

BI-1 enhances Fas-induced cell death through a Na^+/H^+ -associated mechanism

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The role of Bax inhibitor-1 (BI-1) in the protective mechanism against apoptotic stimuli has been studied; however, as little is known about its role in death receptor-mediated cell death, this study was designed to investigate the effect of BI-1 on Fas-induced cell death, and the underlying mechanisms. HT1080 adenocarcinoma cells were cultured in high concentration of glucose media and transfected with vector alone (Neo cells) or BI-1-vector (BI-1 cells), and treated with Fas. In cell viability, apoptosis, and caspase-3 analyses, the BI-1 cells showed enhanced sensitivity to Fas. Fas significantly decreased cytosolic pH in BI-1 cells, compared with Neo cells, and this decrease correlated with BI-1 oligomerization, mitochondrial Ca^{2+} accumulation, and significant inhibition of sodium-hydrogen exchanger (NHE) activity. Compared with Neo cells, a single treatment of BI-1 cells with the NHE inhibitor EIPA or siRNA against NHE significantly increased cell death, which suggests that the viability of BI-1 cells is affected by the maintenance of intracellular pH homeostasis through NHE. [BMB Reports 2014; 47(7): 393-398]

INTRODUCTION

Bax inhibitor-1 (BI-1; also known as 'testis-enhanced gene transcript') is an anti-apoptotic protein that inhibits Bax activation, and its translocation to the mitochondria (1, 2). Functionally, BI-1 affects the leakage of Ca^{2+} ions from the ER (2, 3). In addition to its anti-apoptotic role, BI-1 also enhances cancer/tumor progression (4, 5). Our previous study implicated sodium hydrogen exchanger (NHE) activity as the homeostatic mecha-

nism responsible for maintaining intracellular pH in BI-1-over-expressing cells (6). Increased intracellular H^+ can affect the pH homeostasis system, including NHE (7, 8). The transporters responsible for export of lactic acid, such as monoamine carboxylate transporters (MCT), have also been suggested as playing a role in BI-1-associated cancer metastasis (9). As tumors grow and become more acidic, these transporters prevent lethal decreases in intracellular pH; and their high expression and activation are considered to be characteristics of cancer cells (10).

Fas ligand (FasL) is a member of the TNF superfamily that, upon cross-linking of its receptor, Fas (Apo-1/CD95), induces apoptosis in susceptible cells (11). FasL-induced apoptosis contributes to immune homeostasis and cell-mediated cytotoxicity (12). Defective Fas-mediated apoptosis may lead to oncogenesis and drug resistance in existing tumors (13). It has also been reported that Fas-receptor stimulation inhibits NHE activity, contributing to Fas-induced cytosolic acidification, DNA fragmentation, and cell shrinkage (14). Because BI-1 enhances NHE activity, and is associated with a cancer metastasis phenotype, it may play a role in Fas-induced cell death in cancer cells.

In this study, HT1080 adenocarcinoma cells stably transfected with neomycin-resistant vector (Neo) or BI-1 expression vector were cultured in the presence of a high concentration of glucose, a metabolism-enhancing condition. The induction of cell death by anti-Fas antibody was compared between Neo cells and BI-1-transfected cells. The Fas agonistic anti-Fas antibody Jo2 significantly stimulated cell death more effectively in BI-1-overexpressing colon carcinoma cells, than in Neo cells. Fas enhanced the acidification of cytosolic pH, in association with BI-1 oligomerization, and mitochondrial Ca^{2+} accumulation. The basal activity of the H^+ -endogenous homeostasis protein NHE was highly elevated in BI-1 cells, contributing to their high susceptibility to Fas-induced cell death.

RESULTS

BI-1 enhances Fas-induced cell death with more intracellular acidic pH

Overexpression of BI-1 in HT1080 colon carcinoma cells enhanced cell death stimulated by the Fas-activating antibody Jo2 (Figs. 1A, B), and increased the expression of active cas-

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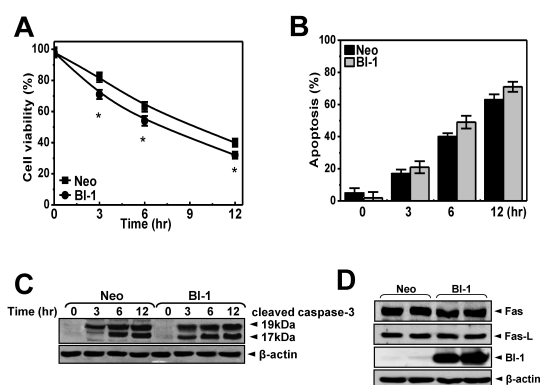


Fig. 1. BI-1 enhances apoptosis induced by Fas. (A) Neo and BI-1 cells were treated for the indicated times with 100 ng/ml anti-Fas antibody. After trypsinization, floating and adherent cells were pooled, and a sample of the pooled cells was subjected to a vital dye trypan blue exclusion assay. (B) Neo and BI-1 cells were treated for the indicated times with 100 ng/ml anti-Fas antibody, and cell death was determined by Hoechst staining analysis. (C) Neo or BI-1 cells were treated as indicated, and subjected to immunoblotting with the appropriate antibodies for cleaved caspase-3 or β -actin. (D) Neo or BI-1 cells were subjected to immunoblotting with anti-FasL, Fas-R, HA, or β -actin antibody. * $P < 0.05$, significantly different from Fas-treated Neo cells, during each time period.

pase 3 (Fig. 1C). To understand the possible mechanisms, endogenous Fas receptor and Fas-L (Fas ligand) expressions were examined. Fig. 1D shows that in these Fas-associated proteins, no difference exists between Neo and BI-1 cells. To confirm the correlation of BI-1 characteristics with cell death, we examined intracellular pH, BI-1 oligomerization and mitochondrial Ca²⁺ accumulation. Fas enhanced acidification of the cytosol in BI-1 cells (Fig. 2A). BI-1, a recently studied Ca²⁺ channel-like protein, was more clearly oligomerized in the presence of Fas (Fig. 2B). The resultant mitochondrial Ca²⁺ accumulation was spiked in BI-1 cells (Fig. 2C).

Fas regulates NHE activity

Because sodium hydrogen exchanger (NHE) is an intracellular pH-modulating protein, we measured NHE activity, through analysis of changes in intracellular pH due to NH₄Cl pulse treatment. The basal NHE activity was higher in BI-1 cells, than in Neo cells (Fig. 3A). Neo and BI-1 cells were cultured in the presence of anti-Fas antibody for 6 hours, and the NHE activity was then measured. With the Fas, the NHE activity was significantly reduced in both Neo and BI-1 cells (Fig. 3B), suggesting that the inhibition of intracellular pH is linked to the regulatory effect of NHE activity. There was no difference in expression of NHE-1 between with or without Fas, which means that NHE activity is not regulated by protein expression.

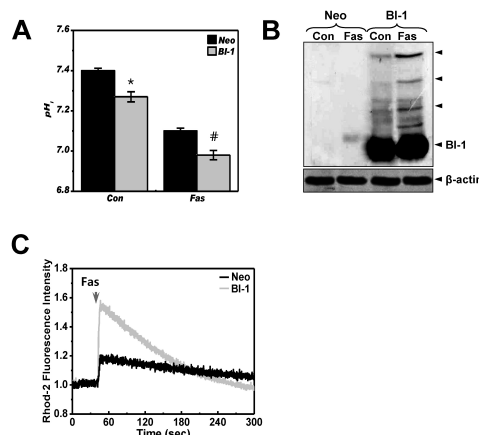


Fig. 2. BI-1 enhances Fas-induced calcium release. Neo and BI-1 cells were treated for 6 hours with 100 ng/ml anti-Fas antibody. (A) After calibrating intracellular pH, as described in Materials and Methods, the cells were incubated with a pH-sensitive dye, carboxy-seminaphthorhodafluor (SNARF-1)/AM (10 μ M) in 150 mM KOH/nigericin buffer, and the intracellular pH was quantified. (B) Fas-treated or untreated Neo and BI-1 cells were fractionated, to collect membrane-bound organelle fractions. Proteins were resolved by gel electrophoresis under non-reducing conditions, followed by immunoblot analysis with anti-HA antibody. Immunoblotting was also performed, with anti- β -actin antibody as a loading control. (C) Neo and BI-1 cells were loaded with Rhod II-AM, followed by treatment with 100 ng/ml anti-Fas antibody. The fluorescence of Rhod II was monitored. * $P < 0.05$, significantly different from Neo cells, # $P < 0.05$, significantly different from Fas-treated Neo cells.

Enhanced NHE activity is a key determinant of the viable condition in BI-1 cells

The NHE inhibitor EIPA more significantly decreased the intracellular pH (Fig. 4A) and cell viability in BI-1-expressing cells, than in control cells (Fig. 4B). NHE1 knockdown by siRNA similarly decreased intracellular pH, and more effectively enhanced cell death in BI-1-expressing cells (Figs. 4C, D), indicating that NHE activity has a greater contribution to cell viability in BI-1 cells, than in Neo cells. To more specifically examine the role of BI-1 in this system, a BI-1 knockdown approach was also used. The BI-1 siRNA-transfected cells reversed the intracellular acidic pH, especially in the Fas-treated condition (Fig. 4C). The reduced cell viability in the BI-1 knock-down condition was consistently significantly reversed (Fig. 4D), also.

DISCUSSION

BI-1 enhances sensitivity against Fas-induced cancer cell death

Any molecules that enhance sensitivity to death receptor stimuli would be important tools for therapeutic adjuncts against cancer. High metabolism is one of the characteristics of cancer. Enhanced glucose metabolism and its subsequent activation of intracellular ionic balances also contribute to cancer

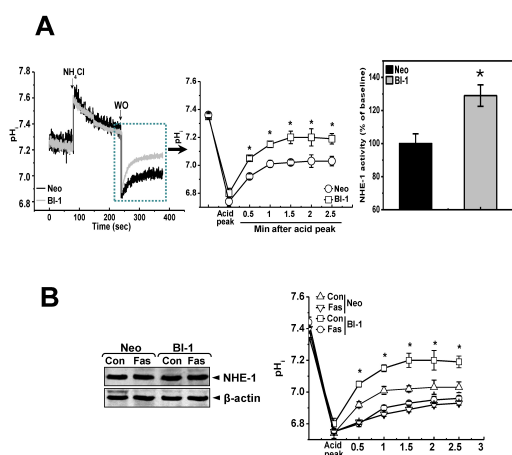


Fig. 3. Fas inhibits NHE activity. (A) After loading Neo and BI-1 cells with 10 μ M SNARF-1/AM dye, internal acid loads were imposed by the NH₄Cl pulse technique, after the application of steady state. After washing the cells, the intracellular pH was measured, as described in Materials and Methods. NHE activity was quantified, through analysis of changes in pHi due to NH₄Cl pulse treatment (right). **P* < 0.05, significantly different from untreated Neo cells. (B) Neo and BI-1 cells were treated with 100 ng/ml anti-Fas antibody, and cultured for 6 hours. Immunoblotting was performed with anti-NHE-1 or β -actin antibody (left). Recovery from internal acid loads was measured in Neo and BI-1 cells (right). **P* < 0.05, significantly different from untreated Neo cells, during each culture time period.

survival and proliferation. In contrast to classical studies of Fas (CD95), a member of the tumor necrosis factor receptor super family, including Fas-associated protein with death domain (FADD), initiates caspase-8 proteolytic autocleavage, activation of the effector caspase, caspase-3, and cell death (12, 13); and the inhibition of Na⁺/H⁺ antiporter activity is suggested as another Fas-associated cell death mechanism (14). Recently, it has been reported that BI-1 enhances glucose metabolism, leading to high activation of the sodium/hydrogen exchange for intracellular pH homeostasis (6). In this study, we showed that BI-1 sensitizes human adenocarcinoma cells, HT1080, against Fas, especially in the high glucose-culturing medium. Culturing cells in low-glucose medium consistently abrogated the high sensitivity against Fas (data not shown). This study provides one example of the enhanced glucose metabolism/ionic balance-associated protein's sensitizing role against Fas.

Enhancement of Fas-induced cell death in BI-1-overexpressing cells may be mediated by inhibition of NHE activity

Overexpression of BI-1 in colon carcinoma cells enhanced cell death stimulated by the Fas-activating antibody Jo2 (Figs. 1A, B), and correlated with intracellular acidic pH and cell death (Fig. 2). Although BI-1 has been established as a specific ER stress regulator (9), an acidic pH-associated Ca²⁺ leak has also been described in BI-1-expressing cells (3). It was also reported that the expression of BI-1 is significantly higher in lymph node

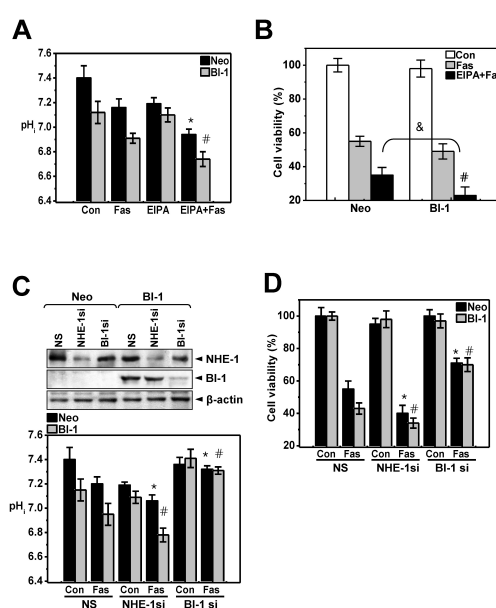


Fig. 4. Enhanced NHE activity is a key determinant of the viable condition in BI-1 cells. Neo and BI-1 cells were exposed to 100 ng/ml Fas antibody for 24 or 48 hours, in the presence of 30 μ M EIPA. Intracellular pH (A), and cell viability (B) were measured, as described in Materials and Methods. **P* < 0.05, significantly different from Fas-treated Neo cells, #*P* < 0.05, significantly different from Fas and EIPA co-treated Neo cells. Neo and BI-1 cells were transfected with NHE siRNA, BI-1 siRNA, or non-specific siRNA. After 16 hours, Neo and BI-1 cells were exposed to 100 ng/ml Fas antibody, intracellular pH was measured (C), and cell viability was analyzed (D). **P* < 0.05, significantly different from Fas-treated non-specific siRNA-transfected Neo cells, #*P* < 0.05, significantly different from Fas-treated non-specific siRNA-transfected BI-1 cells, NS; non-specific siRNA, NHE1si; NHE1 siRNA, BI-1si; BI-1 siRNA.

metastasis models, in which a metastasis mechanism associated with an acidic pH has been suggested (6). Enhancement of the glycolytic metabolism of BI-1 cells seems to be related to the Warburg effect, of high glucose uptake and lactate accumulation (15). The H⁺ extrusion system to achieve endogenous pH homeostasis appears to be highly developed in BI-1 cells. However, this phenotype was clear in the presence of a high concentration of glucose culture medium. Therefore, we used 20 mM glucose medium throughout this study. In this study, Fas inhibited the intracellular H⁺ homeostasis regulator NHE, in both control and BI-1 cells (Figs. 3A, B). However, there was no difference in NHE expression between the cell lines, which would indicate that post-translational modification may be involved in the enhanced NHE activity observed in BI-1 cells (Fig. 3A). Notably, the basal NHE activity was significantly higher in BI-1 cells; while after treatment with Fas, NHE activity was reduced, but compared with control cells, was still relatively high. This study suggests that endogenous NHE activity contributes to the maintenance of intracellular pH homeostasis,

especially in BI-1 cells (Figs. 4B, D). Another intracellular pH regulation system, MCT, is also consistently highly activated in BI-1 cells (6). Fas/CD95-induced apoptosis was previously reported to occur in parallel with cytosolic acidification (14). In Jurkat T cells, Fas/CD95-receptor stimulation caused cytosolic acidification, and blunted the recovery from acidification, following application of butyrate, or an NH₃ pulse. Treatment of Jurkat cells with the NHE inhibitor, HOE694, accelerated Fas-induced DNA fragmentation, suggesting that Fas-induced cell death is dependent on the inhibition of NHE activity (14). Consistent with this, a new Glut inhibitor, fasentin, sensitizes cells against Fas (16). Similarly, another GLUT inhibitor, dipyr-idamole, also inhibited glucose uptake and sensitized cells to FAS, supporting the thesis that targeting glucose uptake and its associated metabolism, including NHE activity, is an attractive therapeutic strategy for the treatment of malignancy. However, other studies have tested whether cell death induced by NHE-1 inhibition is dependent on the Fas/FasL signaling pathway, and found that the inhibition of NHE does not seem to be related to Fas expression (17). Consistent with this, there was no difference of expression of FAS receptor or ligand between Neo and BI-1 cells (Fig. 1D).

Inhibition of NHE activity deregulates intracellular pH homeostasis, and decreases the viability of BI-1 cells

The results of this study also suggest that it is the endogenous NHE activity itself that contributes to the maintenance of intracellular pH homeostasis, leading to protection against cell death in BI-1 cells. In cholangiocarcinoma cells, selective inhibition of ion transport mechanisms that regulate intracellular pH, including NHE, induces apoptosis (18). Intracellular acidification, caused by the inhibition of Na⁺/H⁺ exchanger and H⁺-ATPase, also triggers cell death (19). In addition, inhibition of the intracellular pH regulation system with 5-(N,N-hexamethylene)-amiloride has been proposed as a tumor-selective therapy (20). Tumor metabolism involves complex interactions between oxygenation states, metabolites, ions, and vascular network cascades. Accumulation of lactate within tumors is correlated with poor clinical outcomes (21). As an adaptive/protective mechanism, carcinomas produce high amounts of lactic acid, and up-regulate the H⁺-linked MCT isoform 1. MCT1 mRNA levels in fresh carcinoma biopsy samples are positively correlated with the risk of cancer (22). The intracellular pH-regulating mechanisms seem to be highly activated in oncogenic processes; therefore, BI-1-associated cancer progression might be related to the intracellular pH-homeostatic mechanism, a known cancer characteristic.

Although there is no consensus on the oncogenic characteristics of BI-1, there is accumulating evidence that expression of BI-1 is positively associated with cancer progression. BI-1 has recently been found to be overexpressed in several human carcinomas (5, 23, 24). It was also reported that the expression of BI-1 leads to changes in molecular mechanisms governing normal cellular growth; and consequently, contributes to malig-

nant cell transformation and tumorigenesis (25). Based on this study, we assume that cancer cells that endogenously express BI-1 will show high susceptibility to programmed cell death induced by anti-Fas antibody or Fas ligand, especially in high glucose conditions. Considering that the development of effective therapies targeting the Fas/FasL system plays an important role in the fight against cancer, our findings might contribute to the development of more efficient strategies for cancer therapy.

MATERIALS AND METHODS

Materials

Jo2 antibody against Fas/CD95 was obtained from Fisher Scientific Co. (Waltham, MA). Antibody against hemagglutinin antigen (HA) was purchased from Cell Signaling Technologies (Beverly, MA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, and other tissue culture reagents were supplied by Life Technologies, Inc. (Grand Island, NY). Bicinchoninic acid (BCA) protein assay reagents were obtained from Pierce Biotechnology (Rockford, IL). All other chemicals were of at least analytical grade, and were purchased from Sigma-Aldrich.

Cell lines and cell culture

Human fibrosarcoma cells (HT1080) stably transfected with pcDNA3 or pcDNA3-BI-1 have been previously described (3). Cells were maintained in Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum, 25 mM glucose, 1 mM L-glutamine, and antibiotics.

Cell viability

HT10800 cells were assessed microscopically for dead cells by trypan blue exclusion. Cell viability was calculated, by dividing the non-stained (viable) cell count by the total cell count. The number of cells was determined by averaging the number of cells in four squares, and multiplying this by a dilution factor.

Apoptosis

Cells were fixed for 5 min, in 3.7% paraformaldehyde in phosphate-buffered saline. After air-drying, cells were stained for 10 min in Hoechst 33258 (10 g/L), mounted in 50% glycerol containing 20 mmol/L citric acid and 50 mmol/L orthophosphate, and stored at -20°C, before analysis. Apoptotic cells were quantified using a Zeiss IM fluorescence microscope, after evaluating morphological changes.

Immunoblotting

For immunoblot analysis, cells were lysed in 1% Nonidet P-40, 50 mM HEPES, pH 7.5, 100 mM NaCl, 2 mM EDTA, 1 mM pyrophosphate, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 100 mM sodium fluoride. Lysates were normalized for total protein content with a BCA kit (Pierce), and 40-50-μg aliquots were suspended in an equal

volume of 2× Laemmli buffer containing 2-mercaptoethanol, and then boiled for 5 min, before performing SDS-PAGE, or loaded directly without boiling. Proteins were transferred to Immobilon-P membranes (Millipore, Billerica, MA), blocked with skim milk (5%), and incubated with primary antibodies recognizing hemagglutinin (HA), or actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Antibody detection was accomplished via horseradish peroxidase-conjugated protein A or goat anti-rabbit immunoglobulin G (Bio-Rad), a chemiluminescent substrate (Amersham Biosciences), and exposure to X-ray film (Eastman Kodak Co.).

Subcellular fractionation

To obtain microsome preparations, all procedures were performed at 4°C, using cold reagents. Cells were washed and homogenized, using 20-30 strokes of a motor-driven glass-Teflon homogenizer at 4°C, in an ice-cold pH 7.4 solution containing 250 mM sucrose, 10 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1.0 mM EGTA, 1.0 mM dithiothreitol, and 1× protease inhibitor complex (Roche Applied Science). Homogenates were centrifuged at 1,600 × g for 10 min, to remove the nuclear fraction. The supernatant was then centrifuged at 10,000 × g, for 10 min. The resulting supernatant was finally centrifuged at 100,000 × g for 60 min, to yield the ER-enriched microsomal pellet. Microsomes were immediately used for experiments.

Analysis of BI-1 oligomerization

Endoplasmic reticulum (ER) was isolated from Neo and BI-1 cells. ER pellets were resuspended in buffer B containing 250 mM sucrose, 10 mM HEPES-NaOH, pH 7.5, 2 mM KH₂PO₄, 5 mM sodium succinate, and 25 μM EGTA, and treated at various pH conditions (5.0, 6.0, 7.0, or 7.4) for 30 min, at 30°C. The ER pellets were harvested by centrifugation, and analyzed by immunoblotting, using anti-HA antibody.

Analysis of mitochondrial Ca²⁺

Cells were allowed to adhere to glass coverslips, and incubated with 5% CO₂ at 37°C. All Ca²⁺ imaging experiments were performed using an inverted epifluorescence Nikon microscope, and a digital imaging system, consisting of a TILL polychrome IV monochromator illumination system, a Sencam 12-bit charge-coupled device camera, and TILL VisION acquisition and analysis software, as previously described by Kim et al. (3). Mitochondrial Ca²⁺ uptake was confirmed, by dual loading with MitoTracker Green FM and Rhod-2-TRITC. Cells were excited with light at 488 ± 15 (10 ms) and 550 ± 25 nm (50 ms), and the emitted fluorescence from both dyes was collected through a fluorescein isothiocyanate/TRITC dual emission dichroic beam splitter, and band pass filter (520 ± 15 and 600 ± 25 nm; Chroma). All experiments were performed at 37°C, with 5% CO₂.

Intracellular pH measurements

Cells were loaded with the pH-sensitive dye carboxy-semi-

naphthorhodafluor (SNARF-1)/AM (10 μM) for 30 min at 37°C, washed, and incubated for 30 min with lactic acid in the medium. Internal pH values were determined by flow cytometry analysis, by the ratio of mean FL-2/FL-3. A standard curve was generated, by treating cells with 10 μM nigericin, in calibration buffers ranging from pH 6.0 to 8.0.

NHE activity measurement

After a 20 mM NH₄Cl prepulse, cells were washed with Na-free solution (HEPES buffer with NaCl replaced by N-methyl-D-glutamine). NHE activity was calculated from the initial slope of intracellular alkalization, upon re-addition of Na. To allow for direct comparison, pH change per min was calculated for intracellular pH values in the range of 6.50-6.80. All experiments were performed in pairs, with measurement of NHE activity before, and after, 15 min incubation with 100 ng/ml Fas antibody. Control cells were incubated with standard HEPES solution only.

NHE siRNA transfection

The siRNAs were synthesized in duplex and purified forms, using Bioneer technology (Daejeon, South Korea). The double-strand small interfering RNAs (siRNAs) targeting BI-1 and NHE1 (for BI-1 siRNA, 5'-GUGGAAGGCCUUCUUUCUA-3' (forward) and 5'-UAGAAAGAAGGCCUCCAC-3' (reverse); for NHE1 siRNA, 5'-CGAAGAGAUCCACACACAG-3'; reverse, 5'-CUGUGUGUGGAUCUCUUCG-3'); for non-specific siRNA, CUGAACAAACCAUGCAAU-3' (forward) and 5'-AUUUGCAUUGGUUGUUCAG-3' (reverse) were transfected separately, or together, into Neo and BI-1 cells, using Amaxa Nucleofector (Amaxa, Gaithersburg, MD). Briefly, confluent cells were trypsinized, and resuspended in Amaxa Nucleofector solution at 2 × 10⁵ cells per 100 μl of solution; and BI-1, NHE1, or non-specific siRNA was added. Cells were transfected by electroporation, using the A24 pulsing program.

Statistical analyses

Results are presented as mean ± S.E. of *n* cells, and paired and unpaired Student's *t* tests were applied to test and control conditions, where appropriate. Microcal Origin software (Northampton, MA) was used for statistical calculations.

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