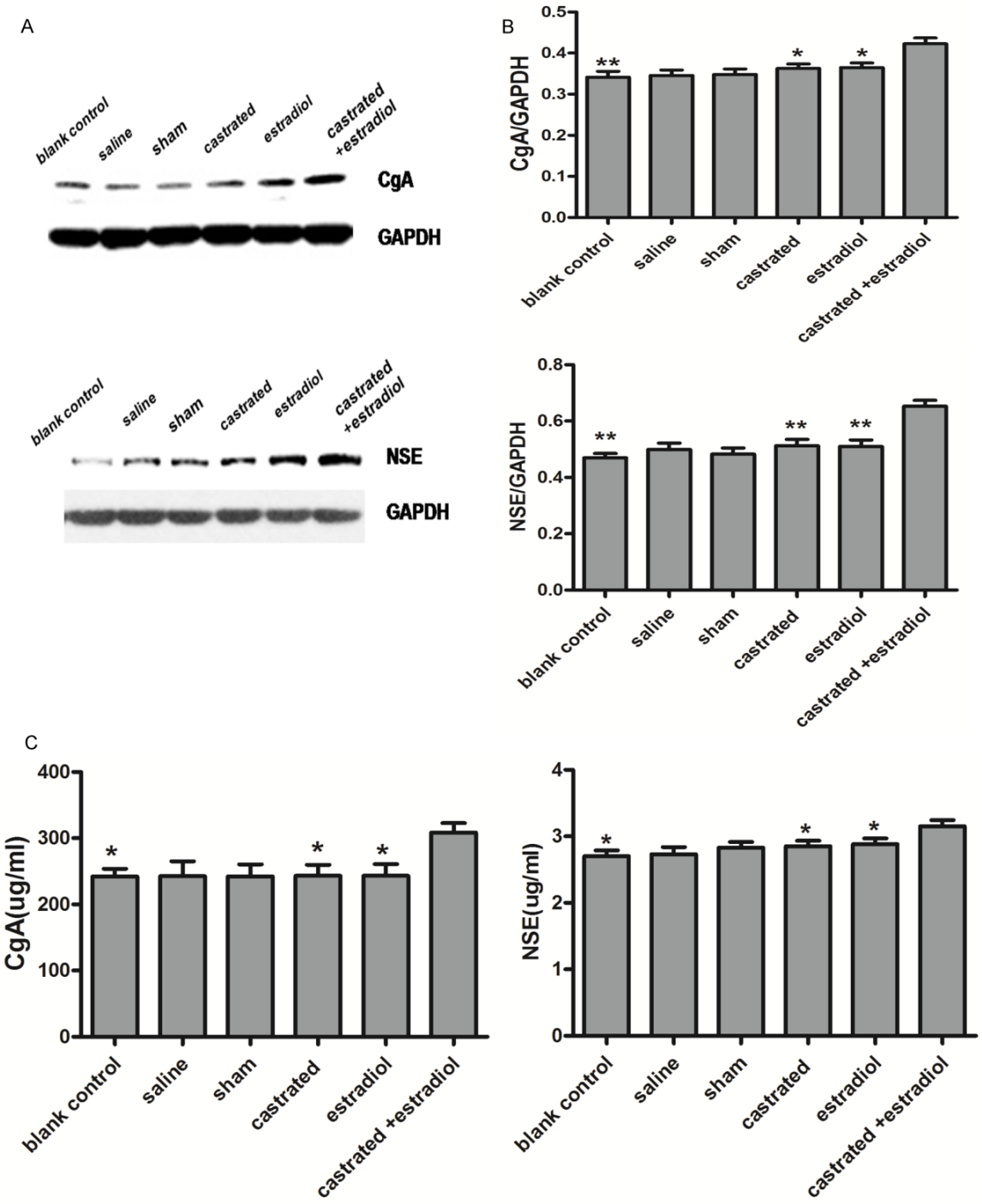

Figure 2. Increased expression of CgA and NSE in chronic nonbacterial prostatitis (CNBP). (A) CgA and NSE were detected in tissue lysates by Western blot analysis using anti-CgA and anti-NSE antibodies. Gels are representative of 10 rats per group. (B, C) Graphs represent quantitative analysis of the band intensity (B) and serum CgA and NSE concentrations, as detected by ELISA (C). Data are expressed as mean ± SE of 10 rats. $*P < 0.05$, $**P < 0.01$ as compared with the CNBP group.

glyceraldehydes-3-phosphate dehydrogenase (1:2000 diluted in TBST, DAKO, Denmark) as an internal control. Excess antibodies were washed three times with TBST for 15 min at room temperature. Membranes were then incubated at 37°C for 1 h with horseradish peroxi-

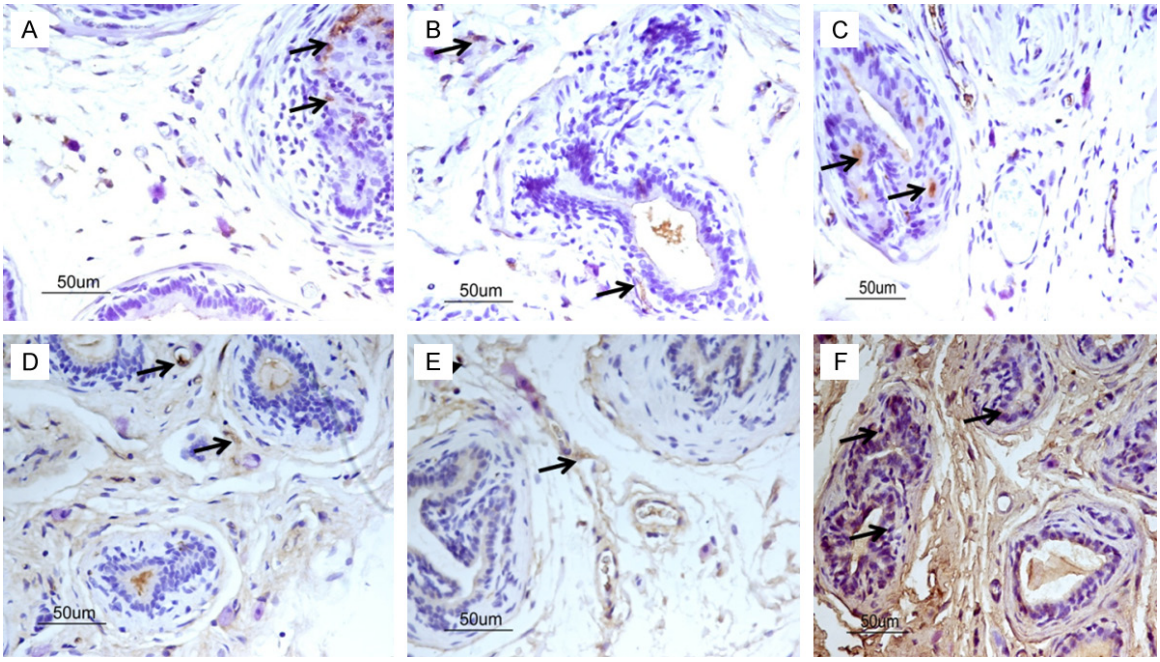

Figure 3. Representative immunohistochemistry findings for CgA subcellular localization (400 ×). A. Vehicle control group. B. Normal saline group. C. Sham group. D. Castration group. E. 17-Beta estradiol group. F. 17-beta estradiol and castration group. Positive staining waspresent in the cytoplasm and cell membrane (Scale bars = 50 μm).

dase-conjugated anti-rabbit secondary antibody (1:2000 dilution in TBST, DAKO, Denmark) and washed three times again with TBST for 15 min at room temperature. The membranes was treated with ECL (Pierce, USA) reagent and exposed on film. The Western blot results were quantitatively analyzed by optical density using Image J software.

Enzyme-linked immunosorbent assay (ELISA) analysis of CgA and NSE

Blood was obtained by the cardiac puncture method, and the serum CgA and NSE concentrations were determined with ELISA using CgA and NSE ELISA kits (R&D, USA) according to the manufacturer's instructions. The absorbance at 450 nm was measured colorimetrically on a microplate reader (BioTek Elx9808, USA).

Immunohistochemical staining

Formalin-fixed, paraffin-embedded prostate tissue sections were deparaffinized in xylene for 15 min, rehydrated with a graded ethanol series, and heated in a microwave oven at 750 W for 10 min in citric acid buffer (0.01 M, pH 6.0) for antigen retrieval. The slides were subsequently washed with PBS for 10 min and treated for 5 min with PBS containing 3% hydrogen peroxide to block endogenous peroxidase activity. After an additional wash with PBS for 5 min, the slides were blocked with blocking buffer for 1 h at room temperature before incubation over night at 4°C with anti-CgA antibody (1:100, Abcam, USA) and anti-NSE antibody (1:200, Abcam, USA). Immune complexes were detected using the SP9000 IHC immunochemistry kit (ZSGB Bio, Beijing, China) and 3,3'-diaminobenzidine (SK-4100, Vector).

Statistical analyses

All results are expressed as the mean ± standard error of the mean. Independent-sample *t*-test or one-way analysis of variance with a post hoc Bonferroni test was used for all statistical analysis. Values of $P < 0.05$ were considered significant.

Results

Histological findings

In Groups 1, 2, and 3, the glandular epithelium and stroma had a normal appearance, with no obvious leukocyte infiltration into the lumina or stroma (**Figure 1A-C**). In groups 4 and 5, no sig-

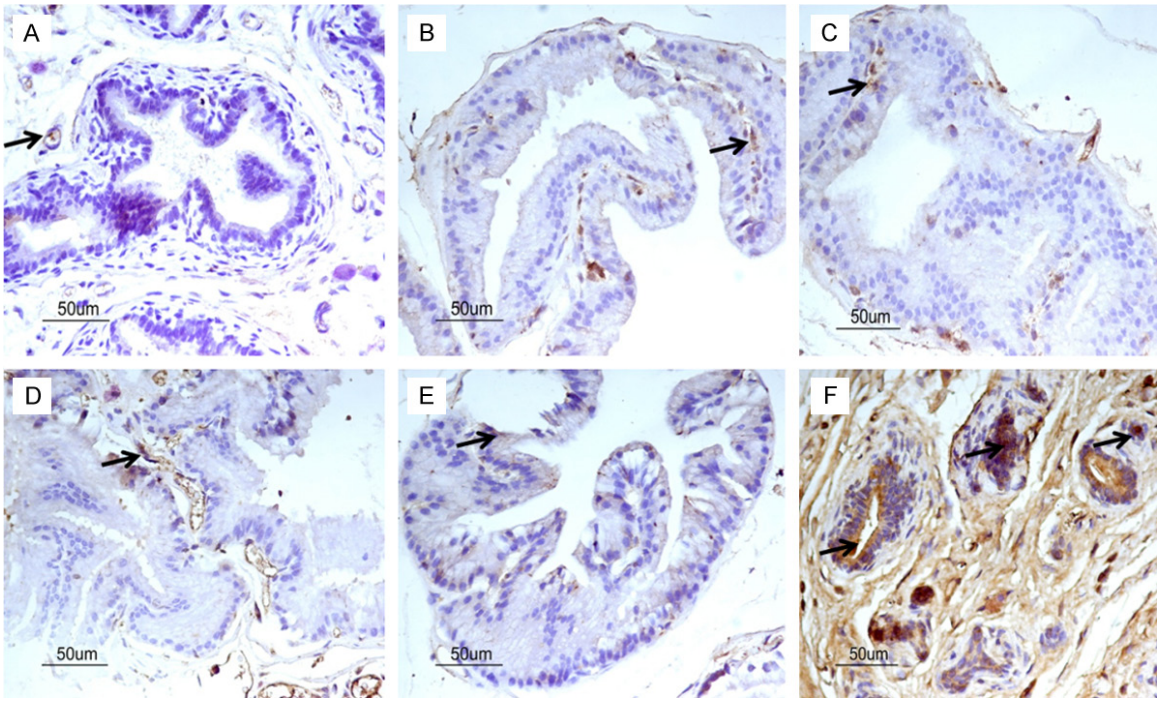

Figure 4. Representative immunohistochemistry findings for NSE subcellular localization (400 ×). A. Vehicle control group. B. Normal saline group. C. Sham group. D. Castration group. E. 17-Beta estradiol group. F. 17-Beta estradiol and castration group. Positive staining was detected in the cytoplasm.

nificant inflammatory lesions were evident (**Figure 1D**, **1E**). By contrast, epithelial degeneration and extensive infiltration of inflammatory cells in the glandular stroma were observed in group 6 (**Figure 1F**). The inflammatory infiltrate was primarily confined to the interstitial space and occasionally presented in the ductal lumen.

Increased CgA and NSE in the prostate of CNBP-induced rats

As shown by Western blot analysis, the expression of CgA and NSE was significantly increased in the prostates of Group 6 rats compared to the expression observed in the other groups (**Figure 2A**). Additionally, the ELISA results showed that the serum concentrations of CgA and NSE were also significantly higher in Group 6 than in the other groups (**Figure 2C**).

Immunohistochemical staining

CgA-positive staining was weakly detected in the cell membrane and cytoplasm of the prostates of Groups 1, 2, and 3 (**Figure 3A-C**) and was slightly increased in these subcellular locations in Groups 4 and 5 (**Figure 3D**, **3E**). By contrast, intense CgA-positive staining was observed in the cytoplasm and cell membrane in Group 6 (**Figure 3F**). Meanwhile, NSE-positive staining was observed in the cytoplasm in all six groups, and was weakly detected in Groups 1, 2, and 3 (**Figure 4A-C**), more strongly detected in Groups 4 and 5 (**Figure 4D**, **4E**), and was strongly detected in Group 6 (**Figure 4F**).

Discussion

Pontari et al. [14] demonstrated that the symptoms of CNBP appear to result from an interplay between psychological factors and dysfunction in the immune, neurological, and endocrine systems. In the present study, we observed increased expression of CgA and NSE in rats with induced CNBP. To our knowledge, no previous studies have investigated CgA and NSE protein levels in the prostate of SD rats with CNBP induced by 17-betaestrogen and castration.

Various animal models, including rat models, have been developed for the study of CNBP. Harris et al. [15] reported that estradiol administered subcutaneously in Wistar rats could induce prostate inflammation, with the inflam-

mation initially occurring in the dorsal prostate and subsequently in the ventral prostate. Wilson et al. [16] adopted a similar approach to establish successfully a chronic nonbacterial prostatitis Wistar rat model. Meanwhile, Kamijo et al. [17] used castration combined with estradiol injections to establish CNBP in Wistar rats for 10 months. In the present study, we were able to induce CNBP in male Sprague-Dawley rats by using 17-beta estradiol combined with castration (**Figure 1F**). The results also showed that neither estrogen applied for the time period used in the present study nor castration alone can induce apparent chronic inflammatory lesions in the Sprague-Dawley rat prostate (**Figure 1D**, **1E**). Within this context, Wistar rats have been reported to have a tendency to suffer from spontaneous prostatitis [18]. To our knowledge, spontaneous prostatitis has not been reported in Sprague-Dawley rats. Hence, the use of Sprague-Dawley rats for inducing CNBP could reduce experimental error in this animal species. In addition, the use of the present CNBP model is suitable for examining prostate neuroendocrine cell function, particularly in relation to hormonal regulation.

Neuroendocrine cells acting in a secretory and autocrine/paracrine fashion are distributed throughout the normal prostatic acini and ducts [19]. CgA and NSE are the most intensively studied prostate proteins and are classic markers of prostatic neuroendocrine differentiation [20-22]. Peptide hormones or pro-hormones are released from neuroendocrine cells by fusion of the granules with the cell membrane and exocytosis of the contents. Our immunohistochemical results showed that CgA was primarily located in the cytoplasm and cell membrane while NSE was located in the cytoplasm. Further, significantly increased CgA and NSE protein levels were observed in the prostate of CNBP rats than in that of the other groups. These results suggest neuroendocrine cells might be associated with the prostatic inflammation induced by the combination of estrogen and castration, although the particular mechanisms are yet to be investigated. Consistent with our findings, Ismail et al. [23] found that androgen ablation promotes neuroendocrine cell differentiation in the prostate of dogs and humans. Furthermore, our ELISA results showed that the serum concentrations of CgA and NSE were increased in the CNBP model compared with other groups. Sidhu et al. [24] reported increased serum CgA in irritable bowel syndrome and inflammatory bowel disease, and suggested that the basis of the pathogenesis might be the same for the two conditions.

Functionally, CgA and the CgA-derived peptides vasostatin-I and catestatin might be involved in regulating various inflammation processes [12]. Meanwhile, NSE is usually used to evaluate damage to the nervous system [25-27]. It is interesting that NSE levels were increased in CNBP rats in the present study. These results might be clinically significant because they suggest the possibility that chronic prostate pain might be caused by neurogenic inflammation.

Based on the collective results, we demonstrated that 17-beta estradiol combined with castration can induce CNBP in Sprague-Dawley rats and that expression of CgA and NSE was increased in CNBP rats. Our findings may provide initial information for further investigation of the role of neuroendocrine cells in the pathogenesis of CNBP. In further studies, we are planning to focus on the analysis of CgA and NSE function in the prostate.

Acknowledgements

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Disclosure of conflict of interest

None.

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