Transcriptional repression of Na-K-2Cl cotransporter NKCC1 by hypoxia-inducible factor-1

Juan C. Ibla,1,2* Joseph Khoury,1,2* Tianqing Kong,2 Andreas Robinson,2 and Sean P. Colgan2

1Department of Anesthesia Perioperative and Pain Medicine, Children’s Hospital, and 2Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesia Perioperative and Pain Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts

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MUCOSAL SURFACES, such as the lung and colon, are lined by a monolayer of epithelia that provides tissue barrier and vectorial ion transport function. Recent reports indicate that mucosal epithelial cells are end point targets for a number of diseases, including ischemia-reperfusion injury, mucosal inflammation, and sepsis (14). It is therefore important to understand mechanisms of epithelial function and adaptation to such adverse conditions. From this standpoint, it is now appreciated that a common feature in a variety of disease processes is diminished oxygen delivery and/or oxygen availability (hypoxia) (31). For example, it was recently shown that epithelial cells lining the colon lack normal oxygenation during modeled colitis and that NKCC1 transcriptional repression by hypoxia. Generally, such episodes of fluid accumulation are self-limiting. At present, little is known about mechanisms to compensate excessive fluid transport. Here we describe an adaptive mechanism to dampen fluid loss during hypoxia. Initial studies confirmed previous observations of attenuated electrogenic Cl– secretion after epithelial hypoxia. A screen of known ion transporters in Cl–-secreting epithelia revealed selective downregulation of Na-K-2Cl cotransporter NKCC1 mRNA, protein, and function. Subsequent studies identified transcriptional repression of NKCC1 mediated by hypoxia-inducible factor (HIF). Chromatin immunoprecipitation analysis identified a functional HIF binding site oriented on the antisense strand of genomic DNA downstream of the transcription start site corresponding to the NKCC1 5′-untranslated region. Additional in vivo studies using conditional Hif1a-null mice revealed that the loss of HIF-1α in Cl–-secreting epithelia results in a loss of NKCC1 repression. These studies describe a novel regulatory pathway for NKCC1 transcriptional repression by hypoxia. These results suggest that HIF-dependent repression of epithelial NKCC1 may provide a compensatory mechanism to prevent excessive fluid loss during hypoxia.

As stated above, a primary function of the epithelium is vectorial ion transport, the transport event responsible for mucosal hydration (1, 13). This process occurs through a coordinated series of membrane transporters. In Cl–-secreting epithelium, the rate-limiting (1) step is entry of Cl– via the Na-K-2Cl cotransporters, a family of proteins that mediate the electroneutral transport of Na+, K+ and Cl– across cellular membranes (13). These cotransporters are distributed over a wide variety of tissues, and, with the exception of renal epithelia, are localized along the basolateral membrane (13). Because of their particular ability to regulate both anionic and cationic fluxes, the Na-K-2Cl cotransporters serve a number of vital physiological functions. In the majority of cell types Na-K-2Cl cotransporters are involved in the regulation of cell volume and are functionally activated by cell shrinkage (13). In coordination with other solute transport pathways, Na-K-2Cl cotransporters play a key role in salt transport by secretory epithelia. Specifically, the apical/basolateral distribution determines whether Na-K-2Cl cotransporters participate in active fluid absorption or secretion depending on the particular organ and their localization (13).

We (37) and others (10, 22, 26) have previously studied the influence of hypoxia on epithelial electrogenic Cl– secretion and water transport. These studies revealed that hypoxia elicits a direct, noncytotoxic attenuation of stimulated Cl– transport. Here we identify molecular mechanisms of attenuated Cl– secretion by hypoxia. Specifically, these studies reveal the transcriptional repression of the major Na-K-2Cl cotransporter (NKCC1). Extensions of these studies identified a functional binding site for HIF-1α in the genomic fraction corresponding to the 5′-untranslated region (5′-UTR) of NKCC1. Such studies provide new insight into adaptive responses of epithelial cells and identify target molecules for their potential therapeutic use in mucosal disease.

MATERIALS AND METHODS

Cell culture. T84 intestinal epithelial cells were grown and maintained as confluent monolayers in 1:1 Dulbecco’s modified Eagle’s medium (DMEM)-Ham’s F-12 medium with 10% FBS at 37°C in 5% CO2 in room air. For electrophysiological measurements described below, T84 cells were plated on collagen-coated permeable supports as previously described in detail (11). Monolayers were grown on 0.33-cm2 or 5.0-cm2 ring-supported polycarbonate filters (Costar, Cambridge MA) unless otherwise noted, and they were used 6–12 days after plating as described previously (21). Epithelial cultures

* J. C. Ibla and J. Khoury contributed equally to this work.

Address for reprint requests and other correspondence: S. P. Colgan, Brigham and Women's Hospital and Harvard Medical School, Thorn Bldg. 704, 20 Shattuck St., Boston, MA 02115 (e-mail: colgan@zeus.bwh.harvard.edu).

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were exposed to hypoxia as described previously (5). Growth media were replaced with fresh media equilibrated with hypoxic gas mixture, and cells were placed in a hypoxic chamber (Coy Laboratory Products, Ann Arbor, MI). Oxygen concentrations were as indicated (normoxia equal to PO2 147 Torr and hypoxia equal to PO2 20 Torr), with the balance made up of N2, CO2 (constant Pco2 35 Torr), and water vapor from the humidified chamber.

Electrophysiological measurements. To measure agonist-stimulated short-circuit current (Isc), transepithelial potential, and resistance, we use a commercially available voltage clamp (Iowa Dual Voltage Clamps, Bioengineering, University of Iowa) interfaced with an equilibrated pair of calomel electrodes and a pair of Ag-AgCl electrodes, as described in detail elsewhere (11). CI− secretory responses are expressed as a change in Isc [peak Isc minus baseline Isc; designated (△Isc)] necessary to maintain zero potential difference across the monolayer.

Transcriptional analyses. The transcriptional profile of epithelial cells subjected to hypoxia was assessed in RNA derived from control epithelia (T84 cells) or epithelia exposed to hypoxia (PO2 20 Torr and Pco2 35 Torr for indicated periods of time) with quantitative genechip expression arrays (Affymetrix, Santa Clara, CA) (20). mRNA expression was quantified by real-time PCR (iCycler; Bio-Rad Laboratories, Hercules, CA), as described previously (27). The primer set containing SYBR green spanning exons 4 and 5 of the human NKCC1 was synthesized with FirstChoice forward primers and NKCC1-specific reverse primers for human β-actin sense 5′-TGACCCGATGATGTTTGAGA-3′ and antisense 5′-AGTCCATACGATGACC-3′.

Protein fractionation, Western blotting, and cell surface biotinylation. Cells were pelleted in PBS, and the pellet was used for protein extraction with a cell lysis buffer (Cell Signaling Technologies, Beverly, MA) containing (mM) 20 Tris-HCl (pH 7.5), 150 NaCl, 1 Na2EDTA, 1 EGTA, 2.5 sodium pyrophosphate, 1 β-glycerophosphate, 1 Na3VO4, and 1 PMSF, with 1% (vol/vol) Triton X-100 and 1 μg/ml leupeptin. For Western blot analyses, samples were subjected to Tricine-SDS gel electrophoresis (7.5% or 10%) and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Blots were sequentially probed with anti-human NKCC1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and horseradish peroxidase-conjugated secondary antibodies. Similarly, blots were probed with anti-human β-actin antibody (Santa Cruz Biotechnology) as a control for loading. Chemiluminescent substrate was detected on X-OMAT film (Kodak). T84 monolayers were exposed to normoxia or 24–72 h of hypoxia, lysed in low-stringency lysis buffer, and immunoprecipitated overnight with NKCC1 antibody. Beads were washed three times in low-stringency lysis buffer, biotinylated (1 mM biotin, 25 min, 4°C), washed again, and resuspended in 150 mM NaHCl (in lysis buffer) for 30 min at 4°C. Washed beads were resuspended in sample buffer, boiled (5 min), and separated on 10% SDS-PAGE. Proteins were transferred to nitrocellulose membrane and probed for biotinylated proteins with avidin-peroxidase.

5′-Rapid amplification of DNA ends, cloning of 5′-UTR, mutation of HIF-1α transcription binding site, and mutation of transcription start site. RNA was isolated from the T84 human epithelial cell line with TRIzol (Invitrogen, Carlsbad, CA) for RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE). 5′-RLM-RACE cDNA was prepared with a FirstChoice kit (Ambion, Austin, TX) per the manufacturer’s protocol. 5′-RACE experiments were carried out with FirstChoice 5′ forward primers and NKCC1-specific reverse primers (outer: 5′-CTGTTCTGGGAAAACCTGGCC-3′; inner: 5′-ACAGCCGCAAGCACCAGTGAGAGACTT-3′) with SuperTaq recombinant DNA polymerase (Ambion). The resulting band was cloned into a TA cloning vector (pCR 2.1, Invitrogen) and sequenced with T7 primer (Biopolymers Facility, Harvard Medical School). Cloning of the 5′-flanking region and adjacent 5′-UTR was performed with genomic DNA extracted from HeLa cells by a commercially available method (Qiagen, Valencia, CA). A PCR fragment was generated extending 510 bp from position −324 upstream of the transcription start site (TSS) (19) to +186 with primers: sense 5′-GATCCCTCGGGGACATCATG-3′ and antisense 5′-TGGCCGACCCCGCGCGCGAAATG-3′. The gel-purified band was then cloned into a pGL3 luciferase reporter vector (Promega, Madison, WI). Transcription factor analysis of this sequence was performed with online software (MatInspector, Genomaxit). The identified HIF-1α transcription factor binding site was mutated with a commercially available kit (QuikChange site-directed mutagenesis kit, Stratagene). The TSS of NKCC1 was identified as described by the 5′-RACE and mutated similar to the HIF-1α transcription binding site mutation.

Plasmid transfection and luciferase reporter assays. HeLa cells were passaged into 24-well plates and allowed to attach for 20 h. Cells were then subjected to hypoxia (PO2 20 Torr), and transient transfection of HeLa cells was carried out at 60% confluence in DMEM (Invitrogen) at a concentration of 0.5 μg of plasmid per well of a 24-well plate with preequilibrated hypoxic medium. Plasmids were transfected with Polyfect (Qiagen) at a concentration of 4 μg of reagents for 1 μg of DNA. Six hours after transfection, plates were either returned to normoxia conditions or maintained in hypoxia for an additional 18–48 h. At the desired time point after hypoxia, transfected cells were lysed in 100 μl of passive lysis buffer (Promega), and 20 μl was used in a luciferase assay with Luciferase Assay Reagent II (Promega). Luminescence readings were obtained with a luminometer (Turner Designs TD-20/20, Sunnyvale, CA). Relative luciferase units were recorded, and values were corrected to the total protein concentration (Lowry method) and verified by cotransfection with Renilla for each sample.

5′-UTR DNA was quantified by standard PCR using primers (sense 5′-CTGTTCTGGGAAAACCTGGCC-3′; antisense 5′-GCACCGGCGCTCATAGCTG-3′) designed to amplify a 217-bp region spanning the HIF-1 binding site located from position 1 bp to +202 bp with respect to the TSS. Chromatin incubated with a nonspecific antibody, beads without antibody, or water alone was used to control for nonspecific binding of DNA.

In vivo assessment of NKCC1. Colonic mucosal scrapings (enriched in epithelial cells) were obtained from 6- to 8-wk-old conditional Hif1α-mutant mice or littermate controls, as described previously (16). Scrapings were homogenized in RNAlater (Qiagen) with a 22-gauge syringe (Becton Dickinson) and a Qiashredder column (Qiagen). RNA extraction including DNAse digestion was performed with an RNeasy kit (Qiagen). Reverse transcription was done with an iScript cDNA synthesis kit (Bio-Rad Laboratories). Amplification was performed on an i-Cycler IQ real-time PCR detection system (Bio-Rad Laboratories) with the gene-specific primers for NKCC1 described above and mouse β-actin: sense 5′-CTAGGCAAGCAAGGTGTAT-3′ and antisense 5′-TGGCCAGATCTTCTGGAACATG-3′. Cycle parameters were 3 min at 95°C and then 40 cycles with 45 s at 94°C, 45 s at 57°C, 45 s at 72°C, and 5 s at 81.5°C (fluorescence data collection point), followed by repetitive melting cycles to establish product specificity. Comparison of gene expression in a semiquantitative manner was performed based on the mathematical model of
Pfaffl (27). All procedures involving animals were performed according to National Institute of Health guidelines for use of live animals and were approved by the Institutional Animal Care and Use Committee at Brigham and Women’s Hospital.

Data analysis. Cl\textsuperscript{\texttt{-}} secretion and luciferase reporter data were compared by two-factor ANOVA or Student’s t-test, where appropriate. Values are expressed as means and SD from at least three separate experiments.

RESULTS

Hypoxia attenuates electrogenic Cl\textsuperscript{-} secretion. Initially, we utilized a cell culture model using Cl\textsuperscript{-} secretory epithelia (T84) grown as monolayers on permeable supports to examine influences of hypoxia on electrogenic Cl\textsuperscript{-} secretion. As shown in Fig. 1, and as we demonstrated previously (37), subjection of epithelia to normobaric hypoxia (Po\textsubscript{2} 20 Torr) resulted in attenuated electrogenic Cl\textsuperscript{-} secretion. Indeed, both receptor-mediated (adenosine, 30 \mu M) and non-receptor-mediated (forskolin, 3 \mu M) stimulation of Cl\textsuperscript{-} secretion, measured as a function of short-circuit current (I\textsubscript{sc}), was attenuated in a time-dependent manner (P < 0.025 by ANOVA for each), with >80% decrease in I\textsubscript{sc} observed at 36 – 48 h of hypoxia. Such attenuated decreases in Cl\textsuperscript{-} secretion are also reflected as a decrease in transepithelial water transport (37). Of note, and as previously shown, such decreases in electrogenic Cl\textsuperscript{-} secretion occur in the absence of a significant fall in transepithelial resistance during normoxic conditions (P < 0.025), a sensitive measure of epithelial toxicity (5). Such data confirm previous work addressing epithelial ion transport properties and provide the basis to define these principles at the molecular level.

Influence of hypoxia on epithelial membrane ion channels. As an initial attempt to gain insight into mechanisms of attenuated Cl\textsuperscript{-} secretion, we transcriptionally profiled epithelial RNA derived from normoxic and hypoxic (6 and 18 h) epithelia. These studies were insightful insomuch as they identified a selective loss of NKCC1 by hypoxia. Indeed, in Cl\textsuperscript{-} secreting epithelium, well-established membrane pathways exist for Cl\textsuperscript{-} uptake through NKCC1 and Cl\textsuperscript{-} efflux through Cl\textsuperscript{-} channels, including the cystic fibrosis transmembrane regulator.

As shown in Fig. 2A, microarray analysis revealed that hypoxia repressed NKCC1 mRNA in a time-dependent fashion (P < 0.025 by ANOVA), RNA transcript downregulation at 6 h (2.1 ± 0.5 fold) and 18 h (8.7 ± 1.2 fold) of hypoxia compared with normoxic controls. This pattern of expression was confirmed with real-time PCR (Fig. 2B), showing a 2.1 ± 0.1- and 3.2 ± 0.2-fold repression at 6 and 18 h of hypoxia, respectively. Notably, mRNA transcript levels of other membrane channels important in coordinated Cl\textsuperscript{-} secretion were not significantly changed by hypoxia (data not shown). These results implicate specific downregulation of NKCC1 by hypoxia.

NKCC1 protein expression during hypoxia. We verified the above findings of hypoxia-mediated NKCC1 repression at the protein level. As shown in Fig. 3A, we examined surface NKCC1 protein with [\textsuperscript{3}H]bumetanide, a method used previously to determine specific cell surface NKCC1 (7). These studies revealed a hypoxia time-dependent loss of surface NKCC1 (as reflected by [\textsuperscript{3}H]bumetanide binding) through 48 h of hypoxia (P < 0.01 by ANOVA). Decreased surface NKCC1 was evident as early as 24 h of hypoxia (53 ± 6.2% decrease; P < 0.01) and was maximal at 48 h (e.g., no further decrease at 72 h; data not shown). In addition, we examined the relative contribution of the bumetanide-sensitive component of Cl\textsuperscript{-} secretion after forskolin (100 nM) stimulation. Our analysis indicated that the bumetanide-sensitive component, measured as an I\textsubscript{sc}, in T84 cells used for these experiments corresponds to −84.2 ± 4.2% of total current generated by agonist stimulation. Importantly, bumetanide was less effective in cells subjected to hypoxia (51.0 ± 4.1% inhibition; P < 0.025) than those subjected to normoxia, thereby supporting our hypothesis that hypoxia results in a functional repression of NKCC1.

Further examination of total NKCC1 by Western blot (representative blot shown in Fig. 3B) revealed a loss of NKCC1 with increasing time of hypoxia. By Western blot, hypoxia-mediated repression of NKCC1 protein was evident at time

Fig. 1. Influence of hypoxia on receptor- and nonreceptor-stimulated Cl\textsuperscript{-} secretion. T84 intestinal epithelia were subjected to normoxia (Po\textsubscript{2} 147 Torr) or hypoxia (Po\textsubscript{2} 20 Torr) for indicated periods of time and examined for forskolin (A, 3 \mu M)-or adenosine (B, 30 \mu M)-stimulated Cl\textsuperscript{-} secretion (measured as a short circuit current, I\textsubscript{sc}). Data are pooled from 8–12 individual monolayers from 3 experiments in each condition, and results are expressed as mean ± SE I\textsubscript{sc} (*P < 0.01 compared with normoxia). Time points in normoxia did not change significantly (P = 0.025).
points beyond 24 h, with maximal decreases at 48 and 72 h of hypoxia exposure compared with normoxia. Additional studies were performed to determine whether NKCC1 repression by hypoxia requires transcriptional activity. To do this, T84 cells were subjected to normoxia or hypoxia (48 h) in the presence or absence of the transcriptional inhibitor 5,6-dichlorobenzimidazole riboside (DRB; 3 μM final concentration) and assessed for changes in NKCC1 expression by Western blot. As shown in Fig. 3B, preincubation of T84 cells with DRB inhibited the hypoxia-dependent loss of NKCC1. Consequently, hypoxia-mediated repression of NKCC1 requires new mRNA synthesis.
Additionally, basolateral biotinylation and immunoprecipitation of NKCC1 during normoxia and hypoxia was performed in confluent, high-resistance (>1,000 ohm-cm²) T84 cells. As shown in Fig. 3C, it is readily evident that surface protein levels decrease with increasing time in hypoxia. Comparison with normoxic controls at the same time points provides strong evidence for NKCC1 dependence on hypoxia, consistent with our results obtained for mRNA and total protein. Although the surface expression of NKCC1 decreases between 12 and 24 h, the total protein expression decreases only after 24 h. In subsets of experiments, we found that with fractionation of membrane, cytoplasm, and nuclear lysates, no differences exist in the percentage of total retrieval of NKCC1 between normoxia and hypoxia (data not shown). From these findings, we concluded that the turnover of surface protein is likely more rapid than intracellular NKCC1.

Transcriptional regulation of NKCC1. In view of the likelihood of a transcription-mediated repression of NKCC1 during hypoxia, attention was directed at the 5′-UTR for potential hypoxia-regulated transcription factor sequences. Our 5′-RACE results confirmed the findings of Liedtke et al. (19) identifying the TSS 190 bp upstream of the start codon. We then cloned a genomic fraction extending from −324 bp to +186 bp (sequence shown in Fig. 4A) into pGL3 luciferase reporter vector and examined transcriptional activity in hypoxia after transient transfection in HeLa cells. As shown in Fig. 4B, analysis of luciferase reporter activity revealed that hypoxia represses NKCC1 activity of the genomic DNA downstream of the TSS corresponding to 5′-UTR by 71 ± 12% (P < 0.025) relative to cells incubated in normoxia, suggesting the possibility that hypoxia acts directly on this genomic fraction of NKCC1. To further characterize the role of the cloned 5′-flanking region and adjacent 5′-UTR for NKCC1 transcriptional control, we created a 5-bp mutation of the TSS (mutation from ACACCT to TGAGA, construct termed NKCC1ΔTSS), using the wild-type NKCC1 construct. Adjusted relative luciferase units relative to the expression of the NKCC1ΔTSS during normoxia showed a significant loss of activity (89 ± 3.7%; P < 0.01) compared with wild-type NKCC1 during normoxia (Fig. 4C). These results corroborate the importance of this region of NKCC1 and the neighboring HIF-1α binding site for the transcriptional control of NKCC1 during hypoxia.

Analysis of our cloned region of NKCC1 revealed the existence of a potential binding site for HIF-1α in reverse orientation (binding site core sequence 5′-GACGT-3′) at position −38 relative to TSS (Fig. 4A). Others have reported that the binding site for HIF can exist within the 5′-UTR (4). On the basis of these findings, and known epithelial expression of HIF-1α (14), we determined whether the genomic DNA downstream of the TSS corresponding to the 5′-UTR of NKCC1 would bind HIF-1α. For these purposes, we used ChIP analysis to examine binding of HIF-1α to the NKCC1 5′ genomic fraction spanning the putative HIF-1α binding site in intact cells. As shown in Fig. 5A, this analysis revealed a prominent band of 217 bp in nuclei derived from hypoxic, but not normoxic, cells. No bands were evident in the beads-only control, and preimmunoprecipitation samples revealed equivalent DNA input. Such results indicate that hypoxia induces HIF-1α binding to the proximal 217 bp relative to the ATG of NKCC1.

To further define the role of HIF-1α in NKCC1 repression by hypoxia, a 5-bp mutation of the central HIF-1α binding site (mutation from GACGT to TGAGG, construct termed NKCC1ΔHIF) was created on the wild-type sequence and confirmed by sequencing. HeLa cells were then transfected with wild-type and NKCC1ΔHIF and exposed to hypoxia. As can be seen in Fig. 5B, although hypoxia repressed activity of the wild-type construct by 76 ± 14% (P < 0.01), mutation of the central HIF binding site resulted in a complete loss of hypoxia-mediated repression (7 ± 3% loss of luciferase activ-
binding site located at position by protein G Sepharose beads alone (B) or beads wash (W). HeLa cell genomic DNA (input), antibody controls, and samples precipitated hypoxic HeLa cells. Reaction controls included PCR performed with whole genomic fraction corresponding to the NKCC1 5’/H11032

A

Fig. 5. Binding and functional role of HIF-1 on NKCC1 expression. A: chromatin immunoprecipitation was used to examine HIF-1α binding to a genomic fraction corresponding to the NKCC1 5’-UTR in normoxic and hypoxic HeLa cells. Reaction controls included PCR performed with whole HeLa cell genomic DNA (input), antibody controls, and samples precipitated by protein G Sepharose beads alone (B) or beads wash (W). B: the HIF-1 binding site located at position +147 to +151 bp with respect to the TSS was eliminated in the wild-type luciferase construct by site-directed mutagenesis. The resulting construct (NKCC1ΔHIF) was cotransfected along with Renilla luciferase vector into HeLa cells, followed by 24 h of hypoxia. Data shown are a representative experiment from n = 3 and are normalized for background vector (empty PGL3, not shown), total protein and verified by cotransfection with Renilla. Data are means ± SD. *P < 0.025 C: real-time PCR analysis of murine epithelial NKCC1 in conditional Hif1a-mutant (+/−) and littermate control (+/+). Data were calculated relative to internal control (β-actin) and are expressed as mean ± SD relative NKCC1 mRNA (*P < 0.025), where transcript levels in control animals were normalized to 1. Results are derived from 3 animals in each genotype.

B

DISCUSSION

Epithelial cells of various mucosal organs are targets for hypoxia-mediated damage in a number of diseases (15). In particular, epithelial cells represent a functional mucosal surface with high turnover and high metabolic activity overlaying a rich vascular bed and a large number of immune-related cell types. At these anatomic sites, even small perturbations of blood flow can result in rapid metabolic changes characteristic of ischemia and resultant hypoxia. Here we address molecular aspects of epithelial adaptation to hypoxia, with particular emphasis on hypoxic regulation of the ion transporter NKCC1.

Initial studies identified functional decreases in agonist-stimulated Cl− secretion in association with selective repression of NKCC1 by transcriptional profiling. In Cl−-secreting epithelia, such as those used here, expression levels of NKCC family members are rate limiting for Cl− uptake and therefore serve as a rate-limiting step for Cl− transport through various membrane Cl− channels (13). These findings of diminished ion transport in hypoxia confirm previous work by us (37) and others (10, 22, 26) in various epithelial cell types. Although our present results clearly demonstrate transcription-dependent downregulation of NKCC1, it is also likely that nontranscriptional mechanisms could also contribute to functional regulation of NKCC1 in Cl−-secreting epithelia. For example, activation of protein kinase C (PKC)-ε results in rapid internalization and degradation of surface NKCC1 (3, 12). Because hypoxia/ischemia has been shown to activate PKC-ε both in vitro and in vivo (9, 23, 33), it is possible that this mechanism contributes to the initial phase of attenuated Cl− secretion. Moreover, NKCC1 function can be directly regulated by threonine phosphorylation at the amino terminus (13). We do not presently know whether NKCC1 phosphorylation patterns differ in response to hypoxia.

Presently, little is known about the transcriptional regulation of NKCC1 family members. A number of studies have addressed the influence of inflammatory and signaling mediators on ion transporters. For example, inflammatory cytokines such as interferon-γ and interleukin-4 have been shown to attenuate Cl− secretion, and such changes are associated with decreased expression of NKCC1 (2, 7, 8, 28, 35). These findings confirm work in the past indicating that select cytokines elicit an epithelial “phenotypic switch” whereby cytokines initiate a loss of epithelial characteristics and a gain of immune acces-
sory function (6). These studies, however, have provided very little insight into mechanisms of NKCC1 regulation. From this perspective, we pursued our microarray findings to address transcriptional repression of NKCC1 by hypoxia. Important initial insight was gained by using the transcription inhibitor DRB, and on the basis of these findings we cloned and functionally characterized a genomic fraction corresponding to the NKCC1 5′-UTR. Studies using these luciferase constructs confirmed initial findings of NKCC1 repression by hypoxia and implicated transcription factor control of NKCC1 repression by hypoxia.

The use of the transcriptional inhibitor DRB provided two lines of evidence. First, the use of DRB demonstrated that the repression of NKCC1 by hypoxia is transcriptionally dependent and provided strong evidence to search for transcription factor that might regulate this function. Second, it revealed that such transcriptional dependence manifested at the protein level was not likely a result of mRNA stabilization. Given the temporal and robust hypoxia response observed in the repression of NKCC1, a candidate regulator was HIF-1, a heterodimeric transcription factor whose activation is dependent on stabilization of an O2-dependent degradation domain (29). It is recently appreciated that HIF can function as both a transcriptional activator and a transcriptional repressor (24). Therefore, a search of the cloned NKCC1 gene 5′-UTR identified one potential HIF-1 binding DNA consensus motif located in the NKCC1 5′-UTR. However, the existence of a HIF-1α binding consensus is not evidence for HIF-1α-mediated response; instead, the hypoxia response element (HRE) is defined as a cis-acting transcriptional regulatory sequence located within 5′-flanking, 3′-flanking, or intervening sequences of target genes (30). Therefore, two strategies were used to define the role of HIF-1 in repression of NKCC1. First, mutational analysis of NKCC1 5′ genomic fraction constructs defined a functional HRE located in the distal 5′ region of the NKCC1 promoter (from +147 bp to +151 bp relative to the major TSS). Second, ChIP analysis confirmed HIF-1α binding to the NKCC1 5′ genomic fraction. At present, we do not know whether this binding is direct or indirect, and we do not know the nature of HIF-mediated transcription. Although previous work with peroxisome proliferator-activated receptor-α gene implicated potential repressor activity with HIF-1α binding sites oriented on the antisense strand (25), implying some degree of transcriptional directionality, there is not direct evidence for such a mechanism. Koshiji et al. (18) recently reported that Sp1 functions as a molecular switch to recruit HIF-1α and repress expression of mutS homolog (MSH-2–6). Our analysis of the NKCC1 5′-UTR and 5′-flanking sequence have revealed at least one Sp1 binding site (positions −87 to −78; not verified experimentally) in the general vicinity of the HRE, and therefore it is possible that Sp1 could contribute to the transcriptional repression of NKCC1 within this region. Moreover, although a recent study comparing transcriptional responses between hypoxia and constitutively active HIF-1α identified a large cohort of transcriptionally repressed genes (24), no unique patterns of HRE expression were noted. Thus more work will be necessary to define the precise nature of HIF-mediated transcriptional repression.

A number of recent studies have implicated epithelial cells in adaptive responses to hypoxia in vitro and in vivo. As an example, we (15) and others have described an adaptive hypoxia response involving a number of barrier-related genes specifically regulated by HIF. This hypothesis was tested in a conditional HIF1a-mutant mouse line and revealed a protective role for murine HIF-1α in a mucosal inflammation model (16). Using this same mouse line, we confirmed our hypothesis that HIF transcriptionally represses NKCC1. Indeed, our findings revealed that colonic epithelial cells lacking Hif1a express increased NKCC1 transcript. Of note, these were unmanipulated mice and these studies reveal, as previously suggested (16, 36), that HIF may regulate homeostatic gene expression (i.e., “physiological hypoxia”) in tissues with normally low Po2 values (e.g., colon) (32). Sequence analysis of NKCC1 in other species revealed similar HIF-1α binding sites consistent with HREs in monkey, rat, and mouse, suggesting that this sequence is evolutionarily conserved. More primitive species (Caenorhabditis elegans, Drosophila) were less convincing, but so too is the expression of NKCC1 in these species. In diseases of the intestine involving a degree of ischemia and/or hypoxia (e.g., ischemic colitis), the most common early and persistent symptom is diarrhea (34). Because electrogenic Cl− secretion, in excess, can result in secretory diarrhea, it is possible that the HIF-dependent repression of NKCC1 represents an adaptive response in the intestine to diminish fluid loss resulting from overstimulation of water transport. Together, the present findings represent a novel, HIF-dependent adaptive response likely to be important in hypoxia compensation of mucosal tissues. Extensions of these studies may provide therapeutic potential to prevent excessive fluid loss during hypoxia.

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