Human intestinal epithelial cell line SK-CO15 is a new model system to study Na⁺/H⁺ exchanger 3

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Yoo BK, Yanda MK, No YR, Yun CC. Human intestinal epithelial cell line SK-CO15 is a new model system to study Na⁺/H⁺ exchanger 3. Am J Physiol Gastrointest Liver Physiol 303: G180–G188, 2012. First published May 2, 2012; doi:10.1152/ajpgi.00069.2012.—The Caco-2 cell line represents absorptive polarized intestinal epithelial cells that express multiple forms of Na⁺/H⁺ exchanger (NHE) in their plasma membranes. Caco-2 cells express the major apical NHE isoform NHE3, but low NHE3 expression together with inefficient transfection often hamper intended studies. In this study, we examined whether SK-CO15 cells could be used to study NHE3 regulation. SK-CO15 cells grown on Transwell inserts developed polarized epithelial cells with microvilli. The transfection efficiency of SK-CO15 cells was markedly higher compared with Caco-2 cells, an advantage in gene transfer and knockout. SK-CO15 cells expressed NHE1, NHE2, and NHE3. NHE3 expression was significantly greater in these cells than Caco-2, and NHE3 comprised more than half of total NHE activity. Apical expression of NHE3 in SK-CO15 cells was confirmed by confocal immunofluorescence and surface biotinylation. NHE regulatory factors NHERF1 and NHERF2, which are important for regulation of NHE3 activity, were expressed in these cells. Stimulatory response of NHE3 in SK-CO15 cells was assessed by dexamethasone and lysophosphatidic acid (LPA). Treatment with dexamethasone for 24–48 h increased NHE3 expression and activity. Similarly to Caco-2 cells, SK-CO15 cells lacked the expression of the LPA receptor LPA1, but exogenous expression of LPA1 resulted in acute stimulation of NHE3. Forskolin acutely inhibited NHE3 activity in SK-CO15 cells, further attesting the validity of these cells. We conclude that SK-CO15 cells with the amenity for transfection and high endogenous NHE3 expression are a new and better cell model for NHE3 regulatory investigation than widely used Caco-2 cells.

POLARIZED INTESTINAL EPITHELIAL cells form an interface separating the internal from external environments and maintaining homeostasis between intestinal lumen and the body interior. The plasma membranes of polarized epithelial cells are divided into apical and basolateral domains with asymmetric distribution of cytoplasmic organelles by vectorial sorting mechanisms into apical and basolateral domains with asymmetric distribution often hampering intended studies. In this study, we examined whether SK-CO15 cells could be used to study NHE3 regulation. SK-CO15 cells grown on Transwell inserts developed polarized epithelial cells with microvilli. The transfection efficiency of SK-CO15 cells was markedly higher compared with Caco-2 cells, an advantage in gene transfer and knockout. SK-CO15 cells expressed NHE1, NHE2, and NHE3. NHE3 expression was significantly greater in these cells than Caco-2, and NHE3 comprised more than half of total NHE activity. Apical expression of NHE3 in SK-CO15 cells was confirmed by confocal immunofluorescence and surface biotinylation. NHE regulatory factors NHERF1 and NHERF2, which are important for regulation of NHE3 activity, were expressed in these cells. Stimulatory response of NHE3 in SK-CO15 cells was assessed by dexamethasone and lysophosphatidic acid (LPA). Treatment with dexamethasone for 24–48 h increased NHE3 expression and activity. Similarly to Caco-2 cells, SK-CO15 cells lacked the expression of the LPA receptor LPA1, but exogenous expression of LPA1 resulted in acute stimulation of NHE3. Forskolin acutely inhibited NHE3 activity in SK-CO15 cells, further attesting the validity of these cells. We conclude that SK-CO15 cells with the amenity for transfection and high endogenous NHE3 expression are a new and better cell model for NHE3 regulatory investigation than widely used Caco-2 cells.

NHE3 (Slc9a3), is the primary brush-border NHE. The functions and mechanisms of NHE3 regulation by hormones and growth factors have been investigated since its cloning in the early 1990s using several cell model systems. Among these, PS120 Chinese hamster lung fibroblasts and AP1 Chinese hamster ovarian cells provide an ideal cell system for reductionist approach to characterize NHE3 (26, 28). However, the nonepithelial origins of PS120 and AP1 cells often led to question the physiological validity of the findings. Originated from human intestines, T84 and HT29 human colon carcinoma epithelial cell lines are not suitable, as these cells represent secretory crypt epithelial cells (5, 6). The Caco-2 human adenocarcinoma cell line, on the other hand, express several brush-border enzymes, such as sucrase-isomaltase, alkaline phosphatase, and aminopeptidase, characteristics of the enterocytes lining the small intestinal villi (25). Caco-2 cells build a polarized monolayer and exhibit epithelial cell characteristics, i.e., transepithelial electric resistance of ~200 Ω·cm², brush-border microvilli, and tight junctions (7). Because of these features, Caco-2 cells have been deployed to study NHE3 regulation; however, Caco-2 cells have several limitations, including a high degree of heterogeneity leading to the nonuniformity in NHE3 activity, low transfection efficiency, and slow rates of cell proliferation (14). A clonal variant of Caco-2 cells, Caco-2bbe, has been used, but as for Caco-2 cells, the endogenous level of NHE3 in Caco-2bbe cells is either low or undetected, and the usefulness of the Caco-2bbe clone is largely limited to the characterization of transfected NHE3 (20, 21). Alternatively, rat intestinal epithelial IEC-6 has been used, but these cells primarily express NHE1 and lack the endogenous expression of NHE3 (35). Isolation of alternative intestinal epithelial cells suitable for the characterization of NHE3 has been sluggish due to the difficulties in isolation and long-term propagation of primary intestinal epithelial cells.

The SK-CO15 cell line derived from human adenocarcinoma of the colon develops polarized epithelial cells that form numerous domes on an impermeable support (19). These cells develop high-resistance monolayers with well-defined adhesion and tight junctions (11, 19). In search of an alternative model system that is more amenable to transfection, we examined the usability of SK-CO15 cells to study NHE3 regulation. In this study, we show that NHE3 is endogenously expressed in the apical membrane of SK-CO15 cells and the activity level of NHE3 in SK-CO15 cells is significantly higher than in Caco-2 cells. The regulation of NHE3 in SK-CO15 cells by selected agonists is consistent with previous reports, demonstrating the practicability of SK-CO15 cells as a suitable epithelial model for characterization of NHE3 regulation.

MATERIALS AND METHODS

Cell culture and treatment. SK-CO15 was a gift from Dr. Rodriguez-Boulan at Weil Medical College, Cornell University, New York, NY. SK-CO15, Caco-2, and Caco-2bbe cells were maintained in DMEM supplemented with 10% FBS, penicillin (50 μM/mL), streptomycin (50 μg/mL), 1 mM sodium pyruvate, 15 mM HEPES, and 1× nonessential amino acids. Caco-2bbe stably expressing NHE3 was previously described (20, 40). When necessary, cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).
Expression of lysophosphatidic acid receptor type 5 (LPA5) in Caco-2be cells was attained by using lentivirus harboring hemagglutinin (HA)-tagged LPA5s, as previously reported (20, 40). **Transfection.** When necessary, Caco-2 or SK-CO15 cells were seeded in six-well plates at a density of 5–6×10^5 cells/well (5–6×10^4 cells/cm^2) and grown overnight. Then 4 μg of plasmid DNA were mixed with 10 μl Lipofectamine 2000 in 500 μl Opti-MEM according to the manufacturer’s recommendation (Invitrogen). Transfection complexes were added to the cells overlayed with 1.5 ml DMEM without antibiotics. After 4 h of incubation at 37°C, the transfection mixture was replaced with fresh DMEM containing antibiotics. For transient expression, cells were harvested after 48 h.

For an estimation of transfection efficiency using enhanced green fluorescence protein (eGFP), cells were transiently transfected with pEGFP as described above. Cells expressing GFP were viewed under an Eclipse T1 inverted fluorescence microscope (Nikon, Melville, NY) and the number of GFP-positive cells was counted using NIS Elements Advanced Research image software (Nikon). The transfection efficiency was expressed as the ratio of number of cells expressing GFP to the total cell number times 100 from more than six fields of vision in each transfection.

**Reagents and antibodies.** Polyclonal rabbit anti-NHE3 serum (EM450) was generated at Covance (Princeton, NJ) against a purified recombinant NHE3 protein corresponding to aa 621–702 of human NHE3. To affinity purify EM450, 1.2 mg of purified NHE3 recombinant protein (aa 621–702) was resolved by 15% SDS-PAGE, followed by transfer onto nitrocellulose membrane. NHE3 protein on the nitrocellulose membrane identified by Ponceau-S stain was excised and transferred onto nitrocellulose membrane. NHE3 protein (aa 621–702) was resolved by 15% SDS-PAGE, followed by transfer onto nitrocellulose membrane identified by Ponceau-S stain was excised and transferred onto nitrocellulose membrane. NHE3 protein on the nitrocellulose membrane identified by Ponceau-S stain was excised and transferred onto nitrocellulose membrane. NHE3 protein corresponding to aa 621–702 of human NHE3 was generated at Covance (Princeton, NJ) against a purified recombinant NHE3 protein. Caco-2bbe cells was attained by using lentivirus harboring hemagglutinin (HA)-tagged LPA5, as previously reported (20, 40). Caco-2bbe cells was attained by using lentivirus harboring hemagglutinin (HA)-tagged LPA5, as previously reported (20, 40).

**Surface biotinylation.** Surface biotinylation of NHE3 was performed as previously described (10). Briefly, cells grown on permeable filters were rinsed twice in PBS and 10 min incubation in borate buffer composed of (in mM): 154 NaCl, 7.2 KCl, 1.8 CaCl₂, and 10 H₂BO₃, pH 9.0. Cells were then incubated for 40 min with 0.5 mg/ml NHS-SS-biotin (Pierce, Rockford, IL) in borate buffer. Unbound NHS-SS-biotin was quenched with Tris buffer (20 mM Tris, 120 mM NaCl, pH 7.4). Cells were then rinsed with PBS, scraped, lysed in the lysis buffer described above, and sonicated for 2 × 15 s. The lysate was agitated for 30 min and spun at 14,000 g for 15 min to remove the insoluble cell debris. Protein concentration was determined and 1 mg of lysate was then incubated with streptavidin-agarose beads (Pierce) for 2 h. The streptavidin-agarose beads were washed three times in lysis buffer and twice in PBS. All of the above procedures were performed at 4°C or on ice. Biotinylated surface proteins were then eluted by boiling the beads at 95°C for 10 min. Dilutions of the total and surface NHE3 were resolved by SDS-PAGE and immunoblotted with anti-NHE3 antibody. Densitometric analysis was performed using Scion Image software (National Institutes of Health, Bethesda, MD).

**Immunofluorescence confocal microscopy.** SK-CO15 cells grown on Transwells were washed twice with cold PBS, fixed with ice-cold ethanol for 10 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and blocked with PBS containing 5% normal goat serum for 45 min at RT. Cells were then incubated with anti-NHE3 EM450, anti-vesicular stomatitis virus glycoprotein PS54, anti-NHERF1, anti-NHERF2, anti-NHERF2, or anti-occludin antibodies (a gift from Dr. Asma Nusrat, Emory University) for 2 h at room temperature. Following three washes, 10 min each, with PBS, the cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) or Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) or Alexa Fluor 555-conjugated goat anti-mouse IgG (Invitrogen) for 1 h or rhodamine-conjugated phalloidin for 30 min at room temperature. After 3 × 10 min washes with PBS, the excised Transwells were mounted with ProLong Gold Antifade Reagent (Invitrogen) and observed under a Zeiss LSM510 laser confocal microscope (Zeiss Microimaging, Thornwood, NY) coupled to a Zeiss Axioplan2e with a Lambda 10–2 filter wheel controller equipped with a multi-wavelength filter set designed for BCFEC. Photometric data was acquired using the Metascope software (Molecular Devices, Sunnyvale, CA). Na⁺/H⁺ exchange rate was described by the ratio of pHi, recovery, which was calculated by determining slopes along the pHi recovery by linear least-squares analysis over a minimum of 9 s.

**RNA extraction, RT-PCR and quantitative real-time RT-PCR.** Total RNA from SK-CO15 or Caco-2 cells was isolated using Trizol (Invitrogen). NHE1, NHE2, NHE3, and NHE8 were amplified using the primer pairs for NHE1 (5'-GCTGGCCTCCAGCTCAGCTCGTTG-3' and 5'-TGGCCTCCAGCTCAGCTGGCCTGTG-3'), NHE2 (5'-AGAGCCGGGCTTCCAGGTTG-3' and 5'-AAGACCCACCCACCGCTGTG-3'), NHE3 (5'-GGCCGATGCGTGAGC-3' and 5'-TGGTCTGGAGGCGCAATGCTT-3'), NHE5 (5'-AGAGCCGGGCTTCCAGGTTG-3' and 5'-AAGACCCACCCACCGCTGTG-3'), and NHE8 (5'-GGCTGGGCGATGCGTGAGC-3' and 5'-GGAGCGGCCAGGCGCAATGGTGGAGT-3'). cDNA was amplified with SYBR Green Supermix (Bio-Rad, Hercules, CA) on Mastercycler Realplex (Eppendorf, Hauppauge, NY). The reaction mix consisted of 1 μl of cDNA, 10 μl of SYBR Green Supermix, 0.5 μM of target primers in total volume of 20 μl. Amplification was carried out at 4 min at 95°C for polymerase activation, and 40 cycles of 95°C for 30 s (denaturation), 60°C for 30 s (annealing), and 72°C for 1 min (extension). The amounts of NHE1, NHE2, and NHE3 mRNA were normalized to β-actin.
Alkaline phosphatase fluorometric assay. All procedures were performed using SK-CO15 and Caco-2 grown for 10 days postconfluence according to the manufacturer’s protocol (Alkaline Phosphatase Fluorometric Assay kit; Abcam, Cambridge, MA). Briefly, cells were scraped in 110 μl of assay buffer, homogenized, centrifuged at 14,000 g for 3 min to remove insoluble material, and added to each well of Fluotrac 96-well plate (Sigma). Methylumbelliferyl phosphate disodium salt (Abcam) substrate was added to each well, incubated for 30 min at 37°C, and stopped by adding 20 μl of STOP solution to each well. Fluorescence intensity was measured at 360 nm for excitation and 440 nm for emission using a fluorescence microplate reader (BioTek, Winooski, VT). Enzyme activity was calculated from the angular coefficient of the linear slope obtained from alkaline phosphatase standard (Abcam) solution, and expressed as 4-methylumbelliferon generated per volume of sample per minute (mU/ml). All experiments were independently performed three times in triplicates.

Statistics. Results were presented as means ± SE. Statistical analyses were performed by Student’s t-test for paired comparison with *P < 0.05* considered significant.

RESULTS

SK-CO15 cells endogenously express NHE1, NHE2 and NHE3. It was reported that SK-CO15 cells lack the expression of sucrase-isomaltase and express villin and alkaline phosphatase at lower levels (19). On the other hand, SK-C015 cells express well-defined apical junctional complexes and form a monolayer with transepithelial electric resistance ≳2,000 Ω·cm² (19). Hence, we sought to compare the ultrastructure of SK-CO15 and Caco-2 cells. SK-CO15 and Caco-2 cells display typical polarized epithelial cell features with junctional complexes and microvilli (Fig. 1A). However, the microvilli were less abundant and disordered in SK-CO15 cells compared with Caco-2 cells. Similarly, the activity of alkaline phosphatase in SK-CO15 cells was significantly lower level (∼33%) compared with Caco-2 cells (Fig. 1B), reflecting less abundant and shortened microvilli in SK-CO15 cells shown in Fig. 1A.

One inherent problem of Caco-2 cells is its relative resistance to transfection. We compared the transfection efficiency of Caco-2 and SK-CO15 by transfecting pEGFP. Typical determination of GFP expression after 48 h of transfection is shown in Fig. 2A where 11 out of 99 Caco-2 cells (11%) expressed GFP. In comparison, ∼71% (78/110) of SK-CO15 cells expressed GFP. To further validate the higher transfection efficiency in SK-CO15 cells, we compared the level of HA-LPAs expression in Caco-2 and SK-CO15 cells transfected with pcDNA-3.1/HA-LPAs. The expression level of LPAs in SK-CO15 cells was markedly greater compared with that in Caco-2 cells (Fig. 2B). We showed previously that LPAs phosphorylates Erk1/2 via transactivation of the epidermal growth factor receptor (40). Consistent with the robust expression of HA-LPAs, LPA markedly activated Erk1/2 in SK-CO15 cells. In comparison, activation of Erk1/2 by LPA in HA-LPAs transfected Caco-2 cells was marginally above control transfected cells. Together, these results demonstrate a significant advantage of SK-CO15 over Caco-2 cells in liposome-mediated transfection.

We next examined expression of NHEs in SK-CO15 cells by RT-PCR. Figure 3A shows the presence of amplicons corresponding to NHE1–3 with predicted sizes (915 bp for NHE1; 669 bp for NHE2; 691 bp for NHE3) in both Caco-2 and
SK-CO15 cells, but NHE8 (515 bp) was expressed in Caco-2 cells and not in SK-CO15 cells. qRT-PCR showed that the expression level of NHE3 mRNA was significantly higher in SK-CO15 cells when compared with NHE1 mRNA levels (Fig. 3, B and C). NHE3 has a molecular mass of 75–80 kDa and a single band of ~75 kDa was detected in SK-CO15 cell lysates by a polyclonal antibody raised against the COOH terminus of human NHE3, EM450 (Fig. 3D). The expression level of NHE3 was markedly higher in SK-CO15 cells than in Caco-2 cells. NHE3 protein in Caco-2bbe cells was not detected, consistent with our previous report that Caco-2bbe cells lack NHE3 expression (20). Lysates from Caco-2bbe cells transfected with NHE3 and opossum kidney (OK) cells, which were used as controls, showed the presence of NHE3 with a similar molecular mass. To confirm the specificity of the 75 kDa band as NHE3 protein, we transiently knocked down NHE3 expression in SK-CO15 cells by NHE3-specific shRNA. Knockdown of NHE3 expression by shRNA decreased the protein band intensity by ~80%, confirming the specificity of the anti-NHE3 antibody and the expression of NHE3 in SK-CO15 cells (Fig. 3E). The transfection efficiency in SK-CO15 cells and the specificity of our anti-NHE3 antibody were further demonstrated by transient transfection of SK-CO15 cells with pcDNA3.1/NHE3V that resulted in a robust increase in NHE3 expression (Fig. 3E).

NHE3 is the major NHE in SK-CO15 cells. We next sought to determine the level of Na+/H+ exchange activity by NHE3 in these cells. NHEs exhibit differential sensitivities to several inhibitors, including amiloride and HOE694, as such different NHEs can be selectively inhibited using these inhibitors (4). Figure 4A shows that 1 µM HOE694 (H), which blocks NHE1 activity (4), decreased the rate of Na+–dependent pH recovery from 0.31 ± 0.02 pH U/min to 0.21 ± 0.03 pH U/min, indicating a significant contribution by NHE1. Increasing HOE694 to 50 µM to block both NHE1 and NHE2 resulted in a small decrease compared with 1 µM HOE694, suggesting that NHE2 is a minor NHE in these cells. Na+/H+ exchange activity was almost completely ablated (0.02 pH U/min) in the presence of 50 µM HOE694 and the NHE3 inhibitor S3226 (S) (Fig. 3, A and B). The complete ablation of Na+/H+ exchange in the presence of HOE694 and S3226 indicates that SK-CO15 cells express Na+/H+ exchanger 3 (NHE3).
cells lack other HOE694-insensitive NHEs than NHE3. NHE8 is reported to be relatively sensitive to HOE694 (38), and it is either absent or expressed at a level undetectable by RT-PCR in SK-CO15 cells (Fig. 3A). Consistently, S3226 alone decreased the NHE activity by >50%, confirming that NHE3 is a major active NHE in SK-CO15 cells. Since Caco-2 cells have been used as a model intestinal epithelial cell system for NHE3 regulation, we compared the levels of NHE3 activity in Caco-2 and SK-CO15 cells. Figure 4C showed that endogenous NHE3 activity in SK-CO15 cells was more than twofold greater than Caco-2 cells, although overexpression of NHE3 in Caco-2bne cells resulted in even greater activity. Moreover, SK-CO15 cells showed markedly more uniform NHE3 activities than Caco-2 cells (Fig. 4D). Together, these studies suggest that SK-CO15 cells represent a cell model that has greater and more consistent NHE3 activity compared with Caco-2 cells, with an advantage of higher transfection efficiency.

NHE3 is localized on the apical membrane in SK-CO15 cells. NHE3 is an apical NHE of intestinal epithelial cells. To determine the cellular localization of NHE3, we performed surface biotinylation of SK-CO15 cells. NHE3 was exclusively detected in the apical surface fraction, but not in the basolateral membrane fraction (Fig. 5A). To corroborate the apical expression, polarized SK-CO15 cells were stained with EM450 (Fig. 5B, middle) and phalloidin (Fig. 5B, left). Figure 5B shows localization of NHE3 on the apical membrane with low NHE3 signal in subapical regions. To determine whether exogenously expressed NHE3 is targeted properly to the apical membrane, we transfected SK-CO15 cells with epitope-tagged NHE3V. Consistent with the cellular expression of endogenous NHE3 shown earlier, NHE3V was localized to the apical membrane without any detectable signal in the basolateral membrane (Fig. 5C).

NHE regulating factor (NHERF) proteins play pivotal roles in the regulation of NHE3 (9, 17). Of four NHERF isoforms characterized to date, NHERF1 is required for cAMP-dependent inhibition of NHE3 in the kidney although it is not obligatory in the intestine (23). NHERF2, on the other hand, is necessary for glucocorticoid- and LPA-induced stimulation of NHE3 (8, 20). Hence, we determined the expression of NHERF1 and NHERF2 in SK-CO15 cells. Figure 5D shows that SK-CO15 cells expressed both NHERF1 and NHERF2. Interestingly, the expression levels of these NHERF proteins in SK-CO15 and Caco-2 appeared a mirror image, with SK-CO15 cells expressing more NHERF2 but less NHERF1 than Caco-2 cells. Figure 5E, left and 5F, left show the expression of tight junction protein occludin in SK-CO15 cells revealing well-defined tight junctions. Confocal immunofluorescence images show that both NHERF1 (Fig. 5E) and NHERF2 (Fig. 5F) were primarily expressed on or near the apical membrane, although NHERF2 exhibited diffused intracellular staining.

Regulation of NHE3 in SK-CO15 cells. Glucocorticoids stimulate NHE3 activity via genomic as well as nongenomic mechanisms (41, 42). To further extend the utility of SK-CO15 cells as an intestinal epithelial cell model for NHE3 study, we determined the chronic effect of a synthetic glucocorticoid, dexamethasone (Dex). Incubation of SK-CO15 cells with 1 μM Dex for 24 or 48 h significantly increased the mRNA levels of NHE3 (Fig. 6A). The changes in NHE3 mRNA expression were paralleled by increased NHE3 protein expression (Fig. 6B) and NHE3 activity (Fig. 6C), consistent with previous studies using Caco-2 or rabbit intestinal brush-border membrane (35, 42).

Previously, we showed that LPA acutely stimulates NHE3 activity in Caco-2 cells when LPA5 is coexpressed with NHERF2 (20). Similarly to Caco-2 cells (20), SK-CO15 cells expressed LPA5 at a low level (data not shown); as such LPA showed no effect on NHE3 activity (Fig. 6D). On the other hand, overexpression of LPA5 in SK-CO15 cells resulted in a marked stimulation of NHE3 activity in response to LPA, consistent with our previous studies (20, 40).

Unlike Dex and LPA, forskolin that activates adenylate cyclase acutely inhibits NHE3 activity via a mechanism involving NHE3 phosphorylation and NHERF proteins (16, 43). Next, we examined the inhibitory effect of forskolin on NHE3 in SK-CO15 cells. Figure 6E shows that forskolin acutely inhibited NHE3 activity by 24%, indicating the presence of NHE3 inhibitory signaling pathways in SK-CO15 cells. Together our results demonstrate that NHE3 in SK-CO15 cells respond to several agonists and antagonists in manners similar to previous studies using other cell lines of intestinal and nonintestinal origins.
The characterization of NHE3 has utilized several cell lines, including fibroblasts, OK, Caco-2, and, to a lesser extent, IEC-6 cells (16, 18, 35, 40, 43). Of these, the OK cell line shows robust expression of NHE3, but its renal proximal tubule origin limits its application in the study of intestinal NHEs. Caco-2 and its clonal Caco-2bbe cells have been used to represent absorptive intestinal epithelial cells. Certainly, these cells are highly polarized and exhibit many characteristics of small intestinal epithelial cells. Caco-2 cells endogenously express NHE1, 2, and 3, but NHE3 expression is relatively low. Consequently, it is often challenging to identify endogenous NHE3 proteins in these cells by immunoblot or immunofluorescence. The Caco-2bbe cell line is reported to have endogenous NHE3 expression and activity (21), but the clonal cell line in our possession does not express NHE3 in a measurable quantity (20). Searching for an alternative cell line has been largely unsuccessful; several immortalized intestinal cell lines, including IEC-6, MSIE (mouse small intestine epithelium), and YAMC (young adult mouse colon), either lack or express NHE3 at a low level (Ref. 37; B. K. Yoo and C. C. Yun, unpublished data). The difficulty in long-term propagation of primary cultures of intestinal epithelial cells further hinders isolation of absorptive epithelial cell cultures. The recent identification of intestinal stem cells and the establishment of long-term culture of intestinal crypt-villus organoids are promising (29), but the development of normal absorptive epithelial cell lines from the intestinal mucosa that are ideal for Na+/H+ exchange and other ion and nutrient transport processes occurring at the brush-border membrane is yet to be achieved.

Herein, we examined whether SK-CO15 cells can be used to study the regulation of intestinal NHE3. SK-CO15 cells have been recently used to investigate cell migration (1), epithelial cyst formation (12), epithelial barrier function (30), and apical junctional proteins (24). Several lines of evidence showed that SK-CO15 cells form a differentiated epithelial cell layer: expression of several junctional proteins including ZO-1, junctional adhesion molecule (JAM)-A, and occludin in SK-CO15 cells have been reported previously (13, 31); SK-CO15 cells rapidly form high-resistance monolayers with well-defined adhesion and tight junctions that regulate the integrity of differentiated epithelial layers (24, 33); the organization and integrity of apical junctional complexes in SK-CO15 cells are shown to be regulated by nonmuscle myosin II and adducin (24, 33); and SK-CO15 cells, like Caco-2 cells, form spherical cysts when imbedded in Matrigel (24, 33).

In this study we showed that 1) SK-CO15 cells form a polarized epithelial monolayer with microvilli formation and alkaline phosphatase activity; 2) SK-CO15 cells endogenously express NHE3 at a level significantly greater than in Caco-2 cells; 3) these cells express NHERF1 and NHERF2; 4) SK-CO15 cells are amenable to transfection; and 5) regulation of NHE3 is identical to previous studies on isolated intestinal tissues and Caco-2 cells.

We showed that SK-CO15 cells express the transcripts of NHE1–3 and that, unlike Caco-2 and Caco-2bbe cells, NHE3 protein expression in SK-CO15 cells was easily detected by immunoblotting. Na+/H+ exchange activity measurement in the presence of selective NHE inhibitors showed that Na+/H+ exchange activity was...
Fig. 6. Regulation of NHE3 in SK-CO15 cells. SK-CO15 cells were treated with 1 μM dexamethasone (Dex) for 24 or 48 h, and the expression levels of NHE3 mRNA (A) and protein (B) were determined by qRT-PCR and Western blot analysis, respectively (n = 3; *P < 0.05). C: NHE3 activity in SK-CO15 cells treated with 1 μM Dex was determined. Results are presented as means ± SE (n = 9, *P < 0.05 compared with control). D: SK-CO15 cells transfected with pcDH or pcDH-LPA5 were treated with 1 μM LPA for 5 min and NHE3 activity was determined as described earlier (n = 9; *P < 0.05 compared with control). E: cells were treated with 10 μM forskolin (FSK) for 30 min prior to NHE3 activity measurement (n = 9; *P < 0.05).

exchange activity by NHE3 in SK-CO15 cells constituted more than 50% of total NHE activity. A caveat of our study is that although polarized cells grown on permeable filters were used, the inhibitors were added from the apical side such that inhibition of NHE1 in the basolateral membrane might have been less efficient than if the inhibitors were added on the basolateral side. However, the combination of 50 μM HOE694 and 10 μM S3226 completely obliterated NHE activity, suggesting that apical HOE694 somehow is able to access NHE1 on the opposite side of the cells. Importantly, NHE3 activity in SK-CO15 cells was significantly higher (>2-fold) than in Caco-2 cells and the rates of Na⁺-dependent pH recovery were markedly more consistent in SK-CO15 cells. It is noteworthy that SK-CO15 cells do not express NHE8 (Fig. 3A). Similar to NHE3, NHE8 is localized at the apical membrane (39), but its expression is more abundantly expressed in young animals during the development and evidence supports its role in electrolyte absorption during early postnatal development (2). In human, NHE8 is ubiquitously expressed but more abundant in the duodenum and ascending colon (38). Caco-2 cells despite its colon carcinoma origin resemble the enterocytes lining the small intestine. On the contrary, the low alkaline phosphatase activity, the formation of tight epithelial layer, and low or absence of NHE8 suggest that SK-CO15 cells exhibits characteristic features of colonocytes. NHE3 is more sensitive to HOE694 than NHE3, inhibited by 10 μM HOE694 but not by 1 μM HOE694 (38). Conversely, NHE8 is less sensitive to S3226 than NHE3. The absence of NHE8 transcript suggests that SK-CO15 cells, in addition to NHE3 studies, could be used as an intestinal epithelial model to characterize acute regulation of NHE8 taking an advantage of differential sensitivity of NHE8 to the NHE specific inhibitors.

In using Caco-2 and Caco-2bbe cells to investigate cellular processes at the molecular levels one major hindrance is the low efficiency of transfection with CaPO₄ or liposomes. To circumvent this limitation, viral transduction or usage of a specialized transfection tool, such as the Nucleofector (Lonza, Allendale, NJ) or Neon (Invitrogen) system, has been introduced. We estimated the transfection efficiency of SK-CO15 cells using liposomes (e.g., lipofectamine 2000) to be about 71% compared with 11% in Caco-2 cells, which eliminates the need for a specialized electroporator. The transfection efficacy of SK-CO15 cells was further demonstrated by knockdown and overexpression of NHE3 expression. Hence, SK-CO15 cells provide a major improvement in transfection over Caco-2 cells.

NHE3 activity is modulated by extrinsic factors via multiple mechanisms that include phosphorylation, trafficking, and interaction with regulatory proteins, which are often inter-dependent (9). We assessed the capacity of SK-CO15 cells as a model cell line for NHE3 regulation by examining the expression of NHERF proteins and regulation of NHE3 by selected agonists. NHERF1 and NHERF2 are two major regulatory proteins interacting with NHE3 and other signaling molecules (9, 17). Indeed, SK-CO15 cells express both NHERF1 and NHERF2, although we did not determine the presence of NHERF3 (PDZK1) in these cells. Evidence emerging from in vitro studies using heterologous expression and in vivo studies using mice deficient in NHERF2 expression has shown that NHERF2 is essential for regulation of NHE3 as well as CFTR by LPA (20, 32). NHERF2 interacts with the LPA₅ receptor and LPA cannot induce Na⁺-dependent fluid absorption in mouse intestine lacking NHERF2 (20, 40). We showed previously that LPA₅ is abundantly expressed in mouse enterocytes and colonocytes, but its expression is low in Caco-2 cells (20). We found that LPA₅ expression was low in SK-CO15 cells and LPA failed to regulate NHE3 in SK-CO15 cells unless LPA₅ was overexpressed. The low expression levels of LPA₅ in Caco-2 and SK-CO15 cells is interesting, and whether the low expression is related to the cancer cell origin of these cell lines is yet to be determined.

Glucocorticoids are a well-known agonist of NHE3 that stimulates NHE3 activity via multiple mechanisms. The effects of 24–48 h treatment of Dex that increased NHE3 mRNA and protein levels in SK-CO15 cells are consistent with the effects on Caco-2 and rabbit intestine (35, 41, 42). In addition to the genomic effects, we have shown previously that the effect of glucocorticoids is also dependent on serum- and glucocorticoid-induced kinase 1 (SGK1) that interacts with NHERF2 such that the extent of NHE3 stimulation is significantly blunted in the absence of SGK1 or NHERF2 (8, 41). Although we did not examine the expression of SGK1 in SK-CO15 cells, we assumed SK-CO15 cells to express SGK1 based on the robust effect of Dex on NHE3 activity (2-fold), which would be expected to be markedly diminished in the absence of SGK1.
Inhibition of NHE3 by forskolin or protein kinase A-dependent mechanisms is another example of well-known regulation of NHE3 (16, 43, 44). Forskolin, which activates adenylate cyclase, inhibits NHE3 activity via multiple pathways involving NHE3 phosphorylation, NHERF proteins, and endocytosis. The absence of NHERF1 ablated cAMP-dependent inhibition of NHE3 in the kidney but not in the intestine (23, 36). Similarly, the absence of NHERF2 did not affect cAMP-dependent inhibition of NHE3 in the intestine (3), although Murtazina et al. (22) reported the NHERF2-dependence of NHE3 inhibition by cAMP in mouse intestine. We found that SK-CO15 cells express NHERF2 at a much higher level but less NHERF1 than Caco-2 cells. Nonetheless, NHE3 in SK-CO15 cells was acutely inhibited by forskolin, further demonstrating the usefulness of these cells in probing the regulatory mechanisms of NHE3.

In summary, we report here that SK-CO15 cells express NHE3 whose regulation is consistent with previous physiological studies. With the high transfection efficiency and the polarized morphology, our studies demonstrate that SK-CO15 cells are an ideal cell model to study NHE3 regulation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: B.K.Y. and C.C.Y. conception and design of research; B.K.Y., M.K.Y., and Y.R.N. performed experiments; B.K.Y. and C.C.Y. analyzed data; B.K.Y. and C.C.Y. interpreted results of experiments; B.K.Y. prepared figures; B.K.Y. drafted manuscript; B.K.Y. and C.C.Y. edited and revised manuscript; B.K.Y. and C.C.Y. approved final version of manuscript.

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