Na-K-2Cl cotransporter inhibition impairs human lung cellular proliferation

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Iwamoto, Lynn M., Naomi Fujiwara, Kenneth T. Nakamura, and Randal K. Wada. Na-K-2Cl cotransporter inhibition impairs human lung cellular proliferation. Am J Physiol Lung Cell Mol Physiol 287: L510–L514, 2004. —The widespread presence of the Na-K-2Cl (NKCC) cotransporter protein suggests that chronic administration of inhibitors may result in adverse effects. Inhibition of the NKCC cotransporter by loop diuretics is felt to underlie the diuretic and the pulmonary smooth muscle relaxant effects of this drug class. However, the fundamental regulation of salt and water movement by this cotransporter suggests that it may also mediate cell volume changes occurring during cell cycle progression. Thus we hypothesized that NKCC cotransporter inhibition by loop diuretics would decrease cellular proliferation. Normal human bronchial smooth muscle cells (BSMC) showed a significant concentration-dependent decrease in cell counts after 7 days of exposure to both bumetanide (n = 5–10) and furosemide (n = 6–16) compared with controls. Proliferation was similarly inhibited in normal human lung fibroblasts (n = 5–9). To determine whether this was due to loss of cells, we performed apoptosis assays on BSMC. Both annexin V-propidium iodide staining (n = 5–10) and single cell gel electrophoresis assays (n = 4) were negative for necrosis and apoptosis in BSMC exposed to 10 μM bumetanide. Subsequent analysis of the cell cycle by flow cytometry showed that bumetanide-exposed BSMC were delayed in G1 phase compared with controls (n = 4–8). This is the first evidence for loop diuretic inhibition of airway smooth muscle cell proliferation. NKCC cotransporter inhibition impeded G1-S phase transition without facilitating cell death. Thus although inhibition by loop diuretics relaxes airway smooth muscle, the NKCC cotransporter may have a more important role in cell proliferation regulation.

furosemide; bumetanide

LOOP DIURETICS, INHIBITORS OF THE Na-K-2Cl (NKCC) cotransporter, are commonly used to improve pulmonary function in premature infants with chronic lung disease. Over the past two decades, clinical and in vitro studies have confirmed airway smooth muscle relaxation responses to these sulfonamide compounds (2, 9, 11, 18, 24), but the exact mechanisms mediating their clinical benefits remain controversial. In addition, the widespread presence of this cotransporter protein in various tissues suggests that chronic, systemic administration of NKCC inhibitors may result in other effects that may or may not be beneficial. For example, although pulmonary compliance improves (4, 23), chronic use of loop diuretics decreases somatic growth in rats (14). Loop diuretics inhibit both isoforms of the NKCC cotransporter. The absorptive isoform of the NKCC cotransporter is found exclusively in the renal epithelium (NKCC2), whereas the secretory form (NKCC1) is nearly ubiquitous (8). Although we (9, 10) have shown that the NKCC1 cotransporter may be involved in the modification of smooth muscle tone, the primary function of this protein appears to be cell volume regulation, (5, 6, 13). Cell volume shrinkage initiated by hyperosmolarity will increase cotransporter function, whereas cellular swelling is associated with decreased function. This regulatory activity of the cotransporter may also play a part in the changes in cell volume observed during progression through the cell cycle (15).

Inhibition of the NKCC cotransporter by loop diuretics is associated with decreased proliferation of human skin fibroblasts, bovine endothelial cells, and rat vascular smooth muscle cells in culture (3, 19, 21). Because we have shown that a functional NKCC cotransporter is present in human airway smooth muscle (9, 10), we hypothesized that inhibition of the cotransporter with loop diuretics will also impair proliferation of different cells within the lung.

In the present study, normal human bronchial smooth muscle cells (BSMC) and lung fibroblasts were exposed to increasing concentrations of the NKCC cotransporter inhibitors bumetanide and furosemide. Assays for apoptosis and cell cycle progression were used to identify the process responsible for the resulting growth suppression. Blockade of cotransporter activity delayed passage of cells through G1 phase but had no effect on apoptosis or necrosis. Chronic use of loop diuretics may thus potentially have an impact on the development and repair of lung parenchyma in growing infants.

METHODS

Cell culture. Normal human BSMC were obtained from Clonetics, Cambrex BioScience (Walkersville, MD) and grown in a modified MCDB 131 medium with 5% FBS (Invitrogen, Carlsbad, CA), recombinant human fibroblast growth factor, insulin, and penicillin-streptomycin (pen-strep; Sigma-Aldrich, St. Louis, MO) at 37°C in 5% CO2. Normal human lung fibroblasts (NHLF) also from Clonetics, Cambrex BioScience, were grown in fibroblast basal media (Clonetics) with 2% FBS, recombinant human fibroblast growth factor, insulin, and pen-strep at 37°C in 5% CO2.

Comparative Western blot. BSMC and NHLF were compared by Western blot for NKCC cotransporter protein content. Equal numbers of cells were lysed, and the proteins were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was probed with the T4 monoclonal antibody (1:1,000) to the NKCC cotransporter (Developmental Studies Hybridoma Bank, Iowa City, IA), and the bands were visualized with a chemiluminescence kit (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). To normalize for protein loading, we also probed the membrane with a monoclonal antibody to β-actin (Sigma) and then finally stained it for total protein with amido-black (Bio-Rad, Hercules, CA).

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NKCC-sensitive transport was determined as the difference between protein. Controls not exposed to bumetanide were run in parallel. and SDS. The lysates were placed in scintillation cocktail (Perkin-Elmer), nonproliferating cells.

From 1 to 100 concentrations of bumetanide (\( \text{B} \)) and furosemide (\( \text{F} \)). Concentrations of bumetanide

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\text{B} = \text{furosemide (Fig. 1.) Bronchial smooth muscle cells (BSMC) exposed to increasing concentrations of bumetanide (A) and furosemide (B). Concentrations of bumetanide from 1 to 100 \( \mu M \) resulted in significant decreases in cell count (n = 5–10, \( P < 0.05 \)). Similarly, at 30 and 300 \( \mu M \) furosemide, there was a significant decrease in cell count after 7 days (n = 6–16, \( P < 0.05 \)).

Proliferation studies. BSMC and NHLF cultures were synchronized in the cell cycle before the experimental exposures. Synchronization was achieved by 48-h incubation in serum deprivation media with minimal FBS (0.2% for BSMC and 0.1% for NHLF) and pen-strep but no growth factors. We verified this by cell cycle analysis using flow cytometry (EPICS XL; Beckman Coulter, Miami, FL).

After synchronization, growth medium was replaced with or without different concentrations of bumetanide (Gensia Sicor Pharmaceuticals, Irvine, CA) or furosemide (Abbott Labs, North Chicago, IL). Medium was changed once at 4 days. Manual counts of viable cells were performed with a hemacytometer at baseline and at 4, 6 (NHLF), or 7 (BSMC) days.

NKCC activity. NKCC activity was determined as a function of ouabain-containing HEPES-buffered medium with and without growth factors (FBS, fibroblast growth factor, epidermal growth factor, and insulin) and allowed to equilibrate for 4 h. Bumetanide (10 \( \mu M \)) was then added for 30 min, followed by 1 \( \mu Ci/ml \) \(^{86}\text{Rb}\). After 30 min, the reaction was stopped with cold MgCl\(_2\), and cells were lysed with 0.1% SDS. The lysates were placed in scintillation cocktail (Perkin-Elmer), and \( \beta \)-emission was measured. The uptake was normalized to total protein. Controls not exposed to bumetanide were run in parallel.

NKCC-sensitive transport was determined as the difference between control and bumetanide-exposed \(^{86}\text{Rb}\) uptake for proliferating and nonproliferating cells.

Annexin V-propidium iodide assay for apoptosis. Synchronized BSMC, exposed to 10 and 100 \( \mu M \) bumetanide for 4 days and negative and positive controls, were trypsinized from the culture flasks and stained with fluorochrome-tagged annexin V (Alexa Fluor 488; Molecular Probes, Eugene, OR) and 100 \( \mu g/ml \) of propidium iodide (Sigma, St. Louis, MO). Fluorescence emission was measured at 530 and 575 nm by flow cytometry. Apoptotic cells stain with annexin only, whereas necrotic cells double stain for both annexin and propidium iodide. Normal cells stain negatively for both. Positive controls were created with 10 \( \mu M \) paclitaxel (LC Laboratories, Woburn, MA).

Single cell gel electrophoresis for apoptosis. Control BSMC and cells exposed to 10 \( \mu M \) bumetanide for 7 days were trypsinized and suspended in low-melting-point agarose. The cells were then lysed, and the DNA was denatured in alkaline solution (pH > 13) and electrophoresed at 25 V for 10 min. The samples were air-dried and stained with SYBR Green fluorescent dye (Trevengen, Gaithersburg, MD). The stained DNA samples were viewed by epifluorescence microscopy (494 nm excitation/521 nm emission), and DNA fragmentation was digitally analyzed with Comet Assay software (Loats Associates, Westminster, MD).

Cell cycle analysis. Synchronized BSMC were exposed to 10 and 100 \( \mu M \) bumetanide. Control cells were grown in the usual growth medium. Experimental and control cells were fixed in 70% ethanol at 4°C every 6 h from baseline to 42 h. The fixed cells were then stained with 20 \( \mu g/ml \) of propidium iodide solution, and fluorescence was measured by flow cytometry. Cell cycle phases were determined by software analysis of the fluorescence data (Multicycle; Phoenix Flow Systems, San Diego, CA).

Statistical analysis. Data are expressed as means ± SE. Control and bumetanide- or furosemide-exposed groups were compared by Student’s t-tests. Multiple-dose comparisons were done by ANOVA with Student-Newman-Keuls post hoc test. Statistical analyses were performed with SigmaStat software (Jandel Scientific, San Rafael, CA).

RESULTS

Effect of loop diuretics on cell counts. BSMC exposed to bumetanide (n = 5–10) and furosemide (n = 6–16) showed dose-dependent decreases in cell counts over 7 days (Fig. 1, A and B, respectively). The diuretic potency of bumetanide is 40 times greater than that of furosemide. At relatively equivalent diuretic concentrations, 1 \( \mu M \) bumetanide exposure resulted in a greater inhibition of cell proliferation compared with 30 \( \mu M \) furosemide (Fig. 2). These drug levels are readily achieved in the clinical setting.

Annexin V-propidium iodide assay for apoptosis. Synchronized BSMC, exposed to 10 and 100 \( \mu M \) bumetanide for 4 days and negative and positive controls, were trypsinized from the culture flasks and stained with fluorochrome-tagged annexin V (Alexa Fluor 488; Molecular Probes, Eugene, OR) and 100 \( \mu g/ml \) of propidium iodide (Sigma, St. Louis, MO). Fluorescence emission was measured at 530 and 575 nm by flow cytometry. Apoptotic cells stain with annexin only, whereas necrotic cells double stain for both annexin and propidium iodide. Normal cells stain negatively for both. Positive controls were created with 10 \( \mu M \) paclitaxel (LC Laboratories, Woburn, MA).

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Comparison of BSMC and NHLF. This concentration-dependent decrease in cell proliferation was also found in NHLF similarly exposed to bumetanide and furosemide (data not shown). However, at equal concentrations of bumetanide, there was a significantly smaller effect on cell proliferation in NHLF compared with BSMC (Fig. 3A).

When equal numbers (100,000) of BSMC and NHLF cells were analyzed for NKCC cotransporter by Western blot \((n = 3)\), there was quantitatively less cotransporter in the NHLF than in the BSMC, normalized to \(\beta\)-actin (Fig. 3B). Total protein staining by amido-black was similar for the two cell types (not shown).

Effect of proliferative state on NKCC cotransport activity. Cells that were actively proliferating demonstrated greater NKCC cotransporter activity compared with cells in which growth was suppressed. NKCC cotransporter activity, as measured by bumetanide-sensitive \(\text{\textsuperscript{86}}\text{Rb}\) uptake, was significantly greater \((n = 5, P < 0.05)\) in growth factor-stimulated BSMC compared with cells maintained in the absence of serum and growth factors (Fig. 4).

Effect of loop diuretics on cell survival. To examine whether the loop diuretic-mediated decrease in cell number is a result of an acceleration of cell death, we performed two assays of apoptosis and necrosis. The single cell gel electrophoresis is based on the differential DNA fragmentation seen in apoptosis and necrosis, whereas the annexin V-propidium iodide assay is based on the membrane destabilization that occurs very early in apoptosis.

With the single cell gel electrophoresis assay, BSMC exposed to 10 \(\mu\)M bumetanide for 7 days \((n = 4)\) showed no difference compared with controls in the amount of DNA fragmentation, expressed as tail length, or in the severity of DNA damage, expressed as tail moment (Fig. 5, A and B).

Similar results were obtained by the annexin V-propidium iodide assay. BSMC exposed to 10 \(\mu\)M bumetanide for 4 days \((n = 5–10)\) showed no differences in early or late apoptosis compared with controls. BSMC exposed to 100 \(\mu\)M bumetanide for 4 days exhibited a significant increase in early but not late apoptosis compared with controls (Fig. 6).

![Fig. 3. The effects of bumetanide were examined in BSMC and normal human lung fibroblasts (NHLF, A). At 10 and 100 \(\mu\)M bumetanide, there was a greater change in cell counts in the BSMC compared with NHLF \((n = 3–4, P < 0.05)\). Western blot of BSMC and NHLF probed for the Na-K-2Cl (NKCC) cotransporter protein, normalized to \(\beta\)-actin and total protein (B). The same number of NHLF \((100,000)\) were lysed and loaded in each lane. A greater amount of NKCC was seen in the BSMC compared with the NHLF \((n = 3)\).](http://ajplung.physiology.org/)

![Fig. 4. Bumetanide-sensitive \(\text{\textsuperscript{86}}\text{Rb}\) uptake in proliferating and nonproliferating BSMC. The cells with growth factors present in the media showed a greater bumetanide-sensitive uptake compared with the cells that were serum starved \((n = 5, P < 0.05)\).](http://ajplung.physiology.org/)

![Fig. 5. Single cell gel electrophoresis assay (Comet assay) performed on BSMC exposed to 10 \(\mu\)M bumetanide for 7 days. There were no differences in tail moment (A) or tail length (B) in control or bumetanide-exposed cells \((n = 4)\).](http://ajplung.physiology.org/)
Effect of loop diuretics on cell cycle progression. To further investigate the effects of NKCC inhibition on cell proliferation, we examined cell cycle progression using propidium iodide staining measured by flow cytometry. Bumetanide exposure of BSMC resulted in a dose-dependent delay in the G1-S phase transition (Fig. 7). At 18 h, cultures exposed to 10 μM (n = 4) and 100 μM (n = 8) bumetanide showed significantly more cells still in G1 compared with controls (P < 0.05). In addition, there was no increase in the number of cells undergoing apoptosis at either concentration.

DISCUSSION

Results of this study demonstrate that loop diuretic-mediated inhibition of the NKCC cotransporter suppresses growth of normal human BSMC and NHLF in vitro. Although these results are consistent with those of Panet and Atlan (20), showing that both loop diuretics inhibit proliferation of normal human skin fibroblasts, pharmacological concentrations of furosemide (1 mM) were used in their study. In the current study, significant inhibition of both BSMC and NHLF growth was demonstrated with 30 μM furosemide, the level achieved after a 1 mg/kg intravenous injection (1).

Although the diuretic potency of bumetanide is 40 times that of furosemide, there was only a half-log greater difference in IC₅₀ for inhibition of cell growth by bumetanide. Interestingly, this half-log difference is also seen in the potency difference between bumetanide- and furosemide-mediated airway smooth muscle relaxation (16). Differences in potencies between diuretics compared with inhibition of cell growth and airway smooth muscle relaxation may be explained by the fact that there are two different isoforms of the NKCC cotransporter: the absorptive isoform, NKCC2, found only in renal epithelia contrasts to the secretory isoform, NKCC1, which is nearly ubiquitous (17). Importantly, the potential clinical relevance of this differing potency is that for the same diuretic effect, adverse effects on cell growth may be greater for bumetanide compared with furosemide.

A second finding in this study supporting the direct role of NKCC cotransporter inhibition with suppression of cell growth is that the proliferative state of the cells correlated with activity of the NKCC cotransporter. In a previous study, we showed that both bumetanide and furosemide inhibited ⁸⁶Rb uptake in BSMC (10). In the current study, NKCC cotransporter uptake correlated with proliferative state of the BSMC. Bumetanide-sensitive ⁸⁶Rb uptake increased in actively proliferating cells compared with growth-suppressed serum-starved cells and is consistent with results obtained previously in human skin fibroblasts (20). Moreover, Guo and O’Brien (7) showed that the mouse BALB/c 3T3 fibroblast cell line deficient in NKCC1 is not responsive to a phorbol ester mitogen, but that transfection with a shark NKCC1 gene restores responsiveness. Together, this suggests that stimulation of the NKCC cotransporter may be an essential part of the mitogenic signal.

Thirdly, our finding that BSMC proliferation was more sensitive to bumetanide inhibition correlated with a greater amount of NKCC cotransporter protein found in BSMC compared with NHLF. Likewise, overexpression of the NKCC cotransporter in mouse fibroblasts results in increased cell proliferation and a transformed phenotype (22). Thus these data provide additional corroborative evidence that the NKCC plays an important role in regulation of cell growth and suggests that growth of normal lung that requires coordinated proliferation among various cell types could be disrupted by loop diuretics.
Mechanisms to explain how inhibition of the NKCC cotransporter may impede cell proliferation could be extrapolated from knowledge that the primary function of the NKCC cotransporter is in cell regulatory volume increase, a step required for cell proliferation (8). Interfering with this response by inhibition of the cotransporter could decrease proliferation but could also theoretically cause enough cell shrinkage to trigger apoptosis. However, while cell shrinkage has been shown to stimulate apoptosis (15), using assays for both early- and late-stage apoptosis, we were unable to find an increase in cell death due to NKCC cotransporter inhibition. BSMC exposed to 100 \( \mu \)M bumetanide for 4 days exhibited a significant increase in early but not late apoptosis compared with control. This suggests that prolonged exposure to very high concentrations of bumetanide does have a toxic effect on the cells. However, annexin V and comet assays demonstrate that the decrease in cell numbers seen at 4 and 7 days with both 10 and 100 \( \mu \)M concentrations of bumetanide is not due to late apoptosis. In addition, no evidence was seen for apoptosis in the propidium iodide staining for cell cycle analysis with either 10 or 100 \( \mu \)M bumetanide.

Finally, data herein provide evidence supporting the notion that cotransporter inhibition may interfere with cell cycle progression rather than via a direct toxic effect on the cells. Specifically, we found the transition from G1 to S phase was affected, supporting previous observations that DNA synthesis is inhibited by loop diuretics using thymidine uptake studies in human skin fibroblasts and bovine vascular endothelial cells (20, 21).

In summary, results of this study demonstrate: 1) loop diuretic-mediated inhibition of normal human airway smooth muscle and lung fibroblast cell proliferation, 2) correlation between the amount and function of the NKCC cotransporter protein with the proliferative state of the cells, 3) that decrease in cell numbers was not due to an increase in apoptosis or necrosis, and 4) a loop diuretic-mediated delay in the G1-S phase progression through the cell cycle.

If these in vitro results were to occur in vivo, altered cell proliferation due to chronic inhibition of the NKCC cotransporter with loop diuretic therapy in premature infants could interfere with maturation and differentiation of the lungs. This disturbance of normal development would be significant as it is consistent with current pathophysiological notions for the “new” bronchopulmonary dysplasia (12). Combined use with other growth suppressors, particularly corticosteroids, could further alter lung development in these high-risk infants.

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NKCC COTRANSPORTER INHIBITION IMPAIRS PROLIFERATION