Role of Na\(^+\)/H\(^+\) exchanger isoform-1 in human inflammatory bowel disease

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I Khan, I Siddique, FM Al-Awadi, K Mohan. Role of Na\(^+\)/H\(^+\) exchanger isoform-1 in human inflammatory bowel disease. Can J Gastroenterol 2003;17(1):31-36.

BACKGROUND: Na\(^+\)/H\(^+\) exchanger (NHE) is responsible for a net uptake of sodium chloride and water from the gastrointestinal tract and maintains electrolyte and water homeostasis. However, its status in human inflammatory bowel disease such as ulcerative colitis (UC) and Crohn's disease (CD) remains poorly understood.

OBJECTIVES: To investigate the role of NHE-1 isoform in human CD and UC.

METHODS: Expression of NHE-1 protein and messenger ribonucleic acid and sodium pump activity were examined in the colonic biopsy samples taken from UC (n=11) and CD (n=13) patients using enhanced chemiluminescence-Western blot analysis, reverse transcription polymerase chain reaction and spectrophotometry. Subjects presenting with abdominal pain and endoscopically normal colon served as normal controls (n=11). Myeloperoxidase (MPO) activity and histology were performed to confirm tissue inflammation.

RESULTS: MPO activity increased significantly (P<0.05) in both UC and CD patients compared with the normal controls. Parallel to MPO activity profile, there was also a significantly higher infiltration of inflammatory cells in both cases. P-nitrophenylphosphatase activity, a marker of the sodium pump, remained unchanged in CD but increased significantly (P<0.05) in UC compared with the normal controls. On the contrary, the level of NHE-1 protein and messenger ribonucleic acid was significantly decreased (P<0.05) in both cases, whereas the internal control, a-actin remained unaltered.

CONCLUSIONS: These findings demonstrate a transcriptionally regulated suppression of NHE-1 in both UC and CD. This NHE-1 suppression may reduce an uptake of sodium chloride and water from the inflamed colonic lumen and thus contribute to diarrhea and neuromuscular alterations in these conditions.

Key Words: Crohn’s colitis; Inflammatory bowel disease; Myeloperoxidase; Na\(^+\)/H\(^+\) exchanger; Sodium pump; Ulcerative colitis

CONTEXTE : L’échangeur Na\(^+\)/H\(^+\) (NHE) est responsable d’une absorption finale de chlorure de sodium et d’eau du tractus gastro-intestinal et il maintient l’homéostasie des électrolytes et de l’eau. Toutefois, on ne comprend pas bien son influence dans les maladies intestinales inflammatoires des humains, telles la colite ulcéreuse (CU) et la maladie de Crohn (MC).

OBJECTIFS : Étudier le rôle de l’isoforme 1 du NHE dans la CU et la MC chez les humains.

MÉTHODOLOGIE : Au moyen de la réaction de Western-Blot, de l’épreuve de transcription inverse-amplification en chaîne par polymérase et de la spectrophotométrie, on a étudié l’expression de la protéine NHE 1 et de l’acide ribonucléique messager, ainsi que l’activité de la pompe à sodium dans des tissus obtenus par biopsie du colon de patients atteints de CU (n = 11) et de la MC (n = 13). Des sujets souffrant de douleurs abdominales dont l’endoscopie du colon était normale ont servi de témoins (n = 11). On a également effectué des examens histologiques et mesuré l’activité de la myélopéroxydase (MPO) afin de confirmer l’inflammation tissulaire.

RÉSULTATS : L’activité de la MPO et, parallèlement, une infiltration de cellules inflammatoires étaient remarquablement plus importantes (P < 0,05) chez les patients atteints de CU et de la MC que chez les témoins normaux. L’activité de la p-nitrophénylphosphatase, un marqueur de la pompe à sodium, est demeurée inchangée chez les patients souffrant de la MC, mais a significativement augmenté (P < 0,05) chez ceux atteints de CU, comparativement aux témoins normaux. Par contre, le taux de la protéine NHE 1 et de l’acide ribonucléique messager a diminué de façon significative (P < 0,05) dans les deux cas, tandis que le témoin interne, l’alpha-actine, est demeuré inchangé.

CONCLUSIONS : Ces observations révèlent une réduction du NHE 1 régulée par la transcription dans la CU et la MC. Cette réduction du NHE peut entraîner une diminution de l’absorption du chlorure de sodium et de l’eau à partir du lumen inflamé du colon et, par conséquent, contribuer à la diarrhée et aux modifications neuromusculaires causées par ces maladies.

Cations such as sodium (Na\(^+\)), potassium and calcium (Ca\(^2+\)) play an important role in several key biological processes. Homeostasis of these ions is compromised in inflammatory bowel disease (IBD), leading to motility dysfunc-

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Alteration in NHE-1 may affect the pH of the intracellular as well as the extracellular milieu and lead to inactivation of proteins and enzymes. In addition, the sodium pump fuels the NHE-1 isoform and may further aggravate electrolyte homeostasis. We hypothesize that alterations in the expression of the NHE-1 and/or sodium pump contribute to the pathophysiology and pathogenesis of CD and UC. Therefore, the primary aim of this study was to investigate the role of NHE-1 in human IBD.

SUBJECTS AND METHODS

Collection of human biopsies

Twenty-seven patients with chronic diarrhea and undergoing initial colonoscopy for suspected IBD were invited to participate in the study from February 1 to June 30, 2002. IBD was suspected on the basis of clinical presentation, laboratory investigations, and in some patients, radiological findings. Twenty-four of these patients were diagnosed with IBD (11 with UC, 13 with CD) on the basis of the typical colonoscopic findings (28) that were confirmed by histopathology. The remaining three patients were diagnosed with conditions other than UC and CD (nonspecific colitis in two, indeterminate colitis in one), and were excluded from the study. Patients with nonspecific abdominal symptoms compatible with irritable bowel syndrome (pain, cramps, bloating, etc.) but without diarrhea served as normal controls (n=11). These patients were undergoing colonoscopy to exclude an organic etiology of their symptoms and were found to have a normal colon. Neither the patients nor the controls were on any medication at the time of the colonoscopy. Biopsies for NHE-1 analysis were collected from the normal appearing mucosa at the splenic flexure of the colon in all patients with CD, nine patients with UC and all of the controls. Two patients with UC had involvement of the colon up to the splenic flexure. In these two patients, the biopsies were taken from normal appearing mucosa in the mid-transverse colon. The biopsies were quickly frozen in liquid nitrogen and transported to the laboratory where they were kept frozen at −70°C until used.

Repeated freezing and thawing of the samples were strictly avoided.

Preparation of crude microsomes

Biopsy samples were homogenized using 2 mL 3-(morpholino)propanesulfonic acid buffer, pH 7.4 containing 20 mM MOPS (Sigma, Germany), 250 mM sucrose, 10 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride and 5 mM dithiothreitol (11,12,29). The lysates were centrifuged at 5,000 x g (Beckman, United Kingdom) for 10 min at 4°C, and the supernatants collected were centrifuged at 110,000 x g for 45 min at 4°C. The pellets were suspended in 100 to 200 uL MOPS buffer and total protein was estimated using a dye binding assay kit (Biorad, Germany). Aliquots containing an equal amount of crude proteins were separated by 8% polyacrylamide gel electrophoresis (30). The proteins then were transferred to a nitrocellulose membrane (Biorad). After blocking with 5% milk solution in phosphate buffered saline, the filters were incubated with anti-human NHE-1 1° antibodies for 1 h. After washing, the filters were incubated with antirabbit 2° antibodies-horseradish peroxidase conjugate (Sigma, Germany) for 1 h. The filters were then washed and the bands were developed using the components of an enhanced chemiluminescence kit (Amersham, United Kingdom). The bands were scanned using a densitometer (Ultrascan, Pharmacia, Sweden). All steps were carried out at room temperature unless specified otherwise.

Sodium pump activity

Potassium-stimulated-ouabain sensitive p-nitrophenylphosphatase is a partial activity of and is used as a marker of the sodium pump (29). Aliquots (10 to 20 ug) of crude microsomes were used to estimate the activity using a buffer containing 3 mM imidazole, pH 7.5, 5 mM potassium chloride, 5 mM magnesium chloride, 1 mM p-nitrophenylphosphate (Sigma) and in the presence and absence of 1 mM ouabain (Sigma). The reaction was performed at 37°C for 1 h and optical density was obtained at 420 nm spectrophotometrically (Beckman 5000). An enzyme unit is defined as mmol of the product released/min/mg protein at 37oC. Units were calculated using the molar extinction coefficient (1.32×10⁴) of p-nitrophenol.

Reverse transcription-polymerase chain reaction amplification

Total ribonucleic acid (RNA) was extracted from human biopsy samples using a Trizol RNA extraction kit (GIBCO, United Kingdom). Quality of total RNA was analyzed by formaldehyde agarose gel electrophoresis. Aliquots (5ug) of total RNA were reversed transcribed using oligo dT primer (500 ng) and superscript kit (GIBCO, United Kingdom) following the standard procedures (31-32). Aliquots of reverse transcribed material were amplified using the NHE-1 specific sense (5’-TCACCATCGACCGGCGT-3’) and antisense (5’-CCTGGCTGCCCCTGGGTTA-3’) primers from 2558 base pairs (bp) to 2575 bp and 2855 bp to 2872 bp from a published complementary (c) DNA sequence (33). Actin specific sense (5’-GTCACTCCAAATATGAGATGC-3’) and antisense (5’-TTCAAAATACAAAACAAAAATTTGGATT-3’).
primers were designed from 1441 bp to 1462 bp and 1612 bp to 1640 bp of actin complementary DNA sequence (accession no. BC001301). These primers were synthesized to amplify a 315 bp (NHE-1) and a 200 bp (actin) fragment. The primers were synthesized and purified in the Department of Biochemistry, Faculty of Medicine, Kuwait University, Kuwait.

**Messenger (m)RNA levels**
Aliquots containing equal amounts of total RNA were reverse transcribed using standard procedures and the levels were amplified using specific primers. As a control, actin was coamplified with the target mRNA. In addition, the amplification was established in linear range for quantitation. The levels were calculated and compared to the controls using the band density obtained by a densitometer (UltoScan, Pharmacia, Sweden).

**Data analysis**
Data are presented as mean ± SD. Significance was calculated using student’s t-test, and a t-value of P<0.05 was considered to be statistically significant.

**RESULTS**

**Subjects**

| Subjects          | Ratio M:F | Mean age (years ± SD) |
|-------------------|-----------|-----------------------|
|                   |           | M         | F         |
| Control           | 7:4 (n=11)| 39±6     | 38±12    |
| Crohn’s disease   | 7:6 (n=13)| 34±13    | 39±14    |
| Ulcerative colitis| 7:4 (n=11)| 32±9     | 35±12    |

F Female; M Male

**Figure 1** Myeloperoxidase (MPO) activity (units) in the normal controls (n=11), Crohn’s disease (CD)(n=13) and ulcerative colitis (UC) (n=11) colonic biopsy samples. Data are mean ± SD.

**Figure 2** Representative micrograph showing hematoxylin and eosin stained sections of the normal control (A), Crohn’s (B) and ulcerative colitis (C) colonic biopsies.

This study used a total of 35 patients that included CD (n=13), UC (n=11) and normal control (n=11) subjects. In each group, both male and female subjects were recruited whose mean age in each group was not statistically (P>0.05) different (Table 1).

**Myeloperoxidase activity**
There was a significant increase in myeloperoxidase (MPO) activity in both CD and UC patients compared with normal controls (Figure 1). In addition, MPO activity in the CD group was significantly (P<0.05) higher than that in the UC group (Figure 1). Furthermore, infiltration of
inflammatory cells in CD (Figure 2B) and UC (Figure 2C) was also (P<0.05) higher compared with the normal IBS control as seen under light microscope (Figure 2A).

NHE-1 protein and mRNA
Crude microsomal yield from the CD and UC patients' biopsies was not significantly (P>0.05) different from the normal control (Figure 3). However, the yield of crude microsomes from the UC was significantly lower (P<0.05) than that from the CD (Figure 3). There was a significant decrease in the level of NHE-1 protein in both CD and UC biopsy crude microsomes (Figure 4A and 4B), whereas actin levels remained unchanged (Figure 4A and 4B).

The amount of total RNA extracted from the biopsy samples was expressed as ug/mg tissue. The yield of total RNA from the CD or UC biopsy samples was not significantly different (P>0.05) from the normal control (Figure 5). NHE-1 mRNA level were also decreased significantly both in the UC and CD patients' biopsies (Figure 6), whereas the internal control level of actin mRNA remained unchanged in these groups (Figure 6). In addition, the quality of total RNA from each group used was comparable with controls (not shown).

Sodium pump activity
The sodium pump plays an important role in regulating the activity of NHE-1. The sodium pump activity measured in terms of p-nitrophenylphosphatase activity in UC was significantly higher (P<0.05) than the normal controls (Figure 7). However, this activity in CD patients was not significantly different (P>0.05) compared with the normal controls or UC patients (Figure 7).

DISCUSSION
The basis of this study came from our recent findings reporting induction of NHE-1 and -3 isoforms in the colon, ileum and kidneys in experimental colitis (11-13). In addition, a recent study has provided evidence of the beneficial effect of NHE-1 blockage in experimental colitis (34). Suppression of sodium pump activity has also been reported previously in an animal model of colitis (14). Because the sodium pump provides a driving force for the activity of NHE-1, these transporters alone or together may contribute to the pathophysiology and pathogenesis of these conditions. To test this hypothesis further, the present study was undertaken to investigate the role of NHE-1 in human IBD. In this study, we focused on the NHE-1 isoform, because this is mainly responsible for pHi regulation and other functions commonly compromised in IBD conditions.

The present findings demonstrate a marked elevation of MPO activity in CD compared with that in UC, indicating the presence of the inflammatory cells, which was supported by our histology data. In addition, there was an evidence of ulcers in the tissue sections from UC patients, whereas granulomas and active edema were present in CD. These findings taken together confirm that the cases selected for this study had inflammatory basis. Furthermore, there was no difference in the age of the male and female patients in each group used in this study, thus ruling out a sex or age relationship of these conditions. Several studies have reported such an association. This discrepancy could be related to demographic, genetic or ethnic differences which remain to be elucidated at least in this part of the world.

Based on animal model studies, we expected an induction of NHE-1 expression in human IBD, but it was suppressed in both CD and UC patients, indicating a pathophysiological disparity between the animal models and human IBD condition. In this context, it is relevant to mention that such a suppression of NHE-1 has been shown by extracellular aci-
dosis in other cell types (35). Although in our population we did not measure intracellular pH, this suppression may contribute to a reported cellular acidosis similar to what has already been reported in IBD (9,10). In addition, activation or no change in sodium pump activity together with suppression of NHE-1 (in the present study) may result in the diminution of intracellular Na+, which might decrease the intracellular Ca²⁺ level. Thus, a reduced Ca²⁺ level may be a factor responsible for decreased contractility reported earlier in these conditions (36,37). Nevertheless, these changes do not seem to be due to a parallel increase in the crude microsomes or total RNA yield in both cases. In addition, invariant expression of actin supports the specificity of the changes reported here. A parallel change was also reflected in the NHE-1 mRNA isoform, indicating a possibility of transcriptional regulation; however, phenomena such as stability and transport of mRNA remain a distinct possibility, which requires further study. Although it is difficult to assign these changes to a particular cell type in this study, based on the fact that the biopsies were mucosal and the major cell population is epithelial in type, we believe that these changes are reflected majorly in the epithelial cells. Nevertheless, it is also possible that the other cell types such as inflammatory cells are also likely to contribute to the reported decrease in NHE-1 levels. It is worth mentioning that the contribution of factors such as presence of diarrhea or intake of medication, which could affect the NHE-1 expression, is also ruled out, because the subjects included...
were not having diarrhea in the controls or taking any medication.

CONCLUSIONS

We demonstrate a decrease in the NHE-1 mRNA and protein levels in the UC and CD patients’ colonic biopsies. Suppression of NHE-1 should leave more unabsorbed Na+ and water in the inflamed lumen, thus contributing to diarrhea and electrolyte disturbance in these conditions. In addition, this suppression may compromise on the normalization of acidic pH and hence contribute to tissue necrosis by altering certain enzymes and proteins. These findings provide the first evidence of the role of NHE-1 in the pathogenesis of Crohn’s disease and ulcerative colitis and may be considered an important parameter in the management of these conditions.

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