Impaired $\text{Na}^+ / \text{K}^+ - \text{ATP} \text{ase}$ Function in Patients with Interstitial Cystitis/Painful Bladder Syndrome

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INTRODUCTION

$\text{Na}^+ / \text{K}^+ - \text{ATPase}$ (NKA; sodium-potassium pump) is abundantly expressed in the basolateral membrane of epithelial cells and maintains cell homeostasis by transporting three $\text{Na}^+$ molecules out and two $\text{K}^+$ molecules into a cell, using the energy from the hydrolysis of adenosine triphosphate (ATP) (1,2). NKA is the primary active transporter that produces the driving force for the activation of other secondary ion transporters on cell membranes (1,2). The function of NKA controls a variety of ion and metabolite transport systems. Moreover, this pump alone accounts for approximately 30% of ATP consumption in animal cells for the purposes of ion regulation to maintain intracellular homeostasis (3,4).

NKA is also a potential regulator of tight junction formation and function (5-7). Tight junctions function as urothelial barriers between urine and the underlying bladder (8,9). They also play a critical role in the biogenesis of polarized epithelial cells during early vertebrate development and during recovery from tissue damage after ischemic or toxic injury (2,10). Previous studies have reported that tight junction protein expression is decreased in the bladder urothelium (11-13) and that increased potassium leakage causes clinical symptoms in patients with interstitial cystitis/painful bladder syndrome (IC/PBS) (14). Therefore, studies of changes in NKA function in bladder tissues may be important for development of appropriate therapeutic strategies in patients with bladder disease.

Here, we aimed to investigate changes in NKA expression and activity in bladder biopsies from patients with IC/PBS. To the best of our knowledge, this is the first study to investigate changes in NKA function in patients with this bladder disease.

MATERIALS AND METHODS

Patients and tissue samples

Bladder specimens were obtained from 85 patients (78 women and 7 men) with IC/PBS who were undergoing cystoscopy under anesthesia for diagnosis of IC/PBS or for therapeutic bladder hydrodistention. All IC/PBS patients were defined with the cystoscopic criteria of National Institute of Diabetes, Digestive, and Kidney Diseases (15) and had experienced clinical symptoms for more than 6 months. The average age of patients in the cohort was 41.2 ± 9.4 years. Patient backgrounds, including clinical symptoms and past therapeutic history, are shown in Table 1. All patients underwent medical history collection, physical examination, urinalysis, and culture for exclusion screening.
Four primary antibodies were used in this study as follows. Mouse monoclonal anti-NKA antibodies (α5; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) were used for immunoblotting (dilution 1:200). Rabbit polyclonal anti-NKA antibodies (EP1845Y; Abcam, Cambridge, MA, USA) were used for immunofluorescence (IF) staining after hydrodistension. Control specimens were obtained from 20 patients (median age: 42.7 years, range: 38-58 years; 18 women and 2 men who had undergone transurethral resection of the prostate more than 1 year prior) undergoing bladder neck suspension for stress urinary incontinence who had no symptoms of IC/PBS and showed no evidence of bladder mucosal disease after hydrodistension. Cytoplasts prepared as stated above were added to the sample buffer and heated to 60°C for 15 minutes. After electrophoresis and transfer, the blots were incubated sequentially in blocking buffer, primary antibodies (α5 or actin), and secondary antibodies. Finally, protein bands were detected using a SuperSignal West Pico Detection Kit (#34082; Pierce) as a standard.
were examined every 15 seconds for up to 20 minutes using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 340 nm and 37°C. The linear rate from 5 to 20 minutes for each pair of triplicate wells was determined. NKA activity was calculated as the difference in the slope of ATP hydrolysis (NADH reduction) in the presence or absence of ouabain, with activity expressed as μmol ADP per μg protein per hour.

**IHC staining**
The staining and microscopy methods were modified from our previous studies (19,23). The formalin-fixed, paraffin-embedded sections (4 µm) of biopsy samples were dewaxed and then rinsed with phosphate-buffered saline. Endogenous peroxidase was inactivated by incubating the sections with 3% hydrogen peroxide. The sections were stained with anti-NKA (α5) or phosphate-buffered saline (as a negative control) before analysis using a commercial kit. Sections were observed by optic microscopy (BX50; Olympus, Tokyo, Japan) with cooling CCD (DP72; Olympus) using CellSens software (CellSens standard version 1.4; Olympus).

**Multiple IF staining**
The method for IF staining was modified from our previous studies (18,20). In brief, paraffin-embedded sections were incubated at 4°C overnight with diluted primary antibodies (α5 or CK7) and then exposed to the appropriate secondary antibodies for 1 hour. Finally, the sections were covered with a coverslip using mounting solution (DAPI Fluoromount G, #0100-20; Southern Biotech, Birmingham, AL, USA). To identify and compare the localization of specific proteins, IF-stained samples were observed with a fluorescent microscope (BX50; Olympus) with cooling CCD (DP72; Olympus) using CellSens software (CellSens standard version 1.4; Olympus). Micrographs were subsequently merged to visualize differently colored labels simultaneously.

**Statistical analysis**
Differences between the groups were analyzed using Mann-Whitney U-tests, and all values are expressed as the mean ± standard error (SE). Differences with P values of less than 0.05 were considered significant. The analyses were performed with SPSS 12.0 software (SPSS, Chicago, IL, USA).

**Ethics statement**
This study was approved by the institutional review board of Tri-Service General Hospital (approval No. TC100-11).

**RESULTS**
The protein levels (1.08 ± 0.06 vs. 2.39 ± 0.29, respectively) and activity (0.60 ± 0.04 vs. 1.81 ± 0.18 μmol ADP/mg protein/hr, respectively) of NKA were significantly lower in the study group (~45% and ~1/3-fold, respectively) than in the control group (P < 0.05; Fig. 1 and 2). Additionally, the bladder urothelium was thinner in patients with IC/PBS (Fig. 3A) compared with patients in the control group (Fig. 3C), and IHC staining for NKA (red) showed weaker immunoreactivity in the study group (Fig. 3B) than in the control group (Fig. 3D). Furthermore, multiple IF staining for NKA (green) and CK7 (uroepithelial marker, red) demonstrated that these two targets colocalized in the bladder urothelium of patients with IC/PBS (Fig. 4). These staining results revealed that NKA was predominantly expressed in the bladder urothelium (Fig. 3 and 4). Taken together, these data showed that the expression and activity of NKA were lower in bladders of patients with IC/PBS than in control individuals.
Fig. 3. Representative tissue sections from patients in the study (A, B) and control (C, D) groups after negative control (A, C) or Na⁺/K⁺-ATPase (NKA) immunostaining (B, D). Magnification: 600 ×. The black double-headed arrow indicates the urothelium. Scale bar = 20 μm.

Fig. 4. Representative tissue sections from patients in the study group after multiple IF staining for Na⁺/K⁺-ATPase (NKA, green; A), CK7 (uroepithelial marker, red; B), and merged image (C). The arrow indicates the urothelium. Scale bar = 20 μm.
DISCUSSION

NKA is expressed in the basolateral plasma membrane of most epithelial cells (1,2) and plays a key role in maintaining cell homeostasis in the epithelium of organs and tissues, including the bladder (2,5). In the present study, our results showed that NKA was predominantly expressed in the bladder urothelium of both the study and control groups, similar to the results of other mammalian studies (24-26). Moreover, the expression and enzyme activity of NKA were decreased in patients with IC/PBS compared with those in the control group. We found no evidence that this pump compensated for potassium leakage in the study group. Thus, these results implied that bladder urothelial NKA was dysfunctional in patients with IC/PBS.

Rajasekaran et al. reported that NKA activity is necessary for tight junction formation in epithelial cells (5,7). The downregulation of tight junction proteins, such as zonula occludens-1, occludin, and E-cadherin, in the bladder urothelium in patients with IC/PBS has been reported previously (19,27). Moreover, inhibition of NKA ion transport function increases the permeability of tight junctions to ionic and nonionic solutes (28,29). The increased permeability and decreased tight junction formation in bladder urothelial cells was confirmed in patients with IC/PBS (11) and was shown to permit the migration of urinary solutes (8,12,14). In particular, high potassium levels in urine (i.e., 24-133 mEq/L) (8,14) could easily lead to leakage into interstitial tissue through dysfunctional bladder urothelium, causing depolarization of nerves and detrusor contractions and resulting in bladder pain, urgency, and increased frequency of urination in patients with IC/PBS (30-32).

Previous studies have reported that NKA is a signaling molecule involved in multiple cell signaling pathways. Inhibition of NKA function activates certain signaling, including Ras, phosphoinositol 3 kinase, mitogen-activated protein kinase (MAPK) and apoptotic pathway (7,33). Higher levels of bladder urothelial apoptosis have been observed in patients with IC/PBS (18,27). Therefore, dysfunction of bladder urothelial NKA may contribute to the observed increase in apoptosis.

In addition, NKA requires direct coupling with metabolic energy (i.e., ATP) (1,2). Our previous study showed that hypoxia-inducible factor-1α expression is increased in the bladder tissues of patients with IC/PBS (20), indicating that hypoxia occurring in these tissues may cause lower energy (ATP) production and impair NKA function (34-36). Taken together, these data suggested that decreased NKA expression and activity may be associated with tissues hypoxia (20,23), which could decrease tight junction formation (19) and increase apoptosis (18). These events would result in the formation of thinner, dysfunctional bladder urothelium in patients with IC/PBS.

There were several potential limitations to this study. First, patients’ clinical symptoms and disease duration may have been subjected to selection bias. Second, there are still no uniform standard criteria and definitions with which to diagnose this disease. Third, the proposed relationships among hypoxia, apoptosis, tight junctions, and NKA are based on the results of our previous studies (18-20,23).

In conclusion, we found that the expression and activity of NKA were decreased in bladder biopsies from patients with IC/PBS and that NKA was predominantly localized on the bladder urothelium. These findings suggest that impaired NKA function in the bladder urothelium may contribute to the diseased state in patients with IC/PBS.

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DISCLOSURE

No contributing authors have any conflicts of interest, including specific financial interests or relationships and affiliations relevant to the subject matter or materials discussed in the manuscript.

AUTHOR CONTRIBUTION

Conception and coordination of the study: Lee JD, Lee MH, and Yang WK. Consultant: Lee MH. Performed the experiments and analyzed the data: Yang WK. Organizer and wrote the paper: Yang WK. Review and revising the manuscript: Lee JD and Yang WK. Manuscript approval: all authors.

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