Na^+/H^+ exchanger blockade inhibits the expression of vascular endothelial growth factor in SGC7901 cells

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Received June 22, 2009; Accepted September 28, 2009

DOI: 10.3892/or_00000608

Abstract. Vascular endothelial growth factor (VEGF) overexpression is critically involved in tumor formation. Na^+/H^+ exchanger isoform (NHE) is elevated in cancer cells. We explored the effect of NHE inhibition on VEGF expression in human gastric cancer SGC7901 cells. VEGF mRNA expression was detected by real-time RT-PCR. VEGF protein expression was measured by Western blotting and immunocytochemistry. For determination of viable cells the MTT colorimetric assay was used. Treatment of SGC7901 cells with NHE inhibitors resulted in significant decrease of VEGF mRNA and protein expression. NHE inhibition decreased intracellular pH (pHi) values and VEGF mRNA expression. These data demonstrate that NHE blockade inhibits VEGF expression in SGC7901 cells.

Introduction

Angiogenesis is tightly regulated by pro-angiogenic and anti-angiogenic balance. In tumorigenesis, this balance is derailed (1), thereby triggering tumor growth, invasion, and metastasis (2). The 'angiogenic switch' (3) is triggered by oncogene-mediated tumor expression of angiogenic proteins such as vascular endothelial growth factor (VEGF) (1). VEGF plays a critical role in many aspects of cancer biology. VEGF and VEGF receptor (VEGFR) expression has been detected in a variety of solid tumors, including those of gastrointestinal tract (4,5).

The acquired ability to maintain elevated intracellular pH (pHi) despite the growth in a progressively acidic extracellular milieu confers selective advantage to transformed malignant cells (6). The microenviroment within solid tumors is slightly acidic, and manipulation of this extracellular acidity to cause intracellular acidification might be used to increase selective antitumor effects of some anticancer drugs (7). An alkaline pHi is a fundamental step in the acquisition of a malignant phenotype (8). On the contrary, cytosolic hyperacidification is a generalized event in programmed cell death at different stages of the apoptotic process (9). The primary regulator of pHi is the Na^+/H^+ exchanger (NHE) of which there are 8 known isoforms (10). The involvement of the ubiquitous pHi regulator NHE in tumorigenesis is well documented; more recently, signaling pathways involved in the activation of NHE have been explored in cells with malignant phenotype (11,12).

Our previous study in leukemia cells indicated that there is a correlation between modulation of pHi and the expression of VEGF (13). Considering VEGF and its receptors play more important roles in solid tumors than in leukemia, it is an agent involved in the relationship between pHi and VEGF expression in solid tumors. However, there is little data on this relationship. In an attempt to integrate these subfields, the study therefore explores the effect of inhibition of NHE on the expression of VEGF mRNA and VEGF protein in human SGC7901 gastric cancer cell line. The purpose of this study was to determine whether NHE inhibition can decrease the expression of VEGF in SGC7901 cell line. If NHE inhibition could decrease the expression of VEGF in SGC7901 cells, it would inhibit tumor angiogenesis. The study allows consideration of NHE inhibitors as potential anti-tumor agents.

Materials and methods

Reagents and chemicals. RPMI-1640 was obtained from Gibco Co. Nigericin, 5-(N-ethyl-N-isopropyl) amiloride (EIPA), 3-(4,5-dimethyl-thiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and amiloride were obtained from Sigma Co. (Vienna, Austria). The pH-sensitive fluorescent probe 2',7'-biscarboxyethyl-5(6)-carboxyfluorescein acetoxymethyl ester Mixed isomers (BCECF-AM) was obtained from Calbiochem Co. Primers were obtained from Shanghai Sangon Biotechnology. TRIzol Reagent, Superscript II

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Key words: vascular endothelial growth factor, Na^+/H^+ exchanger isoform, SGC7901 cell, intracellular pH

reverse transcriptase were obtained from Invitrogen. SYBR-Green PCR kit was obtained from Qiagen. Polyclonal rabbit anti-human VEGF antibodies and horseradish peroxidase-conjugated IgG (HRP-IgG) goat anti-rabbit antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Human gastric cancer SGC7901 cells were obtained from the Wuhan University type culture collection.

Culture. Human gastric cancer SGC7901 cells were grown in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin (100 units/ml), streptomycin (100 μg/ml), and 1% (v/v) L-glutamine and were maintained at 37˚C in 5% CO_2-95% air atmosphere. New cultures were re-established form frozen stocks every 3 months.

Intracellular pH determination. Laser confocal microscope was used to determine the pHi values of SGC7901 cells. SGC7901 cells on coverslips were loaded with 1 μg/ml BCECF-AM for 30 min at 37˚C with gentle shaking in sodium HEPES buffer (25 mmol/l HEPES, pH 7.4, 140 mmol/l NaCl, 5 mmol/l KCl, 0.8 mmol/l $MgCl_2$, 1.8 mmol/l $CaCl_2$, 5.5 mmol/l glucose). After incubation, coverslips were washed three times with the same buffer. Excitation of the probe was performed at 488 nm with a 500 milliwatt ionic argon laser. Emission fluorescence was filtered through a 530 band pass and 640 nm long pass filter. Because the addition of bicarbonate can affect pHi, all incubations and cell washes were performed in bicarbonate-free buffer. pHi value was estimated from the ratio of the emission fluorescence signals obtained at both 530 nm (pH sensitive) and 640 nm (pH independent) by laser confocal microscope. For every experiment, a calibration curve was constructed by incubating BCECF-AM-labeled cells in a high potassium HEPES buffer (25 mmol/l HEPES, 145 mmol/l KCl, 0.8 mmol/l $MgCl_2$, 1.8 mmol/l $CaCl_2$, 5.5 mmol/l glucose) at a specific pH (6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8) and by adding the K^+/H^+ ionophere nigericin (1 μg/ml) for 10 min at 37˚C. Under these conditions, nigericin equilibrates intra- and extra-cellular pH values and calibration curves were constructed by plotting extracellular pH against the corresponding background-corrected fluorescence ratios (530 nm/640 nm).

Measurements of pH in nucleus were analyzed by calculated calibration curves for the cytosol and the nucleus, respectively. Analysis of BCECF distribution in the cells was performed with confocal laser microscopy. Briefly, cells were stained with BCECF-AM as described above and washed with dye-free medium. Samples were analyzed by confocal scanning microscope.

Real-time quantitative reverse-transcription polymerase chain reaction. Total RNA was isolated from cells using TRIzol reagent. Complementary deoxyribonucleic acids (cDNAs) were reverse-transcribed, following the manufacturer's instructions, from 2 μg of total RNA by Superscript II reverse transcriptase. Real-time PCR was performed using the SYBR-Green PCR kit, according to the manufacturer's instructions. Annealing temperature was optimized to create a one-peak melting curve and the productions of PCR were checked by agarose gel electrophoresis for a single band of the expected size. The abundance of each mRNA were detected and normalized to that of GAPDH mRNA. The sequences of the primers used to detect VEGF were 5'-GAAGTGGTGAAGT TCATGGATGTC-3' (forward) and 5'-CGATCGTTCTG TATCAGTCTTTCC-3' (reverse). Amplification of the 'housekeeping' gene, glyceraldehyde-3-phosphodehydrogenase (GAPDH), was used to verify mRNA isolation and real-time PCR techniques. PCR primers used to detect GAPDH gene were 5'-TCACCATCTTCCAGGAGCG-3' (forward) and 5'-AGTGAGCTTCCCGTTCAGC-3' (reverse).

Western blotting. After being washed with PBS, the cells were resuspended in ice-cold lysis buffer. The lysates were transferred to Eppendorf tubes and were centrifuged at 15,000 g. The supernatant was recovered. Protein concentrations were determined using the Bio-Rad protein assay. Ten micrograms of sample were separated on an 8-16% Tris-glycine gel and were transferred to a nitrocellulose membrane. The membrane was incubated with the primary antibody polyclonal rabbit anti-human VEGF (1:200). After the washing steps, the membrane was incubated with IgG-HRP goat anti-rabbit (1:500). The immunoblots were visualized by means of enhanced chemiluminescence.

Immunocytochemistry. SGC7901 cells grown as a monolayer on a slide were treated with different concentrations of amiloride (0, 100 and 300 μmol/l) for 24 h. After being washed twice with PBS, they were then fixed for 15 min with fresh paraformaldehyde (4%) at room temperature. Afterwards, they were washed in PBS and blocked for endogenous peroxidase by 3% hydrogen peroxide. They were then permeabilized with 0.3% Triton X-100 in PBS for 30 min. After washing with PBS, the slides were incubated for 18 h at 4˚C with the appropriate dilution (1:50) of polyclonal rabbit anti-human VEGF antibody. Then samples were incubated for 60 min at room temperature with the appropriate dilution (1:50) of HRP-IgG goat anti-rabbit antibody. The slides were rinsed with PBS and colored with 3,3'-diaminobezidine (DAB) and Mayer's hematoxylin used for counterstaining, and were then dehydrated and coverslipped. The positive cell of VEGF protein showed brown yellow in cytoplasm.

Determination of viable cells using the MTT colorimetric assay. Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial depending on reducing formation of MTT. Cells were seeded at $1x10^5$/ml in 96-well plates and grown with different concentrations of amiloride (or EIPA) for 24 h. Four hours before the end of the culture, the MTT dye was added at a final concentration of 1 mg/ml at 37˚C. During this incubation time, viable cells with mitochondria will reduce the MTT dye to the purple formation, resulting in a shift in the absorption spectrum of the dye. Culture medium was removed by aspiration, and the cells were solubilized in DMSO (100 μl), the extent of reduction of MTT to formation within cells was quantitated by measurement of optical density at 550 nm using a Spectramax 250 microplate reader. Controls were set to 0 μmol/l amiloride (or EIPA). The cells viability = experimental cell OD/control cell OD.

Statistics. Data were presented as mean ± standard error of the mean (SEM). The statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) or Dunnett's test. Some data were analyzed by simple linear regression. P-value <0.05 was considered to be statistically significant. In the experiments involving immunocytochemistry, the figures shown are representative of at least five experiments performed on different days.

Results

Effect of NHE inhibition on SGC7901 cell viability. To elucidate the effects of NHE inhibition on SGC7901 cell viability, the effects of different concentrations NHEs inhibitors amiloride on the cell proliferation for 24 h were observed. Proliferation was assayed by the ability of the cells to reduce MTT. The MTT showed that amiloride did not suppress mitochondrial respiration in the 100-300 μmol/l concentration range, but it did show that amiloride could suppress mitochondrial respiration in the 400-700 μmol/l concentration range, a successive increase in amiloride concentration resulted in a reduction in cell viability (Fig. 1A). The effect of higher concentrations of amiloride on cell viability was not investigated, because amiloride at 1000 μmol/l was toxic to cells (data not shown).

In subsequent experiments, we examined the effect of selective inhibition of NHEs by the amiloride analog EIPA on SGC7901 cell viability. The MTT also showed that EIPA did not suppress mitochondrial respiration in the 0.5-1.5 μmol/l concentration range, but it did show that EIPA could suppress mitochondrial respiration in the 2.0-3.5 μmol/l concentration range, a successive increase in EIPA concentration resulted in a reduction in cell viability (Fig. 2A).

In addition, the MTT showed that low concentrations of NHE inhibitors (100-300 μmol/l amiloride or 0.5-1.5 μmol/l EIPA) did not suppress mitochondrial respiration for 72 h (data not shown).

Effect of NHE inhibition on pHi of SGC7901 cells. To investigate the role of NHE inhibitor on pHi values in SGC7901 cells, we first examined whether the inhibition of NHEs by amiloride influenced the pHi values of SGC7901 cells. pHi values were measured in SGC7901 cells cultivated with different concentrations of amiloride (0, 100, 200 and 300 μmol/l) for 24 h by using the fluorescent dye BCECF-AM as indicated in Fig. 1B1. Cultivation of cells with different concentrations of amiloride resulted in a change in pHi values. By increasing concentrations, pHi values decreased dramatically. pHi values were also measured in cells cultivated with 200 μmol/l amiloride for different time frames (0, 6, 12, 24 and 48 h) as indicated in Fig. 1B2. Cultivation of cells with 200 μmol/l amiloride for prolonged time resulted in a dramatic decline in pHi values.

EIPA also decreased pHi values in SGC7901 cells. pHi values were measured in SGC7901 cells cultivated with different concentrations of EIPA (0, 0.5, 1.0 and 1.5 μmol/l) for 24 h. Fig. 2B shows that cultivation of cells with EIPA resulted in a decrease in pHi. By increasing concentrations, pHi values decreased dramatically.

Effect of NHE inhibition on the expression of VEGF mRNA in SGC7901 cells. To test whether VEGF mRNA expression is inhibited by amiloride, SGC7901 cells were incubated either with different concentrations of amiloride (0, 100, 200 and 300 μmol/l) for 24 h or with 200 μmol/l amiloride for different time frames. As shown in Fig. 1C1 and C2, real-time RT-PCR analysis revealed that the levels of VEGF mRNA first displayed a decrease at 100 μmol/l concentration, and then gradually decreased dramatically with increasing concentrations. Cultivation of cells with 200 μmol/l amiloride for prolonged time resulted in a decline in the VEGF mRNA expression.

SGC7901 cells were cultivated with different concentrations of EIPA (0, 0.5, 1.0 and 1.5 μmol/l) for 24 h. The level of VEGF mRNA showed a decrease at 0.5 μmol/l concentration, and then decreased dramatically as indicated in Fig. 2C. The inhibitory effect of amiloride or EIPA on VEGF expression was not due to a decrease in cell viability, because neither of these agents decreased mitochondrial respiration at the concentrations tested.

Role of pHi on VEGF mRNA. To test whether VEGF mRNA expression is triggered by pHi, cells were incubated with NHE inhibitors. In fact, the levels of VEGF mRNA in SGC7901 cells decreased accompanying pHi values. As shown in Fig. 1B1 and B2, by increasing the amiloride concentration pHi values and the expression of VEGF mRNA decreased after amiloride treatment, they both have positive correlation (r_1=0.986, $^*P<0.05$). By cultivating with 200 μmol/l amilorde for prolonged time, pHi values and the expression of VEGF mRNA decreased, they both have positive correlation (r_2=0.956, $^*P<0.05$).

As shown in Fig. 2B, cultivation of cells with different concentrations of EIPA, pHi values and the expression of VEGF mRNA also decreased, they both have positive correlation (r_3=0.964, $^*P<0.05$).

The inhibitory effects of NHEs blockade on the expression of VEGF protein in SGC7901 cells. SGC7901 cells were treated with different concentrations of amiloride (0, 100, 200, 300 μmol/l) for 24 h or with 200 μmol/l amiloride for different time frames (0, 6, 12, 24 and 48 h). The expression of VEGF protein was detected in SGC7901 cells by Western blotting (Fig. 1D1 and D2). The expression of VEGF protein significantly decreased when cells were cultured with amiloride. With increase in concentrations and time frames, the expression of VEGF protein decreased dramatically.

SGC7901 cells were treated with different concentrations of EIPA (0, 0.5, 1.0 and 1.5 μmol/l) for 24 h. The expression of VEGF protein was detected by Western blotting. Fig. 2D showed that treatment of SGC7901 cells with EIPA resulted in a decrease in the expression of VEGF protein and the best inhibition occurred at 1.5 μmol/l.

SGC7901 cells were treated with different concentrations of amiloride (0, 100 and 300 μmol/l) for 24 h. The expression of VEGF protein was detected in SGC7901 cells by immuno-

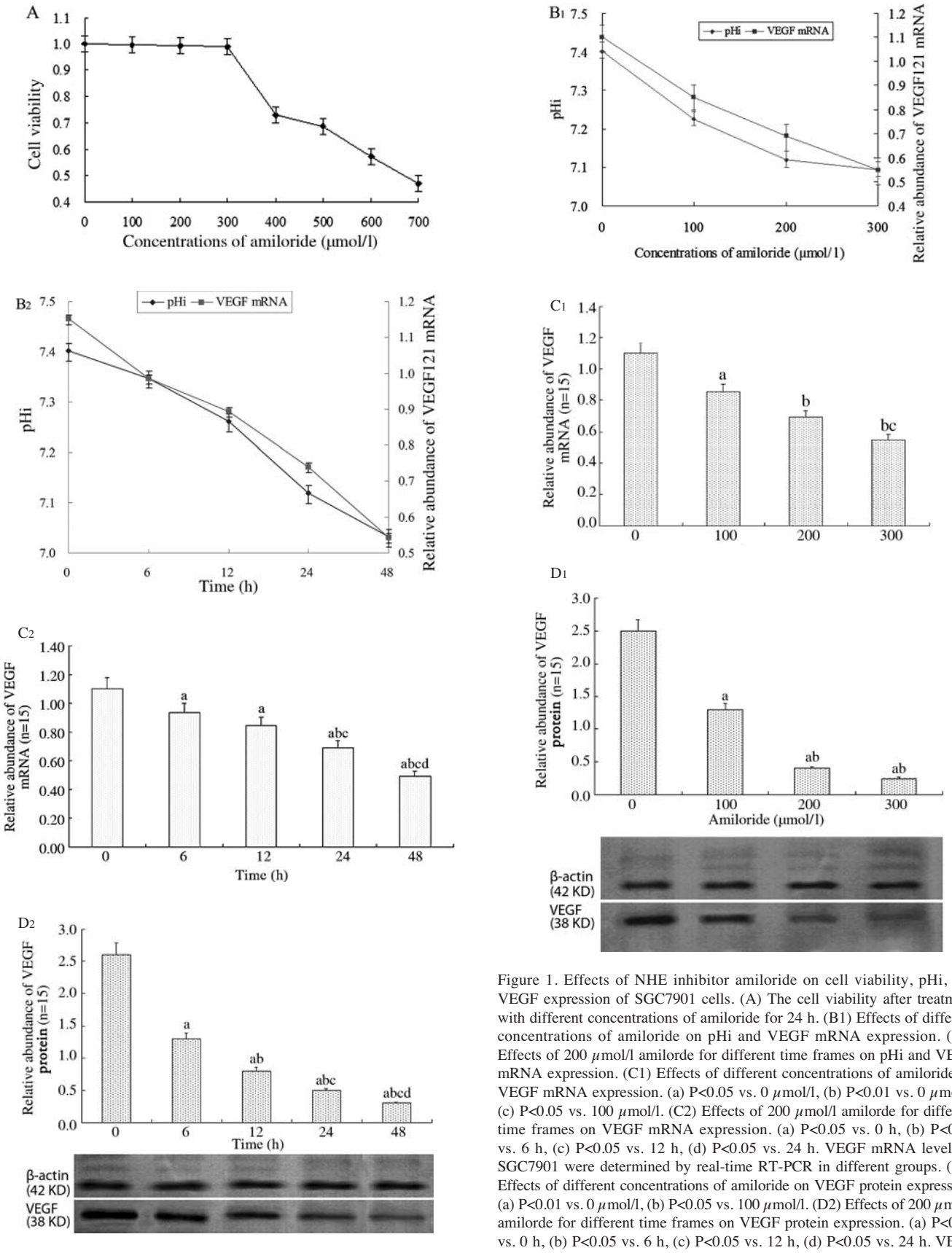

Figure 1. Effects of NHE inhibitor amiloride on cell viability, pHi, and VEGF expression of SGC7901 cells. (A) The cell viability after treatment with different concentrations of amiloride for 24 h. (B1) Effects of different concentrations of amiloride on pHi and VEGF mRNA expression. (B2) Effects of 200 μmol/l amilorde for different time frames on pHi and VEGF mRNA expression. (C1) Effects of different concentrations of amiloride on VEGF mRNA expression. (a) P<0.05 vs. 0 μmol/l, (b) P<0.01 vs. 0 μmol/l, (c) P<0.05 vs. 100 μmol/l. (C2) Effects of 200 μmol/l amilorde for different time frames on VEGF mRNA expression. (a) P<0.05 vs. 0 h, (b) P<0.05 vs. 6 h, (c) P<0.05 vs. 12 h, (d) P<0.05 vs. 24 h. VEGF mRNA levels in SGC7901 were determined by real-time RT-PCR in different groups. (D1) Effects of different concentrations of amiloride on VEGF protein expression. (a) P<0.01 vs. 0 μmol/l, (b) P<0.05 vs. 100 μmol/l. (D2) Effects of 200 μmol/l amilorde for different time frames on VEGF protein expression. (a) P<0.01 vs. 0 h, (b) P<0.05 vs. 6 h, (c) P<0.05 vs. 12 h, (d) P<0.05 vs. 24 h. VEGF protein levels in SGC7901 were determined by Western blotting in different groups, which are consistent with the expression of mRNA. Data were expressed as mean ± SEM. Statistics were performed by Dunnett's t-test. These experiments demonstrate that 100-300 μmol/l concentration range amiloride does not affect cell viability. Amiloride could decrease cells pHi and VEGF expression, and they both have a positive correlation (r_1=0.986, *P<0.05; r_2=0.956, *P<0.05). GAPDH in RT-PCR served as controls. ß-actin in Western blots served as controls.

cytochemistry (Fig. 3). The positive cells of VEGF protein were brown yellow in the cytoplasm. With increase in concentration, the expression of VEGF protein decreased.

Analysis of pH changes in nuclei. BCECF is essentially located in the cytosol and the nucleus as observed by confocal

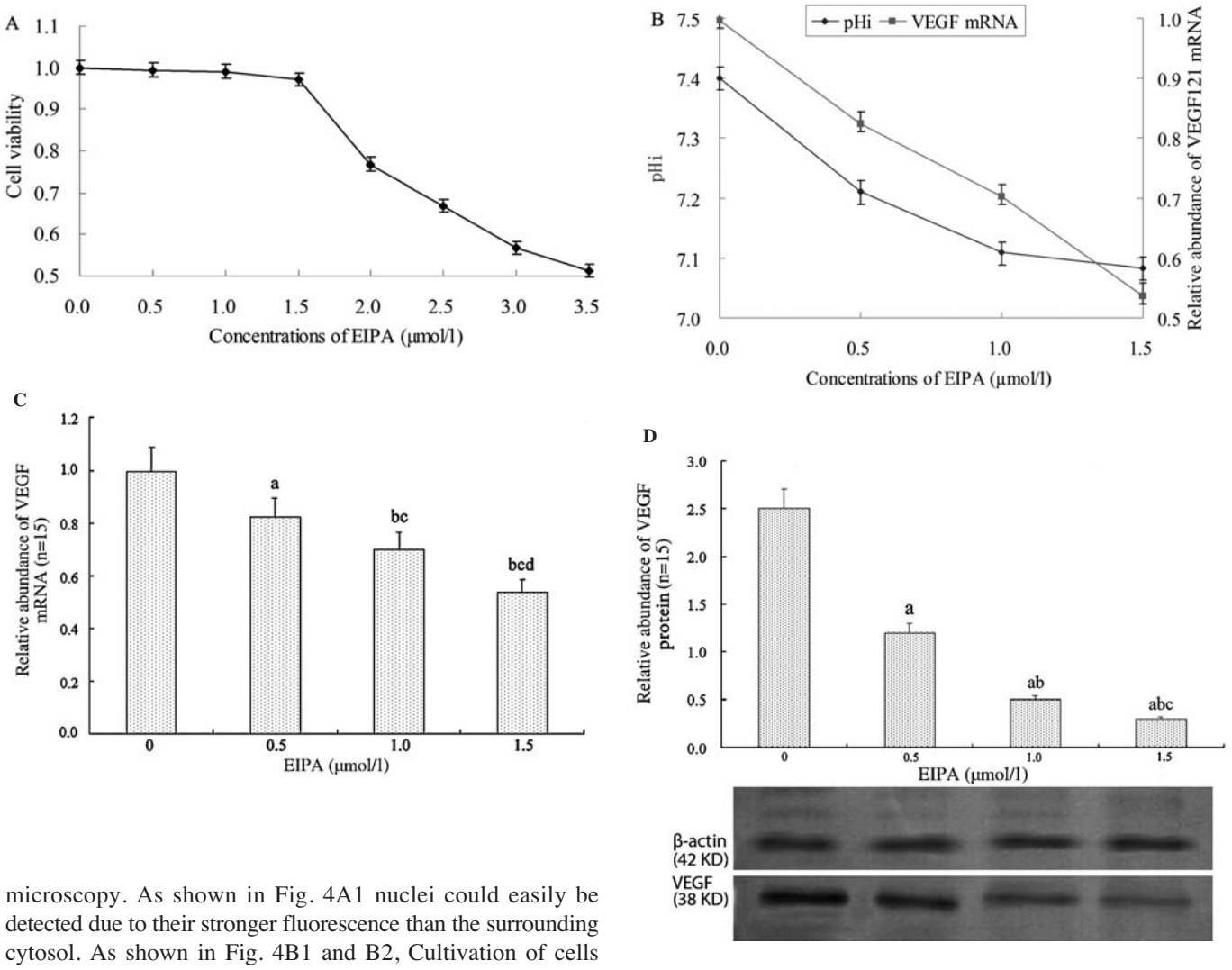

Figure 2. Effects of selective NHE inhibitor EIIPA on cell viability, pHi, and VEGF expression of SGC7901 cells. (A) The cell viability after treatment with different concentrations of EIPA for 24 h. (B) Effects of EIPA on pHi and VEGF mRNA expression. (C) Effects of EIPA on VEGF mRNA expression. VEGF mRNA levels in SGC7901 were determined by real-time RT-PCR in different groups. (a) $P<0.05$ vs. 0 μmol/l, (b) $P<0.01$ vs. 0 μmol/l, (c) $P<0.05$ vs. 0.5 μmol/l, (d) $P<0.05$ vs. 1.0 μmol/l. (D) Effects of EIPA on VEGF protein expression. VEGF protein levels in SGC7901 were determined by Western blotting in different groups, which are consistent with the expression of mRNA. (a) $P<0.01$ vs. 0 μmol/l, (b) $P<0.05$ vs. 0.5 μmol/l, (c) $P<0.05$ vs. 1.0 μmol/l. Data were expressed as mean ± SEM. Statistics were performed by Dunnett's t-test. These experiments demonstrate that 0.5-1.5 μmol/l concentration range EIPA does not affect cell viability. EIPA could decrease cells pHi and VEGF expression, and they both have a positive correlation ($r_3=0.964$, $^*P<0.05$). GAPDH in RT-PCR served as controls. ß-actin in Western blots served as controls.

microscopy. As shown in Fig. 4A1 nuclei could easily be detected due to their stronger fluorescence than the surrounding cytosol. As shown in Fig. 4B1 and B2, Cultivation of cells with different concentrations of amiloride (or EIPA) resulted in a concomitant decline of pH in both cytosol and nucleus to the same pH values indicating a coupled pH regulation in the cytosol and the nucleus.

Discussion

In solid tumors, vascular endothelial growth factor (VEGF) plays an important role in the induction of neovascularization, thereby promoting tumor growth and metastatic potential. The siRNA targeting human VEGF could induce apoptosis in human breast cancer MCF-7 cells, VEGF might be a potential therapeutic target for human breast cancer (14). The study also found that the level of plasma VEGF in peripheral veins was one of the sensitive markers of the status of gastric cancer (15). High VEGF expression showed rapid tumor regression in metastatic gastric cancer (16). High level expression of VEGF-A in gastric cancer correlated with venous tumor infiltration and the presence of isolated tumor cells, and the route of metastatic spread of gastric cancer was determined, at least in part, by the profile of VEGF expressed in primary gastric cancer patients (17).

pHi plays an important role in the regulation of many aspects of cell physiology. pHi was also one of the factors thought to control the rate of cell proliferation and invasion (18,19). In many tumor cell lines pHi was more alkaline than in normal cell lines (9). Because cell alkalinization preceded cell proliferation, a correlation between pHi and cell cycle has been proposed (9). Furthermore, it has been shown that preventing the increase of pHi generally inhibit cell proliferation (20).

The mechanisms by which cells regulate pHi included proton extruders like NHE, the H^+ pump, and/or bicarbonate transporters, which mediated the influx or extrusion of bicarbonate and contribute to the regulation of pHi in response to metabolic activity (21). pHi was a critical factor maintaining

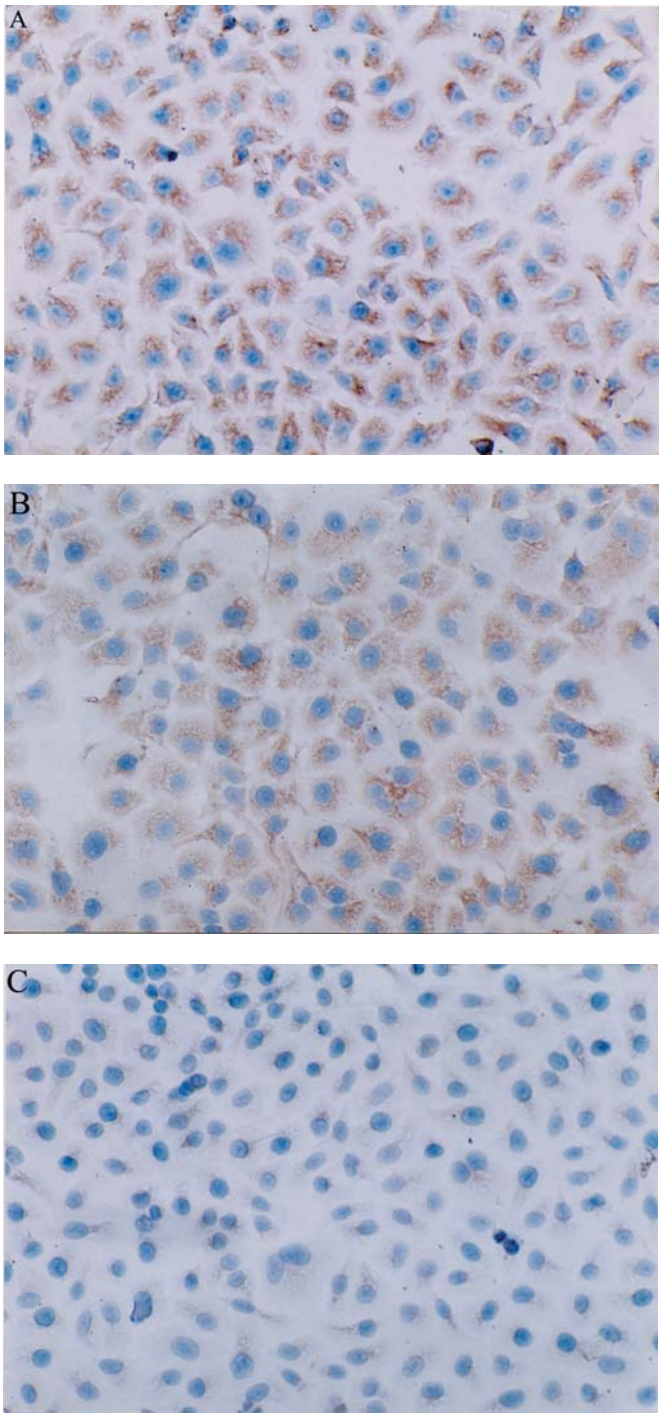

Figure 3. Effects of amiloride on VEGF protein expression of SGC7901 cells by immunocytochemistry. (A) Control; (B) 100 μmol/l; (C) 300 μmol/l (light microscope, S-P technique of immunocytochemistry, stained with hematoxylin again x200). It indicates that SGC7901 cells express VEGF protein and cytoplasm is brown yellow. These experiments indicate that amiloride inhibits VEGF expression.

the intracellular homeostasis. Cytosolic hyperacidification was a generalized event in apoptosis (22). The later studies found that pHi was associated with tumor cells' specific biological significance. For example, reduction of pHi inhibited constitutive expression of cyclooxygenase-2 in human colon cancer cells. The data suggested that an alkaline pHi was an important trigger for constitutive COX-2 expression (18). It is recognized that pathological elevations of pHi induced many specific biological and functional characteristics of malignant cells, such as neoplastic transformation, increases in cell detachment, motility, proliferation, per-meability, and many others (19,20). Indeed, each of these features on its own was an essential step in the formation of a pathological neovasculature network out of pre-existing normal vessels. The NHE was an important mechanism of pHi modulation (22,23). Inhibition of the NHE was able to decrease pHi and induce apoptosis in leukemia cell lines (9), and amiloride was an NHE inhibitor. It has been demonstrated that amiloride is a powerful inhibitor of angiogenesis being able to suppress both *in vitro* and *in vivo* the invasive capacity of human breast cancer cells, at least in part through the inhibition μPA activity (24).

We found an identical pH in the cytosol and the nucleus which decreased in a similar way when cells were grown with NHE inhibitors. Using confocal laser microscopy and BCECF labeled cells Pirkebner *et al* reported identical pH values in the cytosol and the nucleus (18). In contrast, a 0.3-0.5 pH unit higher pH in the nucleus than in the cytosol was found using SNARF-1 as pH indicator (25). First, different behavior of BCECF and SNARF-1 in the nuclear micro-environment may explain the described discrepancy. Second, different nuclear pH regulation in immortalized cells used by Seksek and Board (25) compared to transformed cells also could be responsible for the observed differences. However, the exact factor of described differences in pH nuclear regulation remains to be studied.

Most recently, we have been investigating the effects of NHE inhibitors on the levels of VEGF in cell line derived from acute myelogenous leukemia (AML) knowing that NHE inhibition inhibits VEGF expression in K562 cells (13). It has long been demonstrated that VEGF expression is directly correlated with the activities of solid gastric tumors. Based on the observation that both NHE and VEGF levels were elevated in cancer cells we addressed the question whether NHE was involved in the regulation of VEGF in gastric cancer SGC7901 cells and, if indeed this is the case, whether NHEs inhibition will lead to adverse effects. The study explored the effect of NHE inhibition on the expression of VEGF mRNA and protein in SGC7901 cells.

The results presented here demonstrated that SGC7901 cells exhibited an alkaline pHi and amiloride, EIPA decreased pHi values. With concentrations and time increasing, pHi value decreased dramatically.

Amiloride and its analogues EIPA were known to inhibit NHEs with EIPA being 200-fold as potent as amiloride (26). Thus, we applied 0.5-1.5 μmol/l EIPA (100-300 μmol/l amiloride) to treat SGC7901 cells. Our data documented that amiloride, and EIPA suppressed the expression of VEGF mRNA, respectively. Similarly, our data also documented that amiloride, and EIPA suppressed the expression of VEGF protein, respectively. Therefore, our results showed that inhibition of NHEs suppressed the expression of VEGF.

This study also examined the effect of NHE1 inhibition on cell growth in gastric cancer SGC7901 cells. When amiloride concentrations were ≥400 μmol/l (or EIPA ≥2.0 μmol/l), NHE blockade inhibited cell growth. The possibility that amiloride (or EIPA) suppression of VEGF expression in

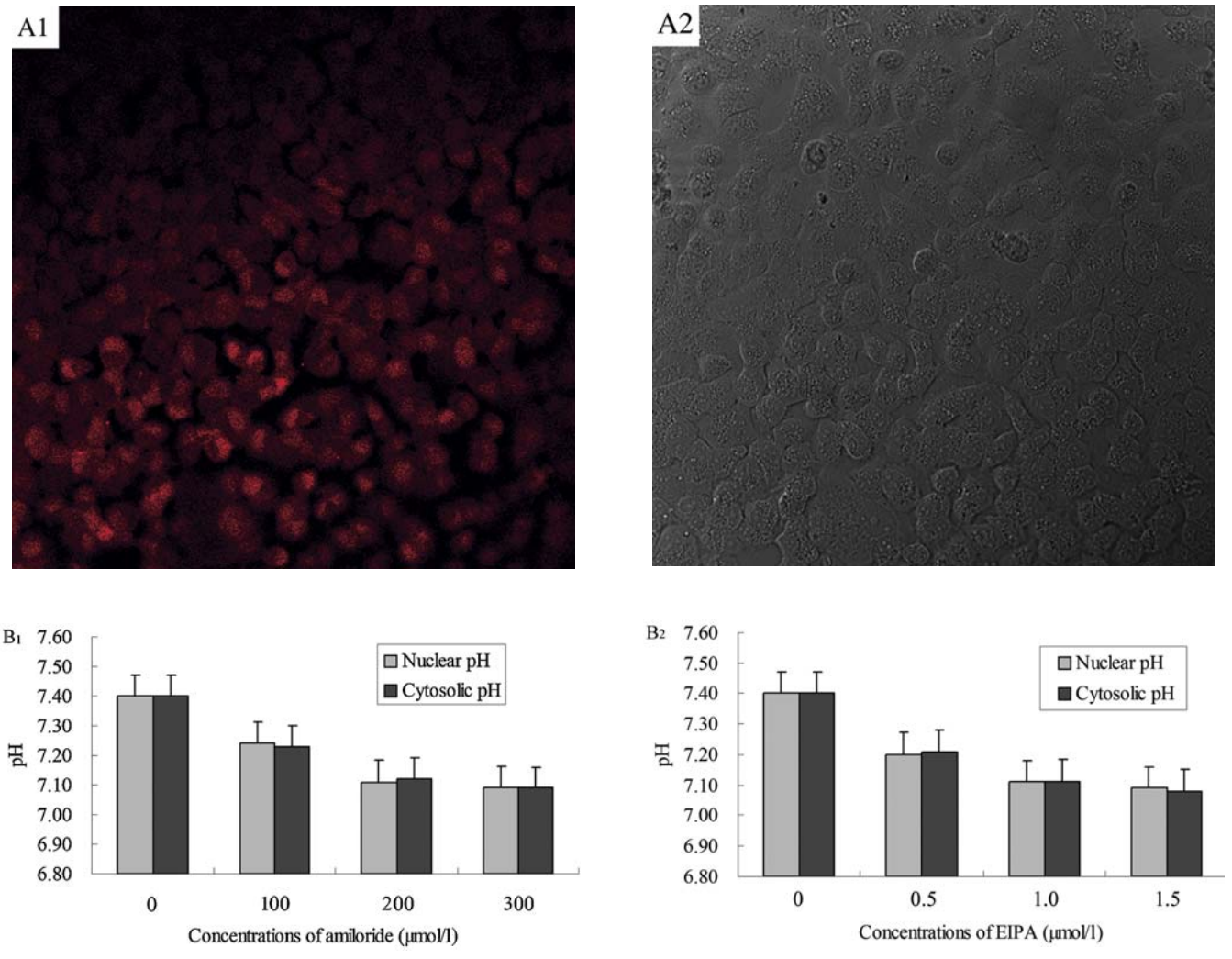

Figure 4. The effect of NHE inhibitors on cytosolic and nuclear pH of SGC7901 cells. (A1) As observed by laser confocal microscope BCECF is located in the cytosol and nucleus of cells, nuclei exhibited a stronger fluorescence than the cytosol. (A2) The corresponding phase contrast micrograph. (B1) Effects of different concentrations of amiloride on cytosolic and nuclear pH. (B2) Effects of different concentrations of EIPA on cytosolic and nuclear pH. These experiments demonstrate identical pH values in the cytosol and the nucleus, respectively.

gastric cancer SGC7901 cells was due to a decrease in cell viability by this inhibitor was excluded using an MTT assay, which showed that amiloride (or EIPA) did not suppress mitochondrial respi-ration in the 100-300 μmol/l (0.5-1.5 μmol/l) concentration range.

In the case of proliferating SGC7901 cells, because a constitutively higher pHi value was demonstrated, we could use NHE inhibition to decrease pHi. The activation of the NHEs suppressed tumor cell apoptosis (27). The activity and expression of NHE, which are involved in the hemeostasis of pHi, were increased in doxorubicin-resistant (HT29-dx) human colon carcinoma cells in comparison with doxorubicin-sensitive HT29-dx cells (28). The NHE-1 antisense gene was able to induce drug-resistant human small cell lung cancer H446/CDDP cells to become acidified and apoptotic, which could provide a novel therapy for multidrug resistance human small cell lung cancer (29). The inhibitor of NHE amiloride potentiated TRAIL-induced tumor cells apoptosis (30). In addition, NHE inhibitor had synergy with chemotherapy to induce apoptosis of tumor cells (12,31). Besides cell growth and proliferation a wide variety of cellular processes and properties such as metabolism, cell volume, were affected by NHEs. Our study demonstrated that treatment of SGC7901 cells with inhibitors of NHEs resulted in a decrease in pHi. In addition, the study first demonstrated NHE inhibition suppressed the VEGF expression in solid cancer SGC7901 cells accompanied by a reduction in pHi values.

Although increased expression of VEGF in gastric cancer has been widely observed, not all of the mechanisms regulating the VEGF expression are well understood. Depending on the stimulus and cell type, a variety of transcription could modulate the expression of VEGF. A number of different agents are now available which target VEGF and its signaling pathways (32). Experimental inhibition of NHEs enabled such studies to find new targets for suppressing levels of VEGF in gastric cancers.

The inhibitory effect of NHE inhibition on the expression of VEGF may be done through decreasing pHi values in SGC7901 cells. The most plausible explanation for the

elevated pHi in cancer would be that there was up-regulation of the signaling pathways that activate NHEs. For example, the activation of NHE by endothelin-1 was partly mediated via protein kinase C (PKC) (33), PKC seemed to play an activating role in Zinc and 17ß-estradiol effects on NHE activity (34). Growth factor such as epidermal growth factor also activated NHE activity via the PKC pathway (35). Moreover, PKC pathway could regulate VEGF products. In serum or tumor tissue of patients with head and neck squamous cell carcinomas (HNSCC), VEGF was suppressed by pharmacologic and siRNA inhibitors of mitogen-activated protein kinase kinase 1/2 (MEK1/2) and PKC pathways (36). Recent data provided evidence that c-Jun NH2-terminal activated by mitogen-activated protein kinase (MAPK) kinase 4 (MKK4) triggered NHE1 activation (37). However, the mechanisms are not known and await further investigations.

In summary, our data demonstrated that NHE inhibition was able to decrease VEGF expression in solid cancer SGC7901 cells, suggesting that the inhibition of NHEs may be a therapeutic approach in gastric cancer.

Acknowledgements

This work was supported in part by a grant-in-aid from Wuhan University of China. The authors sincerely thank Professor Junzhu Wu (Wuhan University) for his considerable contribution in the experimental techniques.

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