Intestinal NHE8 is highly expressed in goblet cells and its expression is subject to TNF-α regulation

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Xu H, Li Q, Zhao Y, Li J, Ghishan FK. Intestinal NHE8 is highly expressed in goblet cells and its expression is subject to TNF-α regulation. Am J Physiol Gastrointest Liver Physiol 310: G64–G69, 2016. First published November 12, 2015; doi:10.1152/ajpgi.00367.2015.—While the intestine plays an important role in digestion and absorption, the mucus lining the epithelium represents a pivotal function in mucosal protection. Goblet cells are scattered in both the crypts and among enterocytes, and they secrete an important component of mucus, mucin. We have reported that sodium/hydrogen exchanger (NHE) 8 is a novel player in mucosal protection, since loss of NHE8 function resulted in reduced mucin production and increased bacterial adhesion. While NHE8 has been shown to be expressed in enterocytes and its expression is reduced during intestinal inflammation, nothing is known about the role of NHE8 in goblet cells. This current study is designed to define the expression of NHE8 and the role of TNF-α in the regulation of NHE8 in goblet cells. Using HT29-MTX cells as an in vitro model, we detected abundant NHE8 mRNA in goblet cells. Immunohistochemical staining localized NHE8 protein on the plasma membrane and in the intracellular compartments in goblet cells. Furthermore, NHE8 expression in goblet cells is regulated by the proinflammatory cytokine TNF-α. The expression of NHE8 in HT29-MTX cells was significantly reduced at both mRNA and protein levels in the presence of TNF-α. This inhibition of NHE8 mRNA expression could be blocked by the transcriptional inhibitor actinomycin D. Promoter reporter assay showed that NHE8 promoter activity was indeed reduced by TNF-α. Mechanistically, TNF-α reduced Sp3 protein binding to the human NHE8 basal promoter region. Therefore, NHE8 is expressed in goblet cells, and the inflammatory cytokine TNF-α downregulates NHE8 expression by a transcriptional mechanism.

The basolateral membrane of gastric parietal cells and is important for acid secretion (8). NHE8 is unique compared with these NHEs. It has roles in the two major cellular phenotypes of the intestinal epithelium, enterocytes that absorb nutrients and goblet cells that make mucin. NHE8 is expressed at the apical membrane of the intestinal epithelial cells, and it mediates sodium-dependent proton exchange and plays important roles in mucosal protection (27, 29, 31). Whereas enterocytes merely form a cellular barrier, goblet cells participate in mucosal immunity by secreting gel-forming mucins that provide an additional layer of protection. Earlier studies have shown that loss of NHE8 resulted in decreased mucin production in the colon (31), suggesting a possible role of NHE8 in the goblet cells. In this study, we identified the expression of NHE8 in goblet cells and studied the effect of TNF-α, a well-known colitis-related proinflammatory cytokine, on NHE8 expression in goblet cells.

MATERIALS AND METHODS

Cell culture. Human intestinal goblet cells (HT29-MTX) were kindly provided by Thécla Lesuffleur (INSERM, France). Cells were cultured in DME medium (HyClone; GE Healthcare Life Sciences, Logan, UT) containing 10% fetal bovine serum (HyClone) and 1% penicillin-streptomycin (HyClone). Human intestinal epithelial cells (Caco-2) were purchased from American Type Culture Collection (Manassas, VA) and were cultured in MEM-NEAA medium (HyClone) containing 20% fetal bovine serum (HyClone) and 1% penicillin-streptomycin (HyClone). Both cell types were maintained at 37°C in a 95% air-5% CO2 atmosphere and passaged every 72 h. For TNF-α experiments, cells were exposed to different concentrations of human recombinant TNF-α (Peprotech, Rocky Hill, NJ) for 18 h before harvest. To study if the effect of TNF-α on NHE8 expression involves transcriptional regulation, cells were treated with actinomycin D (100 nM) for 18 h in the presence or absence of TNF-α.

Functional characterization of NHE8 in HT29-MTX cells. Cells were seeded on glass cover slips and were cultured for 40 h before pHi was measured. The activity of NHE8 was monitored by measuring the rate of Na+-dependent recovery of pHi after acid load in HCO3−−free HBSS as described in previous publications (23, 25). pHi was assessed by monitoring the fluorescence emission of the pH-sensitive dye SNARF 4-AM (Invitrogen, Carlsbad, CA). The ratio of fluorescence intensity (640 nm/570 nm) was measured for individual cells, and these ratios were subsequently converted to pHi by means of an in situ-derived calibration curve.

RNA purification and PCR analysis to detect NHE8 expression. RNA was purified from HT29-MTX cells and Caco-2 cells using Trizol reagent (Invitrogen). TaqMan technology was used to determine the expression of NHE2, NHE3, and NHE8 using human NHE2 (Hs00268166-m1), NHE3 (Hs00188200-m1), NHE8 (Hs00392302-m1), and TATA-binding protein (TBP; Hs00427620-m1) primers purchased from Applied Biosystems (Foster City, CA). Resulting data were analyzed using the comparative cycle threshold (Ct) method. The target gene cycle thresholds were adjusted relative to a calibrator (normalized Ct) value obtained from

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Identification of NHE8 expression in HT29-MTX cells. Real-time PCR was performed on RNA isolated from cultured HT29-MTX and Caco-2 cells to compare NHE2, NHE3, and NHE8 gene expression. As shown in Fig. 1A, only NHE2 and NHE8 were detected by Real-time PCR in HT29-MTX cells. NHE8 expression in HT29-MTX cells was higher than in Caco-2 cells (1.57 ± 0.03 in HT29-MTX cells compared with 1.01 ± 0.03 in Caco-2 cells; n = 4, P = 0.00001), and NHE2 expression in HT29-MTX cells was lower than in Caco-2 cells (0.08 ± 0.01 in HT29-MTX cells compared with 1.01 ± 0.03 in Caco-2 cells; n = 4, P < 0.00001). Immunohistochemical staining using NHE8 antibody also detected plasma membrane and intracellular compartment localization of NHE8 in HT29-MTX cells as well as in mouse colonic tissue (Fig. 1B).

NHE8 functions as NHE in HT29-MTX cells. Using a NHE specific inhibitor, we identified that only NHE1 and NHE8 activity was detected at functional levels in HT29-MTX cells. The rate of pH recovery in HT29-MTX cells was 0.169 ± 0.039 pH/min in the absence of HOE-694 and 0.038 ± 0.008 pH/min in the presence of 1 μM HOE-694. HT29-MTX cells failed to recover from acid load in the presence of 10 μM HOE-694 (Fig. 1C).

Effect of TNF-α treatment on NHE8 protein expression in HT29-MTX cells. NHE8 protein expression in HT29-MTX cells after exposure to standard or TNF-α-containing medium was assessed by Western blot. As shown in Fig. 2, TNF-α treatment reduced NHE8 protein abundance in HT29-MTX cells, and this inhibition is dose-dependent. TNF-α concentration at <10 ng/ml had no effect on NHE8 protein abundance in HT29-MTX cells (0.175 ± 0.004 in TNF-α-treated cells compared with 0.185 ± 0.004 in control cells). When the concentration of TNF-α was 30 ng/ml, NHE8 protein expression was reduced from 0.185 ± 0.004 in control cells to 0.12 ± 0.07 in TNF-α-treated cells. At 100 ng/ml TNF-α, NHE8 protein expression was also reduced, from 0.185 ± 0.004 in untreated cells to 0.115 ± 0.011 in treated cells.

Effect of TNF-α treatment on NHE8 mRNA expression in HT29-MTX cells. RNA was purified from cells exposed to standard or TNF-α-containing medium (30 ng/ml TNF-α) and was used for Real-time PCR analysis. As shown in Fig. 3A, NHE8 mRNA expression in HT29-MTX cells was significantly decreased by TNF-α treatment (1.00 ± 0.03 in control cells compared with 0.46 ± 0.09 in TNF-α-treated cells; n = 6, P = 0.009). In the presence of 100 nM actinomycin D (Act D), TNF-α-induced NHE8 mRNA expression inhibition was completely blocked (0.45 ± 0.03 in Act D-treated cells compared with 0.47 ± 0.01 in Act D- and TNF-α-treated cells; n = 3) (Fig. 3B).

Effect of TNF-α treatment on NHE8 gene promoter activity. To explore if the human NHE8 gene promoter activity is affected by TNF-α, HT29-MTX cells were first transfected with NHE8 gene promoter constructs and then treated with TNF-α (30 ng/ml) for 18 h before promoter activity was studied. The activity of human NHE8 promoter in HT29-MTX cells was decreased in the presence of TNF-α. TNF-α treatment reduced pGL3b−671 promoter activity from 1.02 ± 0.02 in control cells to 0.61 ± 0.06 in treated cells (n = 6, P = 0.00004). TNF-α treatment also reduced pGL3b−32 promoter activity from 1.00 ± 0.02 in control cells to 0.58 ± 0.11 in treated cells (n = 6, P = 0.008) (Fig. 4).

Identification of trans-factor and cis-element involved in TNF-α response of the human NHE8 promoter in HT29-MTX cells. GMSA was used to study the DNA/protein interaction involved in TNF-α-mediated NHE8 regulation. Promoter assay indicated that the shortest human NHE8 gene promoter construct, pGL3b−32, was responsive to TNF-α treatment. Thus
we focused on determining the DNA/protein interaction at this promoter region. Previous studies showed that pGL3b/H11002/H11032 contains a GC-box and that this region recruits Sp3 protein to activate basal NHE8 gene transcription (24). We tested whether TNF-α-mediated NHE8 transcription inhibition in HT29-MTX cells was due to an altered interaction in nuclear protein binding at this DNA region. As shown in Fig. 5A, TNF-α treatment reduced DNA-protein interaction at the basal promoter region of the human NHE8 gene, and this interaction could be blocked by GC-box consensus DNA oligos. Super-shift experiments indicated that this DNA/protein complex could be further shifted by Sp3 antibody in nuclear protein isolated from control and TNF-α-treated HT29-MTX cells (Fig. 5B).
DISCUSSION

The diverse secretory and absorptive function of the intestinal epithelium is constructed by a mixed population of predominantly absorptive cells displaying a brush-border membrane and mucin-producing goblet cells (22). Goblet cells form a significant component of the gastrointestinal tract, comprising ~10% of the small intestinal epithelium and 24% of the total epithelial cell population in the distal colon (7). The main role of goblet cells is to secrete mucin to protect the mucous membranes where they are found. Mucins are large glycoproteins formed mostly by carbohydrates. Their glycans (bound carbohydrates) attract relatively large quantities of water to produce gel-like properties of mucins (12). On the inner surface of the intestine, hydrated mucin forms a thick layer that lubricates and protects the wall of the organ (11). Distinct forms of mucin are produced in different organs. While Muc2 is prevalent in the intestine, Muc5AC and Muc5B are the main forms found in the airway and conjunctiva in the eye (9, 19). Mucins are stored in granules inside the goblet cells before being released to the lumen of the organ (12). Their secretion may be stimulated by irritants such as microbes, dust, and smoke (9).

Because loss of NHE8 expression results in reduced mucin production in the intestine and in the conjunctiva (31, 32), we wondered if NHE8 is expressed in goblet cells and, if NHE8 is expressed there, where it is located in the goblet cells. To address this question, we chose HT29-MTX cells as an in vitro model. HT29-MTX cells are mucin-secreting cells (13) and are widely used to study mucin secretion and bacterial adhesion (6, 14, 21). We first identified what kind of NHE isoforms are expressed in goblet cells using Real-time PCR. Our data showed that mRNA of NHE2 and NHE8 but not NHE3 could be detected in HT29-MTX cells. The expression of NHE8 in the mucin-secreting cells is 1.6-fold higher than in Caco-2 cells. Although PCR detected NHE2 mRNA signals, the expression level of NHE2 in HT29-MTX cells was very low (<10% of Caco-2 cells). Using the pH measurement method, we were able to identify the functional NHEs in HT29-MTX cells. In the presence of 1 μM HOE-694, a concentration inhibiting NHE1 activity, HT29-MTX cells retained ~19% of NHE activity, which indicates functional NHE8 expression. Because pH recovery after acid load was completely blocked by 10 μM HOE-694, the NHE activity detected in the presence of 1 μM HOE-694 was indeed NHE8. These observations suggest that the only functional NHEs in HT29-MTX cells are NHE1 and NHE8.

Furthermore, immunohistochemical stain detected NHE8 protein in HT29-MTX cells as well as in the epithelial cells and goblet cells of mouse colonic tissue sections. Interestingly, NHE8 has different localization in goblet cells compared with enterocytes and Leydig cells. In enterocytes, NHE8 is located at the plasma membrane in the enterocytes (23, 27). In Leydig...
NHE8 expression in the goblet cells is also subject to regulation by the proinflammatory cytokine TNF-α.

Our data showed that NHE8 protein expression was inhibited by TNF-α in HT29-MTX cells and that the inhibition was concentration-dependent. Although a higher concentration of TNF-α was required to inhibit NHE8 expression in goblet cells compared with Caco-2 cells (30 vs. 10 ng/ml) (24), these results suggest that TNF-α impairs NHE8 function by inhibiting NHE8 protein expression in the goblet cells. Further study confirmed that the NHE8 mRNA level was also decreased in HT29-MTX cells after exposure to TNF-α. This inhibition could be blocked by Act D, which suggests that TNF-α-mediated reduction of NHE8 protein was the result of reduced NHE8 mRNA expression in goblet cells. Promoter reporter assay indicated that TNF-α treatment resulted in significant decrease in human NHE8 gene promoter reporter activity in HT29-MTX cells, so TNF-α indeed regulated NHE8 gene expression by inhibiting NHE8 gene promoter activity. Because the pGL3/−32 promoter construct contains the minimal promoter sequence required for basal NHE8 gene expression activation and TNF-α treatment suppressed this promoter construct activity in Caco-2 cells (24), the effect of TNF-α on NHE8 gene expression in HT29-MTX cells was most likely mediated by decreasing basal NHE8 gene transcription.

Sp3 has been shown to be an essential transcriptional factor to activate the basal NHE8 gene promoter in Caco-2 cells (24). Therefore, we focused on Sp3 binding as a possible transcriptional factor involved in NHE8 regulation in goblet cells. Because TNF-α also inhibited NHE8 basal promoter activity in HT29-MTX cells, we suspected that the Sp3-binding region might also be involved. GMSA results showed decreased DNA-protein interaction at the human NHE8 proximal promoter region (−18 bp/+7 bp) in HT29-MTX cells treated with TNF-α. Supershift studies confirmed that Sp3 protein was

cells, NHE8 is located only in the intracellular compartments (26). However, in goblet cells, NHE8 was detected in the plasma membrane and intracellular compartments. The varied NHE8 protein localization suggests different roles of NHE8 in different cells. Whereas NHE8 mediates NHE in enterocytes, NHE8 has a role in luteinizing hormone receptor trafficking in Leydig cells (26). Because NHE8 protein is also detected inside the goblet cells, the role of NHE8 in goblet cells is likely more than mediating the sodium/hydrogen exchange.

The effect of NHE8 deficiency on mucin expression strongly supports an important role of NHE8 in intestinal mucosal protection (15, 29, 31, 32). Loss of NHE8 expression in the intestine resulted in decreased Muc2 expression, increased bacterial adhesion, and elevated inflammatory cytokine expression. The elevated proinflammatory cytokine expression creates a never-ending cycle. TNF-α is a potent proinflammatory cytokine, and it affects many functions of the epithelial cells in the intestine, such as inhibiting NHE3 and NHE8 expression (2, 24), modulating mucin production (5, 21), and damaging tight junctions (2, 16). Because we previously showed that intestinal inflammation and TNF-α exposure reduced NHE8 expression in the intestine and in Caco-2 cells, we wondered if
NHE8 expression is reduced by TNF-α in intestinal goblet cells

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Because Sp3 protein is required for basal NHE8 transcriptional activation in enterocytes and goblet cells in the intestine. Strongly support the conclusion that TNF-α/H9251 HT29-MTX cells in response to TNF-α key transcription factor that regulates NHE8 gene expression in promoter. These observations suggested that Sp3 protein is the bound on this DNA sequence in the human NHE8 proximal promoter. This work adds new insight on understanding the role of TNF-α on NHE8 expression in the intestinal mucosal protection.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author Contributions

Author contributions: H.X. and F.K.G. conception and design of research; H.X., Q.L., Y.Z., and J.L. performed experiments; H.X., Q.L., Y.Z., and J.L. analyzed data; H.X., Q.L., and J.L. interpreted results of experiments; H.X., Q.L., and Y.Z. prepared figures; H.X. drafted manuscript; H.X. and F.K.G. edited and revised manuscript; H.X. and F.K.G. approved final version of manuscript.

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