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Elevated carbon dioxide upregulates NBCn1 Na^+/HCO_3^- cotransporter in human embryonic kidney cells

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Orlowski A, Vargas LA, Aiello EA, Álvarez BV. Elevated carbon dioxide upregulates NBCn1 Na^+/HCO_3^- cotransporter in human embryonic kidney cells. *Am J Physiol Renal Physiol* 305: F1765–F1774, 2013. First published September 4, 2013; doi:10.1152/ajprenal.00096.2013.—The NBCn1 Na^+/HCO_3^- cotransporter catalyzes the electroneutral movement of 1 Na^+:1 HCO_3^- into kidney cells. We characterized the intracellular pH (pH_i) regulation in human embryonic kidney cells (HEK) subjected to NH_4Cl prepulse acid loading, and we examined the NBCn1 expression and function in HEK cells subjected to 24-h elevated P_{CO_2} (10–15%). After acid loading, in the presence of HCO_3^-, ~50% of the pH_i recovery phase was blocked by the Na^+/H^+ exchanger inhibitors EIPA (10–50 μM) and amiloride (1 mM) and was fully cancelled by 30 μM EIPA under nominally HCO_3^--free conditions. In addition, in the presence of HCO_3^-, pH_i recovery after acid loading was completely blocked when Na^+ was omitted in the buffer. pH_i recovery after acidification in HEK cells was repeated in the presence of the NBC inhibitor S0859, and the pH_i recovery was inhibited by S0859 in a dose-dependent manner (K_i = 30 μM, full inhibition at 60 μM), which confirmed NBC Na^+/HCO_3^- cotransporter activation. NBCn1 expression increased threefold after 24-h exposure of cultured HEK cells to 10% CO_2 and sevenfold after exposure to 15% CO_2, examined by immunoblots. Finally, exposure of HEK cells to high CO_2 significantly increased the HCO_3^--dependent recovery of pH_i after acid loading. We conclude that HEK cells expressed the NBCn1 Na^+/HCO_3^- cotransporter as the only HCO_3^--dependent mechanism responsible for cellular alkaline loading. NBCn1, which expresses in different kidney cell types, was upregulated by 24-h high-P_{CO_2} exposure of HEK cells, and this upregulation was accompanied by increased NBCn1-mediated HCO_3^- transport.

NBCn1 Na^+/HCO_3^- cotransporter; human embryonic kidney cells; high P_{CO_2}

THE KIDNEY MAINTAINS ACID-BASE balance by H^+ secretion and HCO_3^- reabsorption. Proximal tubule cells reabsorb HCO_3^- by the apically expressed Na^+/H^+ exchanger (NHE3) in conjunction with the basolaterally expressed Na^+/HCO_3^- cotransporter NBCe1, which mediates Na^+-dependent electrogenic HCO_3^- efflux (8, 19, 35, 36). NBCe1 is expressed at the basolateral plasma membrane domains of S1-S2 segments in proximal tubules of rat kidney (28, 38).

Other members of the NBC family (SLC4) of Na^+/HCO_3^- cotransporters have recently been identified in the kidney. NBCs could potentially play a role in mediating acid-base transport in kidney or in maintaining intracellular pH (pH_i) levels and HCO_3^- homeostasis (1, 6, 12, 33, 34, 47). The electroneutral NBCn1 was cloned from rat smooth muscle cells (12), and its expression was demonstrated in basolateral domains of the thick ascending limb (TAL) cells of the kidney outer medulla (45). Consistent with this, pH_i measurements in the TAL confirmed the existence of a Na^+-dependent amiloride-insensitive recovery after acidosis, in the presence of HCO_3^- (45). Furthermore, NBCn1 immunolabeling was identified in collecting duct intercalated cells in the inner stripe of the outer medulla and in the inner medulla (45).

On the other hand, another electroneutral NBC (also named NBC3), which was isolated from the human skeletal muscle cells (33) and is ~90% identical to NBCn1, was found to be exclusively associated with intercalated cells in connecting tubules and in cortical, outer medullary, and initial inner medullary collecting ducts of rat kidney (23). In connecting tubule and cortical collecting duct, NBCn1 is associated with both type A and type B intercalated cells, suggesting that NBCn1 may participate in the H^+/base transport in the collecting duct (23, 34). A member of the SLC4 HCO_3^- transporter family, the kAE1 Cl^-/HCO_3^- exchanger expresses in basolateral membrane of the α-intercalated cells of the distal nephron (2, 15). In the α-intercalated cells, kAE1 participates in acid secretion by exchanging intracellularly produced HCO_3^- for extracellular $C1^-$, facilitating HCO_3^- reabsorption into the blood (46).

Chronic metabolic acidosis upregulates rat kidney electroneutral Na^+/HCO_3^- cotransporters NBCn1, without changes in the expression of the electrogenic Na/HCO_3^- cotransporters NBCe1 (32). Furthermore, NBCn1 expression increased in the kidney medullary TAL cells exposed to acidic conditions, by varying the [HCO_3^-] (24). In the present study, we therefore examined whether the protein expression levels of NBCn1, which express endogenously in the human embryonic kidney cells (HEK) line, are altered in response to 24-h P_{CO_2} elevation of cultured HEK cells, using immunoblotting analysis. Additionally, we fully characterized the contribution of the NBCn1 Na^+/HCO_3^- cotransporter to pH_i regulation in cultured HEK cells, after cellular acidification or after high-CO_2 exposure of cells.

MATERIALS AND METHODS

HEK293 cells. 293-H cells adapted to suspension culture in 293 SFM II (GIBCO, Invitrogen) were used in our functional and expression studies at *passage 15–20*.

RT-PCR. Total RNA was isolated from cultured HEK293 cells using RNeasy Mini Kits (Qiagen). The RNA was reversed transcribed using 2 U/μl Superscript II Reverse Transcriptase (Invitrogen). PCR (HotStar *Taq* Master Mix, Qiagen) with 10% cDNA and 1 pmol of each primer (human forward and reverse primers for SLC4A and β-actin transcripts, as previously described) (13) was performed for 30 cycles after 15 min at 95°C, after which denaturation was performed for 30 s at 60°C, annealing for 30 s, and elongation at 72°C for 1 min. PCR for β-actin was performed to validate each template. PCR

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products were separated by 2% agarose gel electrophoresis with GelRed Nucleic Acid Stain (Invitrogen) and photographed under ultraviolet illumination. Human primer pairs have been previously validated by nucleotide sequencing representative PCR products (13).

Elevated P_{CO_2} condition assays of HEK cells. HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin in 5% CO_2 air-equilibrated (control) 37°C incubator. For high-P_{CO_2} assays, cells were plated on 100-mm plates at a density of 1×10^6 cells/plate (~30% confluency) and exposed for 24 h to 10% CO_2 air equilibrated, or exposed for 24 h to 15% CO_2 air equilibrated, maintaining oxygen levels of 21%. CO_2 concentrations of 10 and 15% were chosen to provide moderate and severe levels of elevated P_{CO_2}, as described previously (20, 22).

Assay of NBCn1 activity in HEK293 cells. NBCe1 activity was monitored using a fluorescence assay (14). Briefly, HEK293 cells grown to ~30% confluence were incubated with 2 μM BCECF-AM (37°C, 20 min), washed, and resuspended in a HEPES buffer containing (in mM): 133 NaCl, 5 KCl, 1.2 $MgSO_4$, 0.8 $MgCl_2$, 10 glucose, 1.35 $CaCl_2$, and 10 HEPES, pH 7.35, with 5 mM NaOH (138 mM total Na^+). Cells were acid loaded by incubation in a HEPES buffer containing 20 mM NH_4Cl for 5 min, followed by the wash-out of NH_4Cl in a Na^+-free HEPES buffer to maintain acidity (external NaCl was replaced completely with choline Cl). The pH of the Na^+-free HEPES-buffered solution was titrated to pH 7.35 with Tris base. Finally, the cells were placed in a cuvette and perfused with a 5% CO_2 air-equilibrated HCO_3^- buffer (118 mM NaCl, 4.5 mM KCl, 1.35 mM $CaCl_2$, 20 mM $NaHCO_3$, 1.05 mM $MgSO_4$, 10 mM glucose, pH 7.40), or perfused with a 5% CO_2 air-equilibrated Na^+-free HCO_3^- buffer (118 mM choline Cl, 4.5 mM KCl, 1.35 mM $CaCl_2$, 20 mM choline HCO_3, 1.05 mM $MgSO_4$, 10 mM glucose, pH 7.40). HCO_3^- transport activity of NBCn1 was measured during the recovery from transient intracellular acidification in the presence of 10–30 μM EIPA or amiloride (Sigma) to block NHE activity, or measured in the presence of the NBC blocker S0859 (10–100 μM). In other experiments, recovery from transient intracellular acidification was performed under nominally HCO_3^--free conditions (HEPES buffer), in the absence or presence of EIPA (30 μM). Fluorescence was monitored in a Photon Technologies International RCR fluorometer, at excitation wavelengths of 440 and 500 nm and an emission of 530 nm. Fluorescence ratios were converted to pH_i by the nigericin/high-K^+ technique (42) at pH values between 6.5 and 7.5. For the elevated P_{CO_2} experiments, control (5% CO_2) cells plated on 100-mm plates for 24 h, or plated cells subjected to high-CO_2 (10–15%) exposure for 24 h, were loaded with 2 μM BCECF-AM. Cells were acidified by the NH_4Cl pulse, and the pH_i was recorded according to the protocol described above. Initial rate of pH_i recovery for HEK cells subjected to an acid load was calculated by fitting a linear regression of either the first 1 min of the pH_i recovery after maximum acidosis. Rates of HCO_3^- influx were expressed as ΔpH_i/s.

Measurement of intrinsic buffering capacity, proton flux, and extracellular ph in HEK293 cells. Intrinsic buffering capacity (β_{int}) was measured while HEK cells cultured under normal (5%)- or high (10–15%)-CO_2 conditions were superfused in HEPES-buffered solutions (nominally free of CO_2/HCO_3^-) containing various concentrations of a weak base (NH_4^+), as previously described with minor modifications (48). Briefly, solutions were Na^+-free and Ca^{2+}-free and contained 20, 15, 12, 9, 5, 3, or 1 mM NH_4Cl. Cells were superfused in normal HEPES followed by a switch to one of the NH_4^+ solutions. A series of solution changes was then made, in which the extracellular $[NH_4^+]$ concentration was stepped to progressively lower levels. The changes approached a steady state after 2–3 min. The pH_i before and after the NH_4^+ removal step (pH_i^{start} and pH_i^{stop}, respectively) was documented. During NH_4^+ removal, NH_4^+ leaves the cell in the form of uncharged NH_3, leaving behind H^+. The change in $[H^+]_i$ is assumed to be equal to the change in intracellular NH_4^+ concentration ($[NH_4^+]_i$), which can be estimated using the following reorganized Henderson-Hasselbalch equation: $[NH_4^+]_i = [NH_4^+]_o \cdot 10^{pHo-pHi}$, where $[NH_4^+]$ in the extracellular solution $\{[NH_4^+]_o\}$ is calculated from the total $[NH_4^+]$ ($C_{tot} = NHE_3 + NH_4^+$) divided by $[1 + 10^{(pHo-pK)}$, where pH_o is extracellular pH]. β_{int} May then be computed, as previously (37, 48). The pK_a value assumed for NH_4^+ is 9.03. The pH_i dependence of buffering is approximated by referring the value of β_{int} so determined to the midpoint in the change of pH_i induced by the step decrease in $[NH_4^+]_o$. The total buffering capacity of the system (β_{tot}) was then determined as $\beta_{tot} = \beta_{int} + \beta CO_2$, where $\beta CO_2 = 2.3 \cdot [HCO_3^-]$ (37) and the total proton flux (J_{H+}) was calculated as $J_{H+} = \Delta pH_i \cdot \beta_{tot}$ and expressed as H^+ equivalent flux (mM/min).

Extracellular pH (pH_o) was measured for the cultured media of HEK cells maintained in the incubation chamber for 24 h, using a pH Meter Glass Electrode (PHM62 standard pH Meter, Radiometer, Copenhagen, Denmark). The pH meter was calibrated at 37°C before pH determination in the media of cell-containing culture dishes. P_{CO_2} in incubation chamber was increased without modifications in the $[HCO_3^-]$.

Cell surface targeting assays. HEK293 cells were grown at 37°C under normal (5%)- or high (10–15%)-P_{CO_2} conditions in DMEM medium, supplemented with 5% (vol/vol) fetal bovine serum and 5% (vol/vol) calf serum. Assays to assess the degree of cell surface targeting and biotinylation of NBCn1 protein were performed as follows. Briefly, cells were labeled with membrane-impermeant Sulpho-NHS-SS-biotin and then solubilized in RIPA buffer [10 mM Tris·HCl (pH 7.5), 1% (wt/vol) deoxycholate, 1% (vol/vol) Triton X-100, 0.1% SDS, 150 mM NaCl, and 1 mM EDTA] containing protease inhibitors (MiniComplet Tablet, Roche), followed by centrifugation at 14,000 *g* for 15 min to remove insoluble material. Total protein concentration for the lysate was quantified. Immunopure immobilized streptavidin-Sepharose resin (100 μl) was added to the lysate and incubated for 1 h at 4°C to bind biotinylated protein. The beads were washed with RIPA buffer and bound protein was eluted by addition of Laemmli sample buffer containing 5% (vol/vol) 2-mercaptoethanol for 1 h at room temperature, as previously (25). The content of NBCn1 in the bound fraction was determined by immunoblotting, and the amount of NBCn1 on blots was quantified by densitometry, using a Chemidoc Image Station (BioRad).

Immunodetection. HEK293 cell lysates corresponding to cells cultured under normal (5%)- or high (10–15%)-CO_2 air-equilibrated conditions were washed with PBS (140 mM NaCl, 3 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2HPO_4, pH 7.4) and prepared by addition of SDS-PAGE sample buffer [20% (vol/vol) glycerol, 2% (vol/vol) 2-mercaptoethanol, 4% (wt/vol) SDS, 1% (wt/vol) bromophenol blue, 150 mM Tris, pH 6.8]. Before analysis, samples were sheared through a 26-gauge needle (Becton Dickinson) and heated to 70°C for 5 min. Samples were resolved by SDS-PAGE on 8% acrylamide gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes by electrophoresis for 1 h at 100 V at room temperature, in buffer composed of 20% (vol/vol) methanol, 25 mM Tris, and 192 mM glycine. PVDF membranes were blocked by incubation for 1 h in TBST-M buffer [TBST buffer 0.1% (vol/vol) Tween 20, 137 mM NaCl, 20 mM Tris, pH 7.5], containing 10% (wt/vol) nonfat dry milk and then incubated overnight in 10 ml TBST-M [5% (wt/vol) nonfat dry milk], containing rabbit anti-NBCn1 antibody (1:1,000 dilution) (26), or incubated in TBST-M containing rabbit anti-NHE1 antibody (sc-28758; 1:1,000 dilution) (44). Blots were incubated for 1 h with 10 ml of TBST-M containing donkey anti-rabbit IgG conjugated to horseradish peroxidase. Blots were visualized and quantified, using ECL reagent and a Chemidoc Image Station (Bio-Rad).

Statistics. Data were expressed as means ± SE and were compared with Student's *t*-test or one-way ANOVA followed by Newman-Keuls multiple comparison post hoc test. A value of $P < 0.05$ was considered statistically significant (2-tailed test).

RESULTS

Expression of SLC4A transcripts in HEK293 cells. To examine the physiological significance of Na^+-dependent HCO_3^- transporters present in HEK cells, reverse transcription was performed from total RNA extracted from cultured cells and expression of HCO_3^- transporters was verified by PCR analysis. Previously, expression of the Na^+-dependent transporters, the Na^+/HCO_3^- cotransporters NBCe1 (SLC4A4), NBCe2 (SLC4A5), and NBCn1 (SLC4A7), and the Cl^-/HCO_3^- exchanger NDCBE1 (SLC4A8) was detected at the mRNA level in the human kidney cortex and medulla (13). In addition, the Na^+-dependent Cl^-/HCO_3^- exchanger NCBE (SLC4A10) mRNA transcript was also observed in the human kidney cortex (13). Herein, reverse transcription performed in cultured HEK293 cells revealed RT-PCR products obtained only for the NBCn1 Na^+/HCO_3^- cotransporter (Fig. 1). PCR product acquired for β-actin was used as a positive control (Fig. 1). These results suggest a pivotal role for the NBCn1 transporter in this particular kidney cell line.

pH_i regulation in HEK293 cells and role of bicarbonate-dependent and bicarbonate-independent mechanisms. In functional assays (Fig. 2), HEK293 cells were loaded with BCECF-AM fluorescent dye to measure pH_i, maintained in a Na^+-free and HCO_3^--free HEPES buffer and subjected to NH_4Cl acid load. Subsequently, the cells were resuspended into a Na^+-containing HCO_3^- buffer (Fig. 2*A*), or resuspended into a Na^+-containing HEPES buffer (Fig. 2*B*), and the pH_i recovery phase after the imposed acid load was continuously recorded. In the presence of HCO_3^-, inhibition of the Na^+/H^+ exchanger with different doses of the amiloride derivative, EIPA (10–50 μM), or with 1 mM amiloride, reduced the pH_i recovery after acid loading by ~60% (Fig. 2, *A* and *C*). Conversely, 30 μM EIPA fully cancelled the recovery after imposed acid load, in the absence of HCO_3^- (HEPES buffer; Fig. 2, *B* and *D*).

To corroborate whether the EIPA-insensitive HCO_3^--dependant transporter, which endogenously expresses in HEK293 cells, is coupled to Na^+, we next removed Na^+ from the bath in the continued presence of CO_2/HCO_3^- (Fig. 3). Removing extracellular Na^+ (replacing Na^+ with choline) had a profound effect on pH_i after the imposed acid loading, proving the dependence of the transporter to Na^+ (Fig. 3, *A* and *B*). We

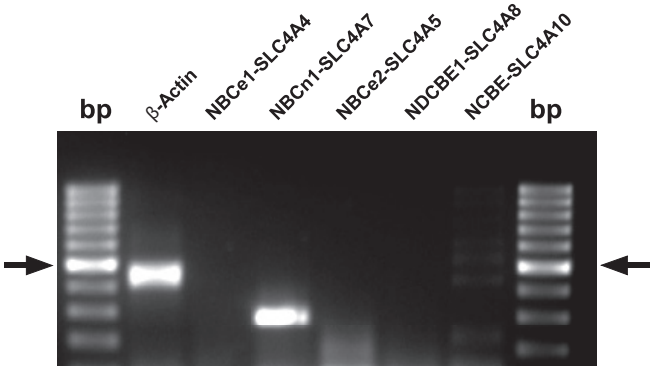

Fig. 1. Expression of Na^+-dependent SLC4A transcripts in human embryonic kidney (HEK)293 cells. RT-PCR analysis of various Na^+-dependent HCO_3^- transporters. Total RNA from cultured HEK cells was reverse transcribed and first-strand cDNA was produced using Superscript II Reverse Transcriptase. Amplicons were analyzed on a 2% agarose gel containing GelRed Nucleic Acid Stain. A 100-bp ladder was used; arrow mark indicates 500 bp.

previously demonstrated that HEK293 cells express endogenous levels of the NBCn1 Na^+/HCO_3^- cotransporter, without expression of the electrogenic NBCe1 or NBCe2 cotransporters (14), which is in agreement with RT-PCR analysis showed in Fig. 1. Finally, we addressed the question of whether this endogenous HCO_3^-- and Na^+-dependent cotransport process can be blocked by the high-affinity generic NBC inhibitor S0859, in the HEK cell system. We briefly pulsed the cells with NH_4Cl to induce cellular acidification and then we studied the recovery phase after acid loading, in the absence or presence of 60 μM S0859, under blockade of the Na^+/H^+ exchange activity with EIPA (30 μM; Fig. 4*A*). Figure 4*B* is a dose-response curve for the effect of S0859 on bicarbonate influx in HEK cells, in experiments similar to that shown in Fig. 4*A*. Control influx values for NBCn1 (that is, nil S0859) are also plotted. The apparent IC_{50} for the effect of S0859 on NBCn1-mediated HCO_3^- transport was 30 ± 3 μM. Previously, S0859 reversibly inhibited NBC-mediated pH_i recovery (K_i = 1.7 μM, full inhibition at ~30 μM) in ventricular myocytes (11). Herein, S0859 fully blocked the pH_i recovery after acid loading in HEK cells exposed to NH_4Cl-induced acidosis, at a higher dose of the compound (<50 μM).

Expression of total NBCn1 and cell surface expression of NBCn1, in HEK cells exposed to high-P_{CO_2} incubation. NBCn1 expression increased after acidic incubation of the TAL cell line ST-1, and the upregulation of NBCn1 was progressively larger with incubation periods longer than 24 h (24). To test whether NBCn1 protein expression is affected by exposure to high CO_2 in the HEK system, we incubated cells at 10 and 15% CO_2 for 24 h and performed immunoblotting using the anti-NBCn1 antibody (26). The antibody used herein is a rabbit polyclonal antibody directed against the COOH-terminal 18 amino acids of human NBCn1-a (also known as NBC3). This amino acid sequence is identical to the NBCn1-b and NBCn1-c splicing variants of the NBCn1 gene, but it is not conserved among other NBC polypeptides (NBCe1, NBCe2; Fig. 5*A*). Same antibody recognized endogenously expressed NBCn1 protein in lysates of HEK293 cells (14). However, specific antibodies against NBCe1 or NBCe2 polypeptides failed to recognize endogenous NBCe1 or NBCe2, respectively, in the HEK system (14) consistent with the RT-PCR analysis showed in Fig. 1. Control HEK cells were incubated at 5% CO_2 for 24 h. Figure 5*B* shows a representative immunoblot analysis of control cells or cells incubated for 24 h at 10% CO_2 or incubated for 24 h at 15% CO_2 (*top*). Expression of NBCn1 increased after incubation at elevated P_{CO_2} conditions. Analyzed by quantitative measurements of NBCn1 relative to GAPDH (Fig. 5*B*, *bottom*), the increase was threefold higher for cells exposed to 10% CO_2 compared with control conditions, and sevenfold higher for cells exposed to 15% CO_2 compared with control.

To investigate the effect of high-P_{CO_2} incubation of cultured HEK293 cells on NBCn1 cell surface targeting, we determined the degree to which NBCn1 could be labeled with the membrane-impermeant Sulpho-NHS-SS-biotin compound. Cells cultured under control (5%) or high (10–15%)-P_{CO_2} conditions were labeled with Sulpho-NHS-SS-biotin and then solubilized in lysis buffer. Samples were incubated with streptavidin resin to remove biotinylated proteins, leaving the unbound fraction. Samples of the eluted fractions removed from resins were subjected to SDS-PAGE and probed on

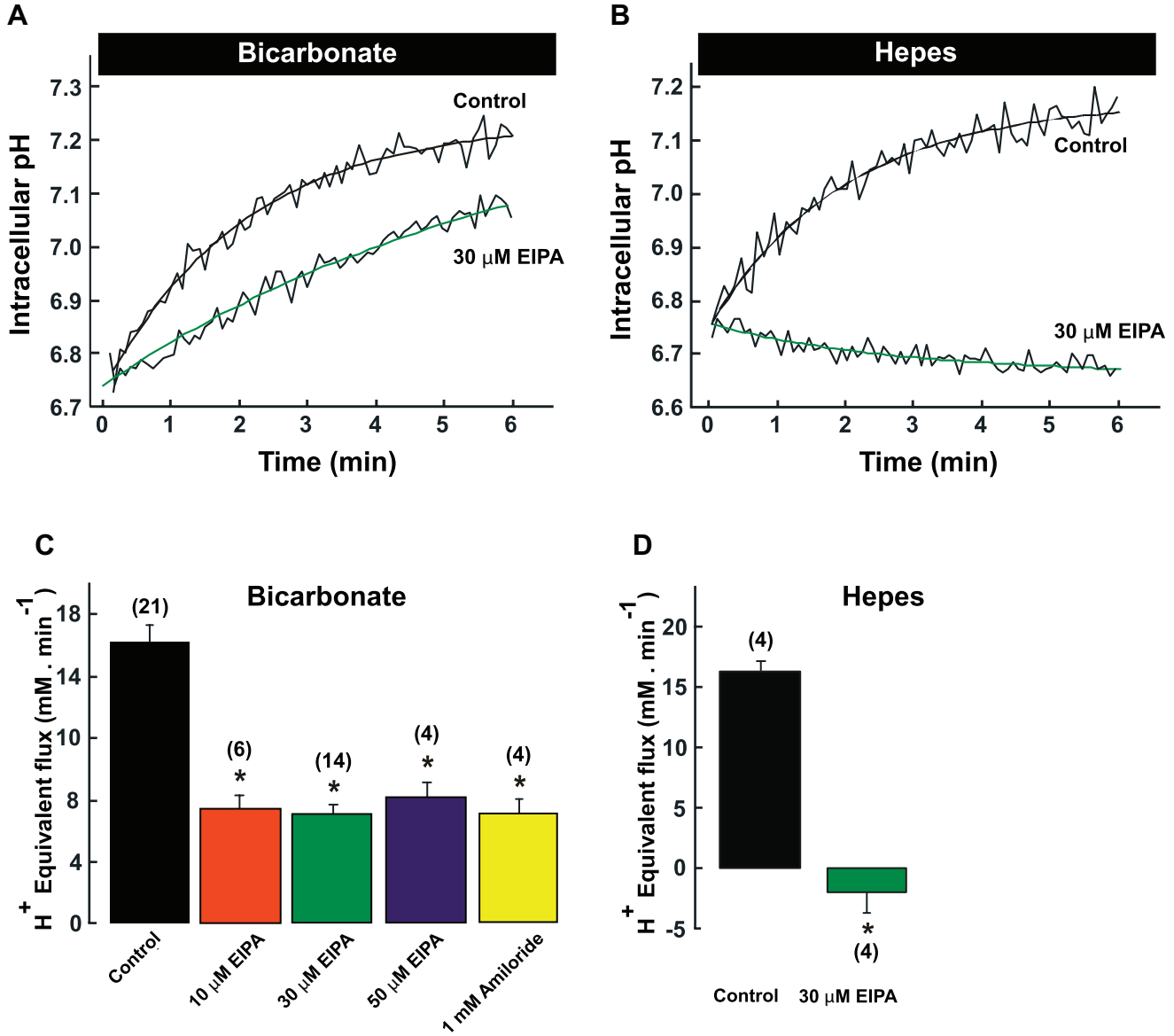

Fig. 2. Effect of Na^+/H^+ exchange activity inhibition on Na^+/HCO_3^- cotransport in HEK cells. HEK293 cells were loaded with BCECF-AM and placed into a fluorometer to monitor intracellular pH (pH_i). Representative traces of cells were perfused with bicarbonate buffer (*A*) or perfused under nominally bicarbonate-free conditions (*B*) acidified with the NH_4Cl pulse method (see MATERIALS AND METHODS) in the absence (control) or presence of the Na^+/H^+ exchanger inhibitor EIPA (20 μM). *C* and *D*: average data of rates of HCO_3^- influx expressed as H^+ equivalent flux (mM/min) and measured under bicarbonate (*C*) or HEPES (*D*) buffer conditions. Initial rates of change of pH_i during the first 60 s were estimated from the slope of the line fitted by the least squares method, in the absence (control) or presence of amiloride (1 mM), or the absence (control) or presence of the amiloride-derivative, EIPA (30 μM), as indicated. Parentheses on *top* of bar indicate number of experiment. *$P < 0.05$ compared with control.

immunoblots for the presence of NBCn1 protein. The amount of NBCn1 on the blots was quantified by densitometry (Fig. 5*C*). The fraction of protein that was biotinylated is interpreted as localized to the cell surface, because it is accessible to labeling by the membrane-impermeant biotinylation reagent. We estimated the fraction of biotinylated protein and found that incubation of cells with 10% P_{CO_2} increased the fraction of NBCn1 at the cell surface (~40%), which further increased by exposing cells to 15% P_{CO_2} (~75%), compared with normal-P_{CO_2} conditions. To assess the validity of this approach, in a separate experiment (not shown), we determined whether a cytosolic marker protein, GAPDH, could be labeled by Sulpho-NHS-SS-biotin. Interestingly, very little GAPDH was labeled with biotin and this labeling was not affected by high-P_{CO_2} exposure of cultured cells, indicating that the biotinylation protocol is reliable in labeling little cytosolic protein.

Functional activity of NBCn1 in HEK cells exposed to high-CO_2 incubation. The rate of NBCn1-mediated pH_i recovery was assessed in cultured HEK293 cells subjected to high-P_{CO_2} conditions for 24 h with the use of the NH_4Cl prepulse technique in the presence of 30 μM EIPA to block Na^+/H^+ exchange, and the H^+ fluxes were expressed as mM/min as a function of the HEK cells pH_i (Fig. 6). HEK cells exposed to 10% CO_2 have faster recovery rates compared with cells cultured under control conditions, which further increased in cells exposed to 15% CO_2.

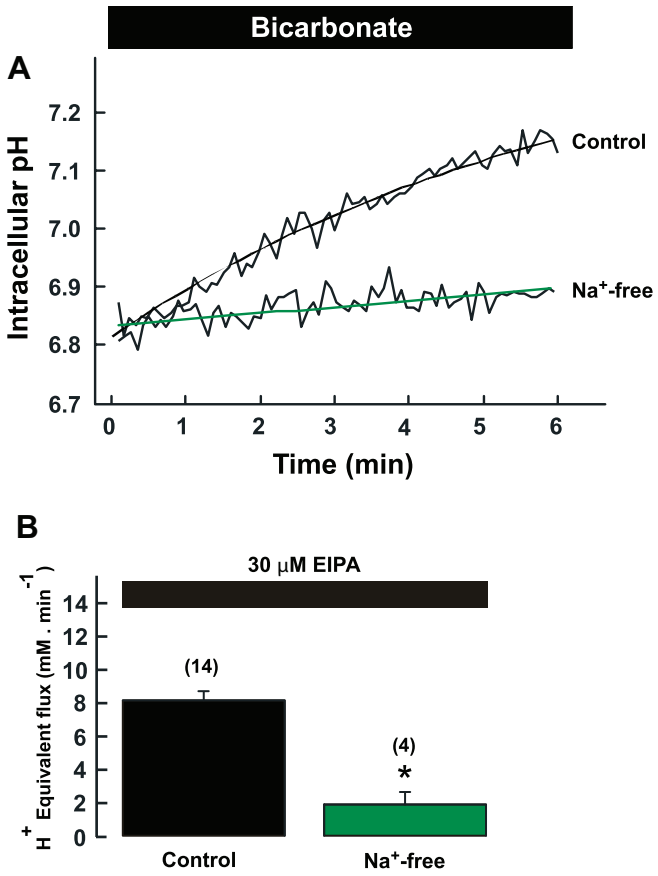

Fig. 3. Effect of Na^+ deprivation on Na^+/HCO_3^- cotransport in HEK cells. pH_i was monitored in HEK293 cells loaded with BCECF-AM and exposed to NH_4Cl to induce acid loading. *A*: representative pH_i traces of cells perfused with bicarbonate buffer solution, or perfused with Na^+-free bicarbonate buffer, in the presence of 30 μM EIPA. *B*: summary of HCO_3^- influx rates measured as H^+ equivalent flux (mM/min). Parentheses on *top* of bar indicate number of experiment. $^*P < 0.05$ compared with control.

Estimating β_{int} and pH_o in HEK293 cells exposed to high-CO_2 incubation. Figure 7, *A* and *B*, illustrates the experimental protocol used for investigating the pH_i dependence of β_{int} in cultured HEK cells exposed to normal (5%) and high (10–15%) P_{CO_2}. A wide range of pH_i values was achieved by superfusing different concentrations of a membrane-permeant weak base (NH_4^+ salt; Fig. 7*A*, *top*). Subsequent reduction in $[NH_4^+]$ in the superfusate results in a step-wise titration of the cells cytoplasm (Fig. 7*A*, *bottom*). The procedure for calculating β_{int} is detailed in MATERIALS AND METHODS. β_{int} Seems to be very similar, calculated for different pH_i values in cells incubated at normal or high (10–15%) P_{CO_2} (Fig. 7*B*).

Interestingly, measurements of pH_o in the media of cultured HEK cells (24 h) showed a small but statistical significant reduction in cells exposed to 10% P_{CO_2} (7.34 ± 0.02, $n = 6$), and cells exposed to 15% P_{CO_2} (7.26 ± 0.03, $n = 6$) compared with cells cultured under normal-P_{CO_2} conditions (7.47 ± 0.05, $n = 6$; Fig. 7*C*). Acidification of pH_o in the media of cultured HEK cells exposed to high P_{CO_2}, which have an impact in the pH_i of cells, could be responsible for the upregulation of the NBCn1 protein, to some extent. Furthermore, the increased NBC-mediated pH_i recovery in HEK cells subjected to high P_{CO_2} may reflect changes in cellular pH_i that underlie acidification of the culture media.

Hypercapnia increases the expression of NHE1 in the brain, heart, and kidney of neonatal mice (22). HEK293 cells express very low endogenous levels of NHE1 protein, under culture conditions (29, 31, 44). Herein, cultured embryonic kidney cells were exposed to high-P_{CO_2} conditions and the expression of NHE1 was evaluated by immunoblots (Fig. 8*A*). The response of NHE1 to hypercapnia followed the same pattern as that of NBCn1. Twenty four-hour exposure of cultured HEK cells to 10% CO_2 led to an increase in the NHE1 normalized protein expression, compared with 5% CO_2 (Fig. 8*B*; $P < 0.05$). Similarly, 15% CO_2 increased NHE1 expression in the kidney cell line by sixfold, compared with control ($P < 0.05$).

Taken together, these experiments demonstrated that NBCn1 total and/or surface expression and function and NHE1 protein expression are upregulated in cultured HEK293 cells subjected to 24-h elevated P_{CO_2} conditions.

DISCUSSION

We have provided evidence that in HEK cells, the Na^+/HCO_3^- cotransport accounts for at least 50% of the pH_i recovery after acid loading, being the NBCn1 cotransporter is the

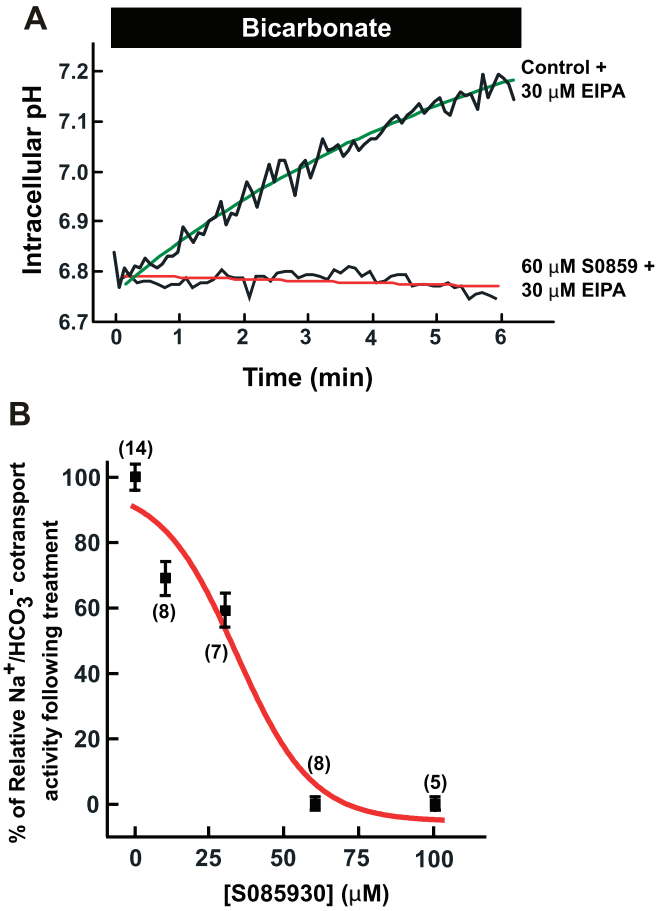

Fig. 4. Effect of S0859 on Na^+/HCO_3^- cotransport activity in HEK cells. *A*: traces of pH_i recovery of HEK cells following an NH_4Cl prepulse in 5% CO_2/HCO_3^--buffered solution, demonstrating the effect of 60 μM S0859 on pH_i recovery from an intracellular acid load, in the presence of 30 μM EIPA. *B*: mean dose-response curve for S0859 inhibition of Na^+/HCO_3^- cotransport in HEK cells, measured as rates of change of pH_i during the first 60 s of the pH_i recovery following acid loading. Parentheses on *top* of bar indicate number of experiments. Dose for half-inhibition, K_i, is 30 μM.

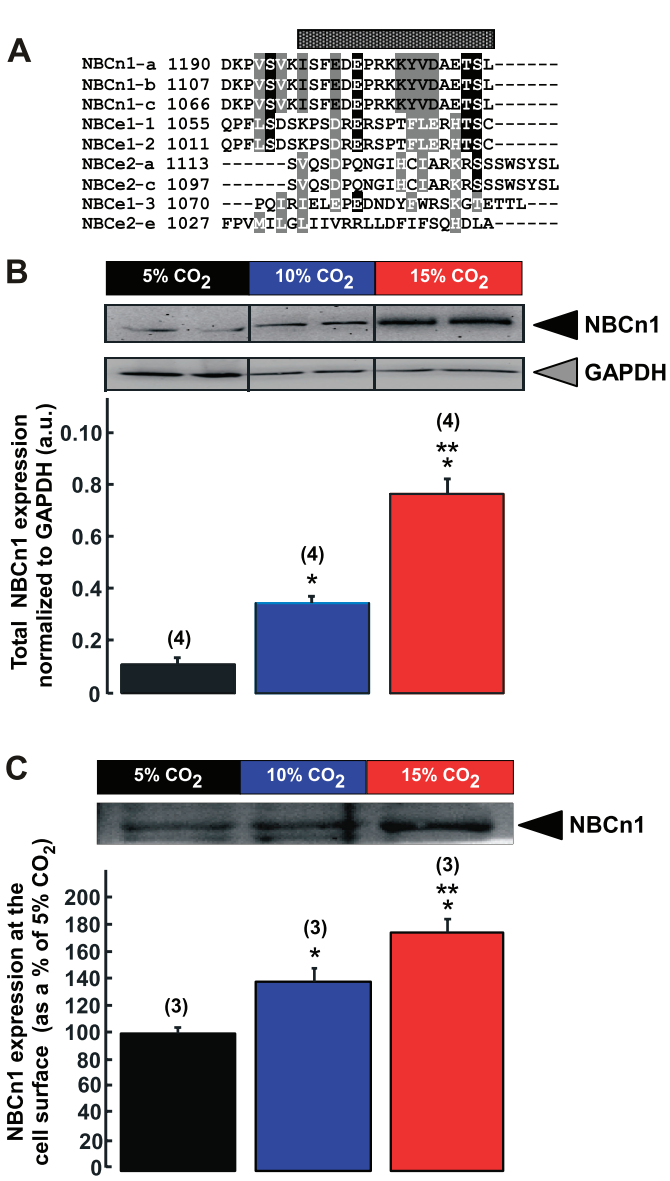

Fig. 5. Alignment of amino acid sequences of NBC Na^+/HCO_3^- cotransporter proteins and effect of 24-h elevated P_{CO_2} incubation on total NBCn1 expression and on NBCn1 surface targeting, in cultured HEK cells. *A*: alignment of amino acid sequences of the COOH terminus of NBC Na^+/HCO_3^- cotransporter proteins (CLUSTALW). Sequence conservation is indicated by black rectangles (identical amino acids) and gray rectangles (similar amino acids). Hatched bar indicates the NBCn1 amino acid epitope recognized by the anti-NBCn1 antibody. *B*: immunoblot for NBCn1. HEK293 cells were incubated under normal (5%)- or high (10–15%)-P_{CO_2} conditions for 24 h. Cell lysates were prepared and subjected to immunoblotting. Protein samples (50 μg) were subjected to SDS-PAGE analysis, transferred to PVDF membrane, and probed with anti-NBCn1 antibody. The blots were stripped and reprobed with the GAPDH antibody. One of four experiments is shown (*top panel*). For quantitative measurements of NBCn1, the pixel intensity of NBCn1 was measured and normalized to GAPDH ($n = 4$). $*P < 0.05$ compared with control (5% CO_2). $**P < 0.05$ compared with 10% CO_2 (*bottom panel*). *C*: HEK cell extracts corresponding to cells cultured under normal (5%)- or high (10–15%)-P_{CO_2} conditions for 24 h were prepared from biotinylated cells and incubated with streptavidin/sepharose resins. The bound fractions (80 μg) were resolved by SDS-PAGE and NBCn1 was detected by immunoblotting (*top panel*). For quantitative measurements of NBCn1, the pixel intensity of NBCn1 was measured in each sample and normalized to the amount of NBCn1 expressed in cell exposed to normal-P_{CO_2} conditions (*bottom panel*). $*P < 0.05$ compared with control (5% CO_2). $**P < 0.05$ compared with 10% CO_2.

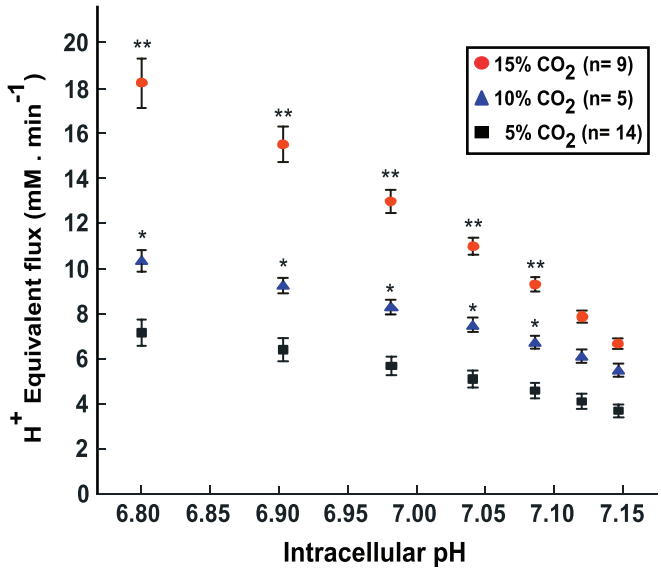

Fig. 6. Na^+/HCO_3^- cotransport activity in cultured HEK cells subjected to 24-h high-P_{CO_2} incubation. Average data of rates of HCO_3^- influx expressed in H^+ equivalent flux (mM/min) as a function of the HEK cells pH_i and measured under bicarbonate buffer conditions, in the presence of 30 μM EIPA. Initial rates of change of pH_i during the first 60 s were estimated from the slope of the line fitted by the least squares method, in HEK cells cultured under control condition (5% CO_2, black) or HEK cells cultured under 10% CO_2 (blue), or under 15% CO_2 (red). $*P < 0.05$ compared with control. $**P < 0.05$ compared with 10% CO_2.

only transporter responsible for this HCO_3^- influx. The remnant 50% recovery was blocked by the Na^+/H^+ exchanger inhibitor amiloride and its derivative, EIPA, and is possibly mediated by NHE1, which expresses endogenously in HEK cells (29, 44), and/or NHE3 (7), and/or NHE8 (17, 18), both of which also express in kidney tissues. Given that the sensitivity of the isoform 1 of NHE to EIPA is greater than any other NHE isoform and that the full blockade of NHE activity in our experimental setting of HEK cells exposed to acid loading was achieved by 10 μM EIPA may suggest that NHE1 is the main NHE express in these HEK293 cells. However, a recent report from Odunewu and Fliegel (31) demonstrated that HEK293 expressed endogenous NHE3 protein and that the S3226 compound, a Na^+/H^+ exchanger inhibitor reported to have specificity for the NHE3 isoform, inhibited up to 60% of the Na^+/H^+ exchange activity in these cells (31).

The HEK cell line has been widely used as an expression tool for recombinant proteins. Despite its epithelial origin, HEK cell's biochemical machinery is capable of carrying out most of the posttranslational folding and processing, generating functional mature protein from a broad spectrum of nucleic acids. The main attributes of the HEK cell system comprise its quick and easy reproduction and maintenance, amenability, and high efficiency of cDNA transfection using a varied range of methods, high competence for protein production, precise translation and processing of proteins, and small cell size with minimal processes appropriate for electrophysiology and pH_i experimentation (43). This will include physiological reasons such as the nature of the endogenous receptors, proteins (e.g., the NBCn1 characterized in this study), or signaling pathways, and also practical considerations. In this context, on the basis of its kidney origin, HEK cells represent a powerful tool to

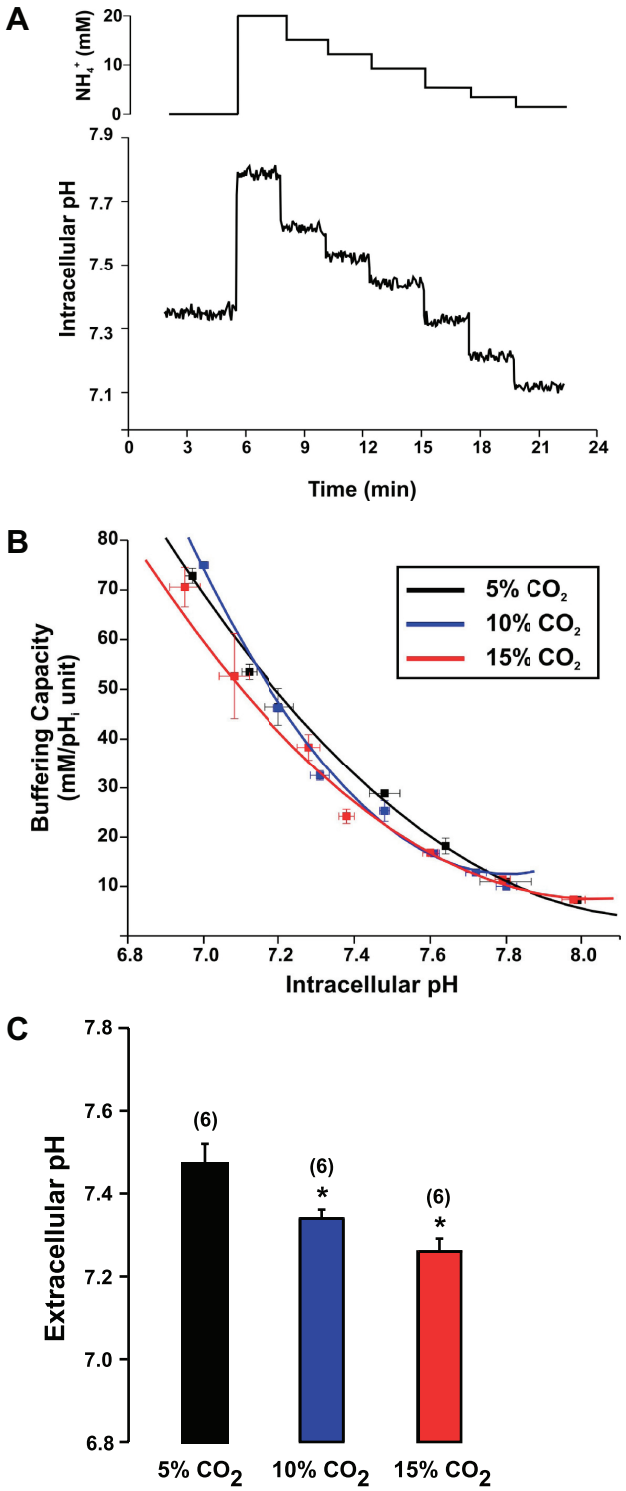

Fig. 7. Intrinsic buffering capacity and extracellular pH, in cultured HEK293 cells exposed to high-P_{CO_2} incubation. HEK cells were superfused with HEPES-buffered solutions, nominally free of CO_2/HCO_3^-. *A*: superfusion of cells with decreasing concentrations of NH_4^+ (from 20 to 0 mM; *top*) produced the pH_i trace measured by epifluorescence (*bottom*). Superfusates were Na^+ and Ca^{2+} free. *B*: pH_i traces obtained in HEK cells subjected to normal (5%, black)-or high (10% blue, 15% red)-P_{CO_2} conditions (24 h, for the NH_4^+ removal method) were analyzed (as outlined in MATERIALS AND METHODS) and the data obtained were plotted to fit exponential curves. *C*: pH_o values obtained in the media of cultured HEK cells exposed to normal (5%) and high (10–15%) P_{CO_2} for 24 h. Parentheses on *top* of bar indicate number of cultures analyzed. *$P < 0.05$ compared with 5% CO_2.

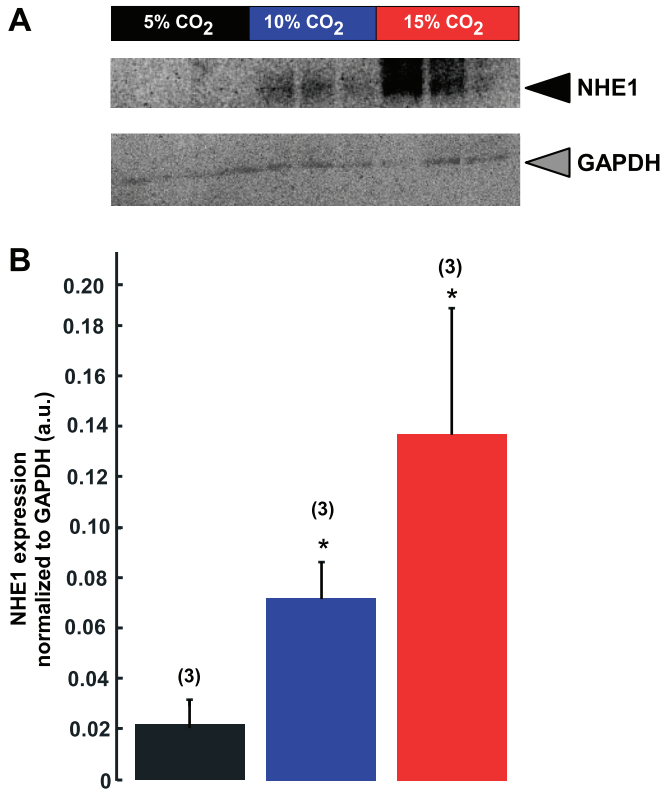

Fig. 8. Effect of 24-h high-P_{CO_2} incubation of HEK cells on NHE1 protein expression. Immunoblot analysis of NHE1 protein expression in cultured HEK293 cells exposed to normal (5%)- and high (10–15%)-CO_2 conditions for 24 h. *A*: HEK293 cell lysates were prepared and subjected to immunoblotting. Protein samples (50 μg) were subjected to SDS-PAGE analysis, transferred to PVDF membrane, and probed with anti-NHE1 antibody. The blots were stripped and reprobed with the GAPDH antibody. Experiments were run in duplicates. *B*: for quantitative measurements of NHE1, the pixel intensity of NHE1 was measured and normalized to GAPDH ($n = 3$). *$P < 0.05$ compared with control (5% CO_2).

study the response of cultured cells to high-P_{CO_2} exposure, and the effect of high-P_{CO_2} conditions to endogenously expressed pH_i regulatory proteins such as the NBCn1 Na^+/HCO_3^- cotransporter and the NHE1 Na^+/H^+ exchanger. Moreover, very recently it was demonstrated that acute sustained intracellular acidosis activates NHE1, in HEK293 cells incubated in reduced serum media and subjected to the NH_4Cl prepulse to monitor the NHE1-dependent pH_i recovery in cells exposed to transient acidosis (31). However, we are fully aware that other culture kidney cell systems such as the Madin-Darby canine kidney (MDCK) cell line from the distal tubule/collecting duct (30), the immortalized rat proximal tubule cells (9), and the M-1 cortical collecting duct cell line (27) are more closely related to native adult kidney cells rather than the HEK cells. Nonetheless, the HEK293 expression system represents a valuable instrument to study membrane transporters, especially anion transporters of the SLC4 and SLC26 families, which did not endogenously express in HEK cells. We and others extensively characterized the molecular identity and functional activity of AE1 (16, 41), AE3 (10, 40), and SLC26A6 (3, 5) by transiently transfecting HEK293 cells with plasmid cDNAs. In addition, members of the NBC family have been transiently expressed in HEK cells to study the physical and functional complexes of NBCs with carbonic anhydrases (4, 26).

However, since HEK cells have noticeable pH_i regulatory mechanisms that respond effectible after cellular acidification, herein we characterized in detail a previously undisclosed observation, the solely functional contribution of the NBCn1 to the HCO_3^--dependent recovery after acid loading of the cells. This is the first reported examination of the NBCn1-mediated Na^+- and HCO_3^--dependent background transport activity in HEK cells. Our report contributes to the understanding of the NBCn1 biology, in particular, and the physiological response of kidney cultured cells to acid loading, in general. In our functional studies, we measured total NBC activity. However, we have good reasons to assume that this activity is largely if not completely mediated by the NBCn1 type of the electroneutral NBC. Recently, using specific antibodies we failed to detect endogenous expression of the NBCe1 (SLC4A4) and NBCe2 (SLC4A5) proteins in cultured HEK293, validated by RT-PCR analysis in the present study (Fig. 1) (14). Conversely, endogenous expression of NBCn1 (SLC4A7) was detected by the same antibodies we are using in the current studies, in HEK293 cells (14). In none of these studies we investigated the expression of an NBCn1 variant (also named NBC2), which is coded by the SLC4A7 gene and is 93% identical to the human NBCn1 (33). This NBCn1 variant was initially cloned from human retina and was detected by Northern blot analysis in several tissues including testis, spleen, heart, ovary, and kidney, among others (21). The NBCn1 variant mentioned above, although the similarity has a very different COOH-terminal domain compared with NBCn1, was analyzed by the CLUSTALW multiple sequence alignment software (not shown). In addition, this NBCn1 variant has not a conserved epitope suitable to be recognized by the anti-NBCn1 antibody utilized in our studies (not shown). Therefore, we could not exclude the possibility that the HEK cells expression system expresses endogenous levels of the NBCn1 variant protein.

In the present study, a portion of the Na^+-dependent and HCO_3^--dependent transport examined in cultured HEK293 cells subjected to acid loading, which was not inhibited by either EIPA or amiloride, was completely blocked by the selective high-affinity generic NBC inhibitor, S0859 (Fig. 4). Therefore, amiloride-sensitive Na^+/HCO_3^- cotransport only plays a partial role, if any in our experimental setting, because the pH_i recovery rates in the presence of CO_2/HCO_3^- were partially inhibited by EIPA/amiloride. Taken together, we cannot entirely exclude that additional NBCs may be functional in cultured HEK cells, but the prevailing evidence suggests that NBCn1 is the most important mediator of the embryonic kidney cell Na^+/HCO_3^- cotransport.

Exposure of mice to chronically elevated CO_2 increases the abundance of acid extruder protein levels in the brain, heart, and kidney of neonatal mice (22). The effects of CO_2 on expression of acid-base transporters increased with the severity of the hypercapnic conditions (12% CO_2 > 8% CO_2), and the different tissues responded differently to hypercapnia; while the brain alters the AE3 Cl^-/HCO_3^- exchanger levels to the greatest degree, the NHE1 Na^+/H^+ exchanger and the NBCn1 and NBCe1 Na^+/HCO_3^- cotransporters suffered the major changes in the heart and the kidney of mice (22). This pattern of changes in transporter expression may be suggestive of an adaptive response to attenuate the deleterious effects of the hypercapnic-induced intracellular acidification. In addition, chronic metabolic acidosis in rats treated chronically with NH_4Cl loading was associated with a significantly increased abundance of the NBCn1 as well as enhanced HCO_3^- transport in medullary TAL (mTAL) and increased abundance of the NBC3 in the intercalated cells of the collecting ducts (23). Moreover, cultured mTAL showed increased NBCn1 expression following acidic incubation of cells (24). Hence, enhanced NBCn1 protein expression may play an important role in maintaining pH_i levels and renal regulation of the acid-base balance in the kidney. However, we are aware that it is excessive to extrapolate knowledge on renal expression of NBCs, NHE, and other membrane transporters in mature animals to the HEK293 cell system we are using in our studies.

In the present manuscript, we have not identified the unequivocally contributory agent responsible for the NBCn1 expression change in the whole lysate or the extracellular surface of cultured HEK293 cells exposed to high CO_2. In this line, many factors ranging from the CO_2 itself, the acidosis, the [HCO_3^-], the release of different cellular factors, or a combination of physiological cell changes could account for the observed NBCn1 upregulation. In addition, NHE1 expression increased in HEK293 exposed to 10 and 15% CO_2. Yet, since NBCn1 has been shown in tissue culture models to be similarly modulated by acidosis (23, 24, 32), we believe that, at least in part, the changes in our study are due to the high P_{CO_2}-induced acidotic stress. We proved that the pH_o slightly acidified in the cell culturing media upon increase in P_{CO_2} (Fig. 7), which might be a factor that contributes to the increased NBCn1 expression and NBC function in response to P_{CO_2}-induced acidosis, in cultured HEK cells.

While the pattern of changes in transporter expression observed in our studies is highly suggestive of an adaptive response to ameliorate severe intracellular acidosis adverse effects, increased expression of both NHE1 and NBCn1 after elevated P_{CO_2} could lead to an increase in intracellular Na^+ which underlies the parallel activation of the Na^+/HCO_3^- cotransport and Na^+/H^+ exchange activities. Yet, the increased NBC function and NBCn1 expression as well as NHE1 increased expression reflect augmented cellular capacity of counteracting intracellular acidosis inflicted by high P_{CO_2}. These interesting findings significantly expand and reinforce previous knowledge on NBCn1 and NHE1 regulation performed in mammalian renal tissues.

We demonstrated that the NBCn1 Na^+/HCO_3^- cotransporter, which is the only NBC transporter and almost certainly the only Na^+-dependent HCO_3^- transporter expressed in HEK293 cells, and which accounts for half of the alkaline loading of cells exposed to intracellular acidification, increased its expression and function after exposure of cultured cells to 24-h high-CO_2 conditions. Further investigation is needed to examine the mechanisms that induce these changes in kidney cells.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: A.O., E.A.A., and B.V.A. conception and design of research; A.O. and L.A.V. performed experiments; A.O., L.A.V., E.A.A., and B.V.A. analyzed data; A.O., E.A.A., and B.V.A. interpreted results of experiments; A.O., L.A.V., and B.V.A. prepared figures; A.O., L.A.V., E.A.A., and B.V.A. approved final version of manuscript; E.A.A. and B.V.A. drafted manuscript; E.A.A. and B.V.A. edited and revised manuscript.

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