Original Article
Expression and subcellular localization of NHE$_3$ in the human gallbladder epithelium

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Abstract: Background: Enhanced gallbladder concentrating function is an important factor for the pathogenesis of cholesterol gallstone disease (CGD), but the mechanism is unknown. Potential candidates for regulation of gallbladder ion absorption are suggested to be Na$^+$/H$^+$ exchanger isoform 3 (NHE$_3$). In this study, we investigated the expression and subcellular localization of NHE$_3$ in both acalculous and calculus human gallbladders. Methods: Adult human gallbladder tissue was obtained from 23 patients (7 men, 16 women) who had undergone cholecystectomy. The patients were divided into two groups: Group A (acalculous group) and Group B (calculus group). Gene expression of NHE$_3$ was quantitatively estimated by real-time PCR. Protein expression was studied by Western blotting assays. Furthermore, expression of immunoreactive NHE$_3$ was investigated by immunohistochemistry. Results: There was no significant difference in the NHE$_3$ mRNA expression between calculous and acalculous human gallbladders. NHE$_3$ protein expression in gallbladders from patients with cholelithiasis is increased compared to those without gallstones. Immunohistochemistry studies prove that NHE$_3$ is located both on the apical plasma membrane and in the intracellular pool in human GBECs. Conclusions: NHE$_3$ may play a role in the pathogenesis of human CGD. Additional studies are required to further delineate the underlying mechanisms.

Keywords: Cholesterol gallstone disease (CGD), Na$^+$/H$^+$ exchanger isoform 3 (NHE$_3$), concentration

Introduction

Cholesterol gallstone disease (CGD) is one of the most common biliary tract diseases worldwide in which both genetic and environmental factors play roles in its pathogenesis. Currently, biliary cholesterol supersaturation is regarded as the principal cause of CGD when there is excess cholesterol or not enough solubilizing bile salts and phospholipids. However, despite its higher cholesterol saturation index (CSI), hepatic bile from patients with cholesterol gallstones usually nucleates more slowly than associated (more concentrated) gallbladder bile. On the other hand, dilution of model bile leads to progressively longer nucleation time. These phenomena demonstrate that bile concentration is another important pathogenic factor for CGD [1].

The gallbladder absorbs large amounts of biliary water. The concentrating function of gallbladder, which is abnormally enhanced during the early stage of CGD, acts to continuously increase the lithogenicity of the gallbladder bile [2]. The removal of water from the gallbladder bile involves different epithelial ion transport systems that lead to net NaCl apical absorption [3]. Previous investigations in a variety of species demonstrate that gallbladder Na$^+$ and Cl$^-$ absorption is predominantly mediated by the parallel Na$^+$/H$^+$ exchanger (NHE) and Cl$^-$/HCO$_3^-$ anion exchanger (AE) on the apical membrane of gallbladder epithelial cells (GBECs). However, little attention has been given to investigating the involvement of these proteins in CGD. Recently, Narins et al. [4] presented data showing that NHE$_3$ is expressed in the gallbladder epithelium. These findings point to a possible involvement of the protein in pathogenesis of CGD. We, therefore, hypothesized that altered expression of NHE$_3$ may be a precondition for the enhanced gallbladder concentrating function and an incline for cholesterol monohydrate to crystallize, an important pathogenic factor for CGD.

To investigate this hypothesis of a potential association between NHE$_3$ expression and CGD, we studied the expression of NHE$_3$ in

specimens from calculous and acalculous human gallbladder epithelia. Gene expression was quantitatively estimated by real-time PCR. Protein expression was studied by western blotting assays. Furthermore, expression of immunoreactive NHE$_3$ was investigated by immunohistochemistry.

Materials and methods

Human gallbladder specimens

Adult human gallbladder tissue was obtained from 23 patients (7 men, 16 women) who had undergone cholecystectomy for clinically-indicated reasons. The patients were divided into two groups. Group A (acalculous group; 3 men, 6 women; mean age 52.0 ± 8.5 years) comprised patients who had cholecystectomy for the reasons, including gastrectomy, adenomatous polyp and adenomyomatosis of gallbladder.Group B (calculous group; 4 men, 10 women; mean age 53.6 ± 10.2 years) comprised patients who suffered from cholelithiasis. All the patients from Group B had cholesterol-type gallstones. Tissues of Group A were all carefully selected from normal portions of the gallbladder mucosa, and were checked postoperatively by a pathologist to confirm that they were normal. Informed consent for surgical procedures and use of the specimens for research was obtained from all patients. Ethical approval for the research protocol was obtained by the Ethical and Scientific Committee of Shengjing Hospital of China Medical University.

Portions of the freshly excised tissue specimens were snap-frozen at -80°C for subsequent RNA and protein extraction, as described below. The remaining portions were fixed in formalin, embedded in paraffin, sectioned at 4 μm for immunohistochemistry.

RNA isolation and real time PCR

Total RNA was extracted from gallbladder tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA concentration and purity were assessed by spectrophotometry at 260 nm/280 nm. 2 μg of total RNA was reversetranscribed into cDNA using the TaKaRa RNA PCR kit (DRR037, TaKaRa, Dalian, China) following the manufacturer's protocol. Real time PCR was performed on Roche LightCycler (Roche, Basel, Switzerland). Sequences for the pri-

mers used were as follows: NHE$_3$: (forward) 5'-GTGGTGCTTCTGGATGGAGACA-3', (reverse) 5'-CTCCTCTTCACCTTCAGCCACT-3'. The relative level of mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method after normalization with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Results are expressed as the mean from duplicate values of gene expression in relation to GAPDH in the same RNA preparation.

Western blotting

Gallbladders obtained from both groups were homogenized in ice-cold buffer that contained complete ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail (Beyotime), and centrifuged at 14000 rpm for 10 min at 4°C. Protein concentrations were measured by using the bicinchoninic acid protein assay. Proteins (60 μg/lane) were separated by 8% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes and probed with anti-NHE$_3$ antibody (sc-16103-R, Santa Cruz Biotechnology, Santa Cruz, CA, 1:200 dilution). Membrane were then washed four times with TBST and incubated with a 1:2,000 dilution of the appropriate secondary antibody for 2 h at room temperature. Protein expression was normalized against GAPDH. All immunoblots were run at least in triplicate. Immunodetection was carried out using the ECL system. Quantity One software (Bio-Rad) was applied for the analysis of the optical density of the protein bands.

Immunohistochemistry

Human gallbladder tissue sections were deparaffinized and rehydrated by standard methods, treated with 0.3% H$_2$O$_2$ for 20 min at room temperature to reduce endogenous peroxidase activity, and then blocked by normal goat serum for 30 min. Slides were incubated for overnight with a polyclonal rabbit antibody for NHE$_3$ (sc-16103-R, Santa Cruz Biotechnology, Santa Cruz, CA, 1:100 dilution), followed by biotinylated goat anti-rabbit IgG antibody (SP-9000, ZSGB-BIO, Beijing, China) according to the manufacturer's protocol. Slides were incubated for another 30 min in horseradish peroxidase. As negative controls, tissue sections were stained as above but using irrelevant isotypic immunoglobulins or without the inclusion of
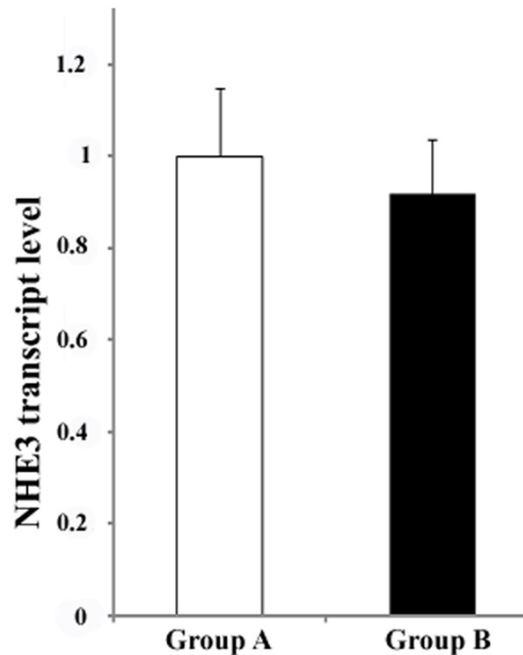
Figure 1. Real-time PCR was performed to measure the changes in NHE$_3$ mRNA levels. Transcript levels were calibrated based on GAPDH levels. All data is presented as the fold-change in NHE$_3$ gene expression.

the primary antibody. Finally, the slides were observed by microscopy.

Statistical analysis

Data were expressed as $\bar{x} \pm$ s. d. and analyzed using SPSS version 19.0 (SPSS Inc., Chicago, IL). Student's t test was used for independent samples and $P < 0.05$ was considered statistically significant.

Results

NHE$_3$ mRNA expression

In order to quantitatively estimate the comparative abundance of mRNA transcripts, real-time PCR was performed using total RNA preparations from seven acalculous and eleven calculous gallbladders. No significant differences were observed in the levels of NHE$_3$ mRNA between acalculous and calculous human gallbladders (**Figure 1**).

Western blotting assays of NHE$_3$ in human gallbladder

To determine whether CGD is associated with altered NHE$_3$ expression, western blotting as-
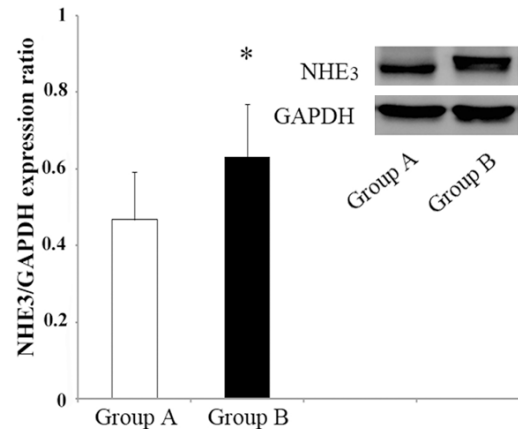

Figure 2. Protein concentrations of NHE$_3$ in acalculous and calculous human gallbladders. NHE$_3$ protein expression is increased in patients with CGD. Densitometry values were normalized to the signal from the housekeeping gene GAPDH.

says was performed. The results revealed a significant increase in NHE$_3$ protein expression in gallbladders from patients with cholelithiasis compared to those without gallstones (**Figure 2**). The discrepancy between the NHE$_3$ protein expression and mRNA levels may reflect enhanced stability of NHE$_3$ protein or increased translation of NHE$_3$ mRNA. The above results suggest possible relationship between altered gallbladder NHE$_3$ protein expression and CGD in the human.

Immunohistochemical distribution of NHE$_3$ in human gallbladder

Immunohistochemistry was used to evaluate the expression and subcellular localization of NHE$_3$ protein in calculous and acalculous human gallbladder epithelial sections. Overall, the immunohistochemical pattern of calculous gallbladders was similar to that of the acalculous gallbladders (**Figure 3**). The simple columnar epithelial cells that line the gallbladder prominently express NHE$_3$ protein. Immunostaining for the protein was apparent throughout the GBECs, with higher levels of stain evident in the apical aspects of the cells. In addition, intracellular NHE$_3$ signal was also noted, consistent with a report that NHE$_3$ shuttles from the plasma membrane (PM) to intracellular compartments in other cell types [5]. Little or no expression of the protein was detected in the underlying lamina propria of areolar tissue. NHE$_3$ expression is slightly increased in gallbladders with cholesterol gallstones. No immu-
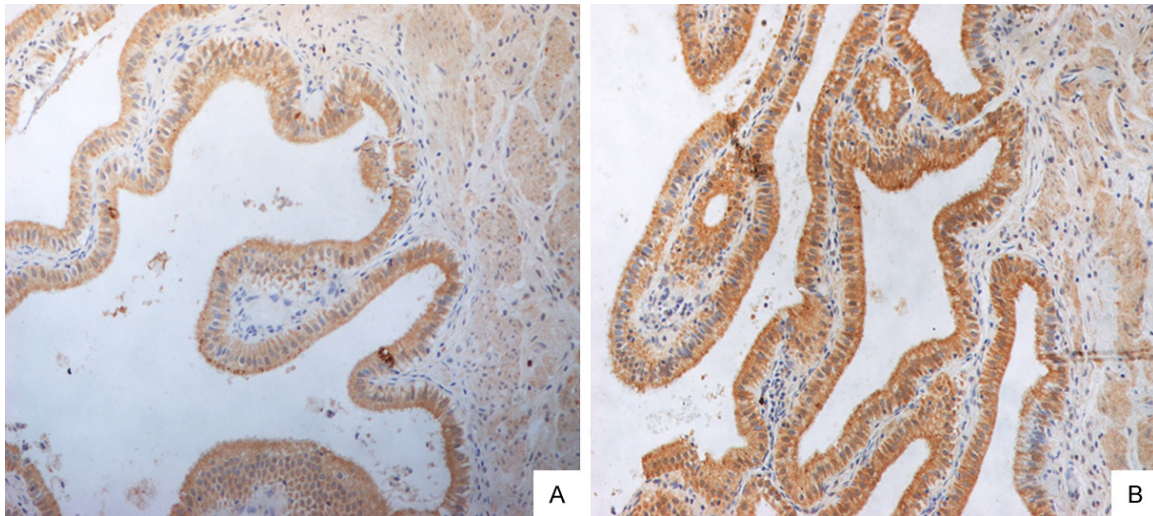
Figure 3. Gallbladder samples were stained for NHE$_3$. The protein appeared to be confined to the epithelial cells lining the lumen and no significant staining in any of the deeper cell layers was detected. NHE$_3$ expression is slightly increased in gallbladders with cholesterol gallstones. A: Normal gallbladder. B: Gallbladder with cholesterolosis. Original magnification × 200.

nostaining was observed when the NHE$_3$ antibody was omitted (results not shown).

Discussion

Although there have been previous studies on ion exchanger NHE$_3$ in *in vitro* murine cell models [6], prior to our study no data existed on comparative expression of NHE$_3$ in normal and diseased human gallbladders. Moreover, there was no research that studied in detail the subcellular localization of the protein in gallbladder tissue. In the present study, we have demonstrated that NHE$_3$ is diffusely distributed in human GBECs, with a focal intracellular pattern on the apical side of the cells. In addition, NHE$_3$ protein expression was significantly higher in human gallbladders with cholesterol stones than in normal gallbladders. This supports the idea that enhanced gallbladder concentrating function, which is a common finding during the process cholesterol gallstone formation, is the result of increased NHE$_3$ protein expression.

It has been long established that enhanced gallbladder concentrating function is an important factor in the pathogenesis of many gallbladder diseases, especially CGD. However, the underlying mechanisms remain mostly unclear. In healthy animals, protein kinase C (PKC), Ca^{2+}-calmodulin (CaM) and protein kinase A (PKA) hinder gallbladder concentrating function by inhibiting the rate of Na$^+$ and Cl$^-$ absorption.

In response to a lithogenic diet, ion transport is released from these inhibitory factors, resulting in enhanced gallbladder absorption [7]. A study by Giurgiu et al. has demonstrated an increased net Na$^+$ absorption during the early pre-crystal stage of gallstone formation [8]. The observed increase in Na$^+$ absorption was shown to be the result of an increase in gallbladder NHE activity [9]. These findings are in accordance with increased NHE$_3$ protein expression in calculous human gallbladders as demonstrated in the present study. Mobilization of preexisting NHE$_3$ transporters between intracellular pool and PM is a well-known mechanism of NHE$_3$ activity modulation [10-12]. The results of immunohistochemistry studies prove that NHE$_3$ is located both on the apical PM and in the intracellular pool in human GBECs. Our findings may add human gallbladder epithelium to the many other tissues in which trafficking mechanism plays an important role in the regulation NHE$_3$ activity.

The initiating factors that lead to the observed enhanced gallbladder concentrating function are not clear. Sine these changes occur only when cholesterol-supersaturated bile is present in the gallbladder, direct exposure of the gallbladder mucosa to lithogenic bile is highly suspected to be the trigger event [13]. Exposure of the gallbladder epithelium to cholesterol-supersaturated bile may increase the cholesterol content of GBECs and affect PM fluidity. A

link between PM cholesterol content and NHE$_3$ activity has been suggested by numerous studies, which have shown that reducing cholesterol content of lipid raft with methyl-β-cyclodextrin (MβCD) may decrease cellular Na$^+$/H$^+$ exchange by influencing NHE$_3$ trafficking [14, 15]. This effect of MβCD can be completely reversed by repletion of cholesterol. It is then entirely reasonable to suppose that high PM cholesterol content could result in increased NHE$_3$ activity and enhanced gallbladder absorption. However, additional investigations are required to identify the underlying mechanisms.

Of course, increased NHE$_3$ protein expression is not necessarily translated into enhanced ion exchange activity. Regulation of NHE$_3$ also involves at least another two mechanisms: regulation by altering the number of active transporters on the PM and the turnover number of each exchanger. A limitation of our study is that functional data with respect to NHE$_3$ transport activity in human GBECs were not gathered. This was due to the technical difficulties associated with studying ion transport in human GBECs. The ion transport of GBECs is influenced by a variety of factors including humoral, neural influences, lipids concentration and composition of gallbladder bile. Of all the tissues in the body, the gallbladder epithelium is uniquely positioned to face the highest concentrations of bile lipids for the longest time that has complex influence on its ion transport. We are not aware of a reliable *in vitro* functional assay for Na$^+$/H$^+$ exchange mediated by NHE$_3$ that can mimic this physiological state. Moreover, unlike dog or mouse GBECs, which can be readily cultured and passaged, human GBECs culture is much more technically demanding. Subsequent *in vitro* functional assays for NHE$_3$ would provide a clearer view into gallbladder ion transport and its relation to cholesterol gallstone formation.

In the present study, we used tissue samples from patients who did not have gallstones, as revealed in both preoperative and intraoperative imaging, but who underwent cholecystectomy for other reasons. These patients were used as a control study group for the gallbladders with cholelithiasis. We cannot rule out the possibility that the expression pattern of NHE$_3$ in this group of patients was not related to the coexisting pathologies of the stomach or gallbladder. However, it would not be expected for all these different pathologies to have the same effect.

In this study, we demonstrate that NHE$_3$ protein expresses both on the apical membrane and in the cytoplasm of human GBECs. NHE$_3$ expression is increased in protein level in gallbladders of patients with CGD. The results of our study suggest that NHE$_3$ may play a role in the pathogenesis of human CGD. Additional studies are required to further delineate the underlying mechanisms.

Disclosure of conflict of interest

None.

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