Original Paper

An Inhibitor of Na⁺/H⁺ Exchanger (NHE), Ethyl-Isopropyl Amiloride (EIPA), Diminishes Proliferation of MKN28 Human Gastric Cancer Cells by Decreasing the Cytosolic Cl⁻ Concentration via DIDS-Sensitive Pathways

Shigekuni Hosogi^a Hiroaki Miyazaki^a Ken-ichi Nakajima^a Eishi Ashihara^a
Naomi Niisato^{a,b} Katsuyuki Kusuzaki^{a,b} Yoshinori Marunaka^{a,b}

^aDepartment of Molecular Cell Physiology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto; ^bJapan Institute for Food Education and Health, Heian Jogakuin (St. Agnes') University, Kyoto

Key Words

MAPK • Rb • p21 • Cell cycle • Anion exchanger

Abstract

Background/Aims: Tumor cells produce a large amount of acidic metabolites due to their high metabolic condition. However, cytosolic pH (pH_c) of tumor cells is identical to or even slightly higher than that of normal cells. To maintain pH_c at a normal or higher level, tumor cells would have to have higher expression and/or activity of H⁺ transporting systems than normal cells. The purpose of the present study was to identify effects of ethyl-isopropyl amiloride (EIPA, an inhibitor of Na⁺/H⁺ exchanger (NHE)) on proliferation of human gastric cancer MKN28 cells.

Methods: Effects of EIPA on proliferation, pH_c, [Cl⁻]_c and expression of proteins regulating cell cycle and MAPKs were studied in MKN28 expressing NHE exposed to EIPA for 48 h. **Results:** EIPA suppressed proliferation of MKN28 cells by causing G₀/G₁ arrest without any significant effects on pH_c but associated with reduction of [Cl⁻]_c. Although EIPA alone had no effects on pH_c, EIPA co-applied with DIDS (an inhibitor of Cl⁻/HCO₃⁻ exchangers; i.e., anion exchanger (AE) and Na⁺-driven Cl⁻/HCO₃⁻ exchanger (NDCBE)) reduced pH_c, suggesting that DIDS-sensitive Cl⁻/HCO₃⁻ transporters such as AE and/or NDCBE keep pH_c normal by stimulating HCO₃⁻ uptake coupled with Cl⁻ release under an NHE-inhibited condition. EIPA-induced lowered [Cl⁻]_c up-regulated expression of p21 associated with phosphorylation of MAPKs, suppressing proliferation associated with G₀/G₁ arrest. **Conclusions:** EIPA suppressed proliferation of MKN28 cells through up-regulation of p21 expression via reduction of [Cl⁻]_c as a result from DIDS-sensitive Cl⁻/HCO₃⁻ exchanger-mediated compensation for keeping pH_c normal under an NHE-inhibited condition. This is the first study revealing that an NHE inhibitor suppressed the proliferation of cancer cells by reducing [Cl⁻]_c but not pH_c.

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Dr. Shigekuni Hosogi, MD, PhD
Prof. Yoshinori Marunaka, MD, PhD

Department of Molecular Cell Physiology, Kyoto Prefectural University
of Medicine, 465 Kajii-cho, Kamigyo-ku, Kyoto 602-8566 (Japan)
Tel, +81-75-251-5311, Fax +81-75-251-0295,
E-Mail hosogi@koto.kpu-m.ac.jp or marunaka@koto.kpum.ac.jp

Introduction

Tumor cells predominantly produce energy with high-rate glycolysis followed by lactate formation in their cytoplasm due to their fast proliferation under hypoxic, hyponutrient conditions [1] differently from most normal cells producing energy with relatively low-rate glycolysis followed by oxidation of pyruvate in mitochondria [2]. This high-rate glycolysis with lactate formation of tumor cells provides a large amount of H^+ leading to acidic microenvironments. Even in environments that tumor cells produce a large amount of H^+ due to high-rate glycolysis, the cytosolic pH (pH_c) of tumor cells is maintained at a level identical to or even slightly higher than that of normal cells [3, 4]. However, if pH_c of tumor cells is lowered by production of a large amount of H^+ , tumor cells would induce cell cycle arrest and apoptosis [5, 6]. Therefore, regulation of pH_c is critical for survival of tumor cells producing a large amount of H^+ . To maintain pH_c at a normal level under conditions with production of a large amount of H^+ , tumor cells should have higher expression and/or activity of H^+ transporting systems than normal cells.

Four major transporters contributing to pH_c regulation have been identified in tumor cells: 1) Na^+/H^+ exchanger (NHE), 2) Na^+/HCO_3^- cotransporter (NBC), 3) Cl^-/HCO_3^- exchanger including anion exchanger (AE) and Na^+ -driven Cl^-/HCO_3^- exchanger (NDCBE), and 4) H^+ pumps (v-type H^+ -ATPase, etc.) [7-9]. On the other hand, acidic microenvironments surrounding tumor cells produced by activation of H^+ transporters provide tumor cells with advantages for migration and invasion. NHE family, which is ubiquitously expressed in most cells, is a major regulator of pH_c and 10 isoforms of NHEs have been identified [10, 11]. NHE1 is the most ubiquitously expressed one among 10 isoforms, and its activity is regulated by numerous growth factors, mitogens, integrins, tyrosine phosphatases and cytokines [12-18]. NHE in tumor cells is a key transporter to maintain pH_c at a normal level [19], and regulation of NHE activity is one of the most important factors for proliferation and migration of many tumor cells [20-22].

Among many anticancer agents, NHE inhibitors as intelligent agents against proliferation of cancer cells in acidic environments are the most investigated ones [23]. However, the mechanism of NHE inhibitors diminishing proliferation of cancer cells is still unclear, although NHE inhibitors are generally thought to lower pH_c resulting in a decreased proliferation rate. In the present study, we investigated the effect of 5-(N-ethyl-N-isopropyl) amiloride (EIPA, an NHE inhibitor [24]) on the proliferation of cancer cells regarding the intracellular ionic environment in general and not exclusively the H^+ concentration. This is the first report indicating that an NHE inhibitor induced G_0/G_1 arrest via up-regulation of MAPKs/p21 by reducing the cytosolic Cl^- concentration ($[Cl]_c$).

Materials and Methods

Cell culture

The moderately differentiated human gastric adenocarcinoma cell line, MKN28, were seeded into 25 cm^2 flasks at a density of 2.5×10^5 cells/flask and incubated for 24 h in RPMI1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% fetal bovine serum (FBS) in a humidified incubator at 37°C with 5% CO_2 in air. We defined this time point as time zero (0 h). Then, cells were cultured in a culture medium containing ethyl isopropyl amiloride (EIPA, Sigma-Aldrich) or DMSO (Sigma-Aldrich) as a solvent control for EIPA.

Assay of cell proliferation

After culture under each experimental condition for 48 h, cells were detached from the flasks with trypsin-EDTA and counted with a hemacytometer. EIPA was dissolved and stocked in DMSO at concentrations 1,000 times higher than those finally applied to the cells in the culture medium. The concentrations of EIPA used in the present study were 5, 10, 25, 50, and 100 μM , and the concentration of DMSO (solvent control) was 0.1%.

Analysis of cell cycle

After culturing the cells in the culture medium containing EIPA or DMSO alone for 48 h, we detached the cells from the flasks with trypsin-EDTA treatment, and centrifuged the detached cells. Nuclear Isolation Medium of 0.5 ml (NIM-DAPI 10; Beckman Coulter, Fullerton, CA, USA) was added to cells in the pellets [25]. We determined cell cycle phases from 10,000 cells using FlowJo software (Tree Star, Inc., Ashland, OR, USA) by the Cell Lab Quanta (Beckman Coulter) with an excitation at 365 nm and emission at 450 nm for DAPI.

RT-PCR and real-time quantitative RT-PCR

At 48 h after culturing cells, we prepared total RNA from cells by using RNeasy Mini Kit (Qiagen, Venlo, the Netherlands). Total RNA was first transcribed with Superscript TM III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) by using random hexamers for priming in accordance with the manufacturer's instruction. The expression levels of human NHE1 mRNA were quantified by real-time quantitative PCR, using the ABI Gene Amp 5700 (Applied Biosystems, Foster City, CA, USA). We generated standard curves by using total RNA of human stomach (Cell Applications, San Diego, CA, USA) as template. The ratio of the signal of NHE to that of GAPDH was calculated for each sample. The PCR primers and probes used in the present study were as follows: TaqMan Gene Expression Assays; human NHE1 (Hs00300047_m1), human NHE2 (Hs00268166_m1), human NHE3 (Hs00188200_m1), human NHE4 (Hs00962493_m1), and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) (Applied Biosystems).

Measurement of cytosolic pH (pH_c)

We measured pH_c of MKN28 cells using carboxy-seminaphthorhodafluor-1 (carboxy-SNARF-1) (Molecular Probes, Eugene, OR, USA), a pH-sensitive fluorescent dye, with an inverted confocal laser microscope, LSM510META (Carl Zeiss, Jena Germany). Cells were seeded into 35 mm glass bottom dishes at a density of 1.0×10^5 cells/dish and incubated for 24 h in RPMI1640 medium supplemented with 5% FBS in a humidified incubator at 37°C with 5% CO_2 in air. Then, cells were cultured in the culture medium containing 25 μM EIPA with/without 50 μM 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) (Sigma-Aldrich) or DMSO alone as a solvent control. After culture under each experimental condition for 48 h, cells were equilibrated at 37°C in air with 5% CO_2 for 30 min in saline solutions (115 mM NaCl, 25 mM $NaHCO_3$, 11 mM glucose, 4.4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.4 adjusted by CsOH) containing 10 μM carboxy-SNARF-1 of the acetoxymethyl ester form. The culture dishes were placed in an incubator; ZILCS (Tokai-Hit Co., Shizuoka, Japan), on the stage of the confocal microscope and allowed to adapt for at least 20 min before starting pH_c measurements. The excitation laser beam of 514 nm (Ar laser) was directed to the sample via a Plan-Apochromat 100 $\times$ oil-immersion objective lens (NA 1.4, Carl Zeiss). Emitted fluorescence was simultaneously collected using a gating, and the separated fluorescences were detected with 24 photomultiplier tubes (PMTs). Each PMT detected fluorescence in each wavelength range of 10.7 nm, and we collected two PMTs centered at 645 nm and 592 nm. The intensity of fluorescence was digitized with a META system. Several regions of interest (ROI) with a diameter of 1 μm were then randomly selected excluding nuclear regions. The emission ratio was calibrated using solutions (110 mM KCl, 25 mM $KHCO_3$, 11 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES) with varying pH adjusted by CsOH that contained 10 μM nigericin (K^+/H^+ ionophore). The fluorescence emission ratio (645 nm / 592 nm) was calculated and used to estimate pH_c from the calibration curve.

Measurement of cytosolic Cl^- concentration ($[Cl^-]_c$)

We measured $[Cl^-]_c$ of MKN28 cells using N-(Ethoxycarbonyl methyl)-6-methoxy quinolinium (MQAE, Invitrogen, Carlsbad, CA, USA) at 37°C in air with 5% CO_2 in saline solutions (115 mM NaCl, 25 mM $NaHCO_3$, 11 mM glucose, 4.4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.4 adjusted by CsOH) as previously reported [26]. The MQAE is insensitive to physiological changes in pH [27]. Calibration of the fluorescence based on $[Cl^-]_c$ was accomplished by 5 μM nigericin (Wako Pure Chemical Industries, Osaka, Japan), 5 μM valinomycin (K^+ ionophore, Sigma-Aldrich), and 10 μM tributyltin chloride (Cl^-/OH^- ionophore, Wako Pure Chemical Industries) in 140 mM- K^+ calibration buffers (pH 7.4) with various concentrations of Cl^- , which was replaced with NO_3^- . The ionophores were used in order to adjust the $[Cl^-]_c$ to a level identical to the extracellular Cl^- concentration at constant cytosolic K^+ and H^+ concentrations. We prepared calibration buffers by mixing 0 and 100 mM Cl^- buffers. In the 140 mM K^+ buffer with these ionophores, $[Cl^-]_c$ depends solely on the extracellular Cl^- concentration. Cellular fluorescence values observed in the 0, 50, and 100 mM

Cl⁻ buffers were processed as the fluorescence intensity (F) and incorporated into the Stern-Volmer equation as follows: $F_0/F = 1 + K_{sv} [Cl]_c$, where F_0 is the fluorescence at $[Cl]_c = 0$ mM, F is the fluorescence intensity of the cells equilibrated to a defined $[Cl]_c$ and K_{sv} is the Stern-Volmer constant. K_{sv} was determined by performing a linear regression on a plot of F_0/F vs. $[Cl]_c$. MKN28 cells were cultured in a glass bottom dish with the culture medium containing 5 mM MQAE with EIPA or DMSO for 45 min at 37°C in a CO₂ incubator. MQAE-loaded cells were washed with the culture medium containing EIPA or DMSO, and incubated for 20 min at 37°C. Then, the intensity of MQAE in the chamber (ZILCS) on the stage of the microscope was measured by LSM510 META system. The sample was excited at 780 nm using a 2-photon excitation laser system (MaiTai®, Spectra-Physics, Tokyo, Japan). The laser beam was directed to the dish containing the cells via a C-Apochromat 40x water-immersion objective lens (Carl Zeiss). Emitted fluorescence was simultaneously collected by a gating, and the separated fluorescences were detected by 24 PMTs. We collected one PMT at 460 nm. Then, the intensity was digitized with a META system. Several regions of interest (ROI) with a diameter of 1 μm were then randomly selected excluding nuclear regions. The $[Cl]_c$ was estimated by using the Stern-Volmer equation with the determined value of K_{sv} .

Western blotting

We cultured the cells in the culture medium containing EIPA or DMSO alone for 48 h. Immunoblotting was performed as previously reported [28]. The blots were incubated with a primary antibody (anti-ERK, anti-phosphorylated-ERK (Thr202/Tyr204), anti-p38, anti-phosphorylated-p38 (Thr180/Tyr182), anti-JNK, anti-phosphorylated-JNK (Thr183/Tyr185), anti-p21, anti-p53, anti-phosphorylated-p53 (Ser15), and anti-GAPDH obtained from Cell Signaling Technology (Beverly, MA, USA), and anti-NHE1, and anti-NHE2 from Millipore (Temecula, CA, USA)), all of which were detected by using the ECL plus (GE Healthcare, Buckinghamshire, UK). We measured the band densities with Image Lab (BIO-RAD, Hercules, CA, USA) after scanning with Chemidoc XRS Plus system (BIO-RAD).

Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was carried out using Student's *t*-test. The differences were considered significant when the *P* value was less than 0.05.

Results

mRNA transcriptions of NHE isoforms

We first investigated the mRNA transcriptions of NHE isoforms 1 to 4 in MKN28 cells by RT-PCR, and detected mRNA expression of NHE1, NHE2 and NHE4, but not NHE3 in MKN28 cells (Fig. 1A). Then, we performed real-time RT-PCR to determine the mRNA expression levels of NHE1, NHE2, NHE3 and NHE4. NHE1 was the most expressed one among them, and NHE 2 was also relatively largely expressed compared with NHE3 and NHE4 (Fig. 1B). We further observed expression of NHE1 and NHE2 proteins (Fig. 1C), although NHE1 protein was much easier to be detected than NHE2 protein (Fig. 1C).

Effects of EIPA on cell proliferation

We studied the effects of a potent NHE inhibitor, EIPA, on the cell proliferation. EIPA is an amiloride-derivative NHE inhibitor. We exposed the cells to EIPA for 48 h and counted the cell numbers. The exposure of the cells to EIPA inhibited the proliferation of MKN28 cells in a dose- and time-dependent manner (Fig. 2A and B). These results suggest that NHE plays an important role in cell proliferation of MKN28 cells.

Effects of EIPA on the cell cycle

We next investigated effects of EIPA on cell cycle using flow cytometry. The cells were exposed to 25 μM EIPA [29, 30]. Cell cycle analysis indicated that the application of EIPA for 48 h significantly increased the G₀/G₁ fraction, and reduced the S phase (Fig. 3A). The representative data on cell cycle analysis is shown in Figure 3B. These data suggest that EIPA inhibits the cell proliferation by suppressing the transition from the G₁ phase to the S phase.

Fig. 1. Expression of NHE in MKN28 human gastric cancer cells. A: The representative data of RT-PCR analysis of NHE isoforms 1 - 4 mRNA by RT-PCR. B: Real time RT-PCR of NHE isoforms 1 - 4 mRNA. The amount of expression is expressed as the NHEx/GAPDH ratio. A greater amount of NHE1 mRNA was expressed in MKN28 cells. C: Western blots of NHE1, NHE2, and GAPDH. n = 6.

Fig. 2. Effects of EIPA on proliferation of MKN28 cells. A: Cell numbers were counted at 24 and 48 h after application of EIPA (25 μ M) or DMSO (a solvent control). EIPA significantly inhibited proliferation of MKN28 cells. B: Cell numbers were counted 48 h after application of EIPA or DMSO alone. EIPA of each dose significantly inhibited cell proliferation in a dose-dependent manner. n = 6. * $P < 0.05$ (compared with DMSO).

Effects of EIPA on pH_c

We further studied if EIPA shows its inhibitory effects on cell growth and cell cycle by changing pH_c . Interestingly, the treatment with EIPA for 48 h did not significantly change pH_c of MKN28 cells (EIPA, 7.41 ± 0.01 ; control (DMSO alone), 7.43 ± 0.01 ; Fig. 4A), although EIPA induced a transient decrease in pH_c within 5 min after application of EIPA (data not shown). These data suggest that pH_c of MKN28 cells was regulated not only by NHE but also by other ion transporters compensating for changes in pH_c due to the EIPA-caused inhibition of the NHE-mediated H^+ transport. Therefore, we next studied possible roles of Cl^-/HCO_3^- exchangers (anion exchanger (AE, Cl^-/HCO_3^- exchanger) and Na^+ -driven Cl^-/HCO_3^- exchanger (NDCBE)) in pH_c regulation of MKN28 cells. To clarify roles of Cl^-/HCO_3^- exchangers, AE and NDCBE, in pH_c regulation in the presence of EIPA, we applied DIDS, an inhibitor of AE and NDCBE. EIPA co-applied with DIDS for 48 h significantly decreased pH_c to 7.33 ± 0.02 from 7.41 ± 0.01 (EIPA alone) or 7.41 ± 0.01 (DIDS alone) as shown in Figure 4A. DIDS alone had no significant effects on pH_c (Fig. 4A). These results strongly suggest that AE and/or NDCBE would compensate for a change in pH_c caused by EIPA-induced NHE-inhibition.

Effects of EIPA on $[Cl^-]_c$

Based on the result that EIPA co-applied with DIDS, but neither EIPA nor DIDS alone, decreased pH_c , we speculated that AE and/or NDCBE would prevent cells from EIPA-induced accumulation of H^+ in the cytosolic space (a decrease in pH_c) by stimulating uptake of HCO_3^- coupled with release of Cl^- . If so, $[Cl^-]_c$ should be decreased due to an elevation of Cl^- release exchanged with HCO_3^- uptake to maintain pH_c normal under the NHE-inhibited condition with EIPA. To study if this speculation is correct, we next measured $[Cl^-]_c$ using a Cl^- -sensitive

Fig. 3. Cell cycle analysis in MKN28 cells exposed to EIPA. A: The percentage of MKN28 cells in each phase of the cell cycle. The population of cells in the G_0/G_1 phase was increased and that of cells in the S phase was decreased by exposure to EIPA for 48 h in a dose-dependent manner. B: Flow cytometry analysis of MKN28 cells exposed to EIPA or DMSO alone for 48 h. EIPA caused G_0/G_1 arrest in MKN28 cells. $n = 6$. $*P < 0.05$ (compared with DMSO).

Fig. 4. Measurements of pH_c and $[Cl^-]_c$ in MKN28 cells exposed to EIPA. A: pH_c of MKN28 cells exposed to DMSO, EIPA, DIDS, or EIPA + DIDS. EIPA or DIDS alone caused no significant change in pH_c , but co-application of EIPA and DIDS significantly decreased pH_c in MKN28 cells. DMSO, $n = 35$; EIPA, $n = 31$; DIDS, $n = 27$; EIPA + DIDS, $n = 27$. B: $[Cl^-]_c$ of MKN28 cells exposed to EIPA or DMSO for 48 hrs. $[Cl^-]_c$ was significantly decreased by EIPA ($n = 13$) compared to DMSO treatment ($n = 13$). Co-application of EIPA and DIDS or DIDS alone caused no significant change in $[Cl^-]_c$ compared to DMSO treatment. $*P < 0.05$.

fluorescent dye MQAE. Indeed, as shown in Figure 4B, $[Cl^-]_c$ was lower (28.12 ± 3.03 mM) in EIPA-treated cells than that (42.88 ± 4.98 mM) in control cells. Like the case of pH_c , we next studied possible roles of Cl^-/HCO_3^- exchangers (anion exchanger (AE, Cl^-/HCO_3^- exchanger) and Na^+ -driven Cl^-/HCO_3^- exchanger (NDCBE)) in $[Cl^-]_c$ regulation of MKN28 cells. To clarify roles of Cl^-/HCO_3^- exchangers, AE and/or NDCBE, in regulation of $[Cl^-]_c$ in the presence of EIPA, we applied DIDS, an inhibitor of Cl^-/HCO_3^- exchangers, AE and NDCBE. Co-application of DIDS with EIPA significantly increased $[Cl^-]_c$ from the level induced by EIPA alone (Fig. 4B), while DIDS alone had no effects on $[Cl^-]_c$. These results strongly suggest that AE or NDCBE alone or together would not contribute to regulation of $[Cl^-]_c$ in the absence of EIPA (i.e., under a condition that NHEs function) but extrude Cl^- from the cytosolic space to the extracellular space for uptake of HCO_3^- in order to maintain pH_c normal under the lowered pH_c condition caused by EIPA-induced NHE-inhibition.

Effects of EIPA on the expression of cell cycle-associated proteins, p21, p53 and Rb

We have previously reported that expression of p21 increases under a low $[Cl^-]_c$ condition in a p53-independent manner, diminishing Rb phosphorylation [31]. Based on this observation, we speculated that EIPA would elevate expression of p21 in a p53-independent manner by lowering $[Cl^-]_c$, resulting in diminution of cell growth. Indeed, p21 expression in EIPA treated cells is 2.5 times larger compared to that in control cells (Fig. 5A and B),

Fig. 5. Expression of p21 and p53.

A: Expression of p21, p53 and phosphorylated-p53 (p-p53) and GAPDH by Western Blotting using anti-p21, p53 and phosphorylated-p53 at Ser15 antibodies. B: The expression level of p21 protein relative to GAPDH. Application of EIPA significantly increased protein expression of p21 at 48 h after its application. C: The expression level of phosphorylated p53 relative to GAPDH. Application of EIPA did not significantly increase the expression level of phosphorylated p53 compared with DMSO. D: The expression level of total p53 relative to GAPDH. Application of EIPA significantly decreased protein expression of total p53 at 48 h after its application. * $P < 0.05$ ($n = 5$).

Fig. 6. Expression of the phosphorylated Rb (p-Rb) and total Rb in MKN28 cells exposed to EIPA or DMSO. A: Expression of phosphorylated and total Rb detected by Western Blotting using anti-Rb and anti-phosphorylated-Rb (807/811, 780) antibodies. B: The relative expression level of total Rb to GAPDH. $n = 5$. C: The expression level of phosphorylated (807/811) Rb relative to GAPDH. $n = 5$. D: The expression level of phosphorylated (780) Rb relative to GAPDH. $n = 5$. Application of EIPA significantly decreased the expression of phosphorylated Rb. * $P < 0.05$.

while EIPA, having no effects on phosphorylated-p53 (Ser15; an activated form of p53; Fig. 5A and C), caused a decrease in total p53 (Fig. 5A and D). Induction of p21 expression is mediated by p53 including both phosphorylated and dephosphorylated forms [32], although phosphorylation of p53 at Ser15 elongates the life-time of p53 protein contributing to the total amount of p53 protein expression. If the EIPA action on p21 expression would be mediated via a p53-dependent pathway, EIPA should increase the total amount of p53 regulating p21 expression. However, EIPA decreased the total amount of p53 protein expression. This suggests that the stimulatory action of EIPA on p21 protein expression would not be mediated via a p53-dependent pathway, although EIPA had a regulatory (diminishing) action on expression of p53 protein without any effect on the amount of phosphorylated p53 at Ser15. We further studied the action of EIPA on Rb (Fig. 6), indicating that EIPA induced a decrease

Fig. 7. Expression of the phosphorylated and total MAPKs in MKN28 cells exposed to EIPA or DMSO. A: Membrane images of total p38 and phosphorylated p38 (p-p38) detected by Western blots (an upper panel) and the expression level of phosphorylated p38 relative to GAPDH (a lower panel). Application of EIPA significantly increased the phosphorylation level of p38. $*P < 0.05$ ($n = 5$). B: Membrane images of total ERK and phosphorylated ERK (p-ERK) detected by Western blots (an upper panel) and the expression of phosphorylated ERK relative to GAPDH (a lower panel). Application of EIPA significantly increased the phosphorylation level of ERK. $*P < 0.05$ ($n = 5$). C: Membrane images of total JNK and phosphorylated JNK (p-JNK) detected by Western blots (an upper panel) and the expression of phosphorylated JNK relative to GAPDH (a lower panel). Application of EIPA significantly increased the phosphorylation level of JNK. $*P < 0.05$ ($n = 5$).

in phosphorylated-Rb (Fig. 6A, C and D) without any significant effects on expression of total Rb (Fig. 6A and B).

Effects of EIPA on the expression and phosphorylation of MAPKs

We have previously demonstrated that mitogen-activated protein kinases (MAPKs; p38, JNK, and ERK) are phosphorylated (activated) under a lowered $[Cl]_e$ condition [31]. Therefore, we performed western blotting to study if EIPA phosphorylates (activates) MAPKs. As shown in Figure 7, EIPA phosphorylated (activated) all of them (p38, JNK, and ERK) without any significant effects on expression of MAPKs. Taken together, the observations in our present and previous [28] studies suggest that in MKN28 cells EIPA treatment inactivated Rb via up-regulation of p21 expression by activating MAPKs, resulting in the inhibition of cell proliferation.

Discussion

The present study shows that EIPA, a potent NHE inhibitor, diminished the proliferation of gastric cancer MKN28 cells by decreasing $[Cl]_e$, but not affecting pH_e . Unlike the microenvironments in normal tissue the extracellular microenvironments of cancer cells are more acidic than their intracellular environments. This pH gradient is created and maintained by the secretion of H^+ from cancer cells into their extracellular spaces. NHE1 plays an important role in regulation of pH_e , and activity of NHE1 is indicated to be co-related to tumor aggressiveness [33]. In the present study, we show that an amiloride-derived potent NHE inhibitor, EIPA, suppressed the proliferation of gastric cancer MKN28 cells in

a dose-dependent manner. Many investigators have previously demonstrated that NHE is a target for regulating cancer proliferation; i.e., NHE inhibitors decrease pH_c [18, 20, 34, 35], regulating the expression of check point proteins (i.e., cyclinB1 and cdc2) for cell cycle or signal transductions (i.e., Wee1 kinase) [36]. On the other hand, our previous reports [25, 28, 31, 37, 38] indicate cytosolic Cl^- plays an important role in cell proliferation. Therefore, we should consider two important factors, 1) pH_c and 2) $[Cl]_c$, regarding regulation of cell proliferation. Putney and Barber [36] have reported that diminution of NHE function induces G_2/M arrest in fibroblasts associated with a decrease in pH_c . On the other hand, our present study showed that EIPA induced G_0/G_1 arrest without any effect on pH_c . Therefore, these apparently different effects of NHE blockade on cell cycle would be caused by different effects on pH_c ; i.e., blockade of NHE induces a decrease in pH_c in the study by Putney and Barber [36], but not in our present study. These observations lead us to hypothesize that: 1) in the case that blockade of NHE decreases pH_c blockade of NHE diminishes cell proliferation associated with G_2/M arrest, and 2) in the case that blockade of NHE decreases $[Cl]_c$ without any changes in pH_c blockade of NHE diminishes cell proliferation associated with G_0/G_1 arrest. A study [39] shows a suppressive action of NHE inhibitor on the proliferation and migration of cancer cells without measuring pH_c , although the suppression mechanism is unclear. L'Allemain et al. [40] have demonstrated that the inhibition of NHE1 has no effects on pH_c of fibroblasts in a bicarbonate-buffered medium, suggesting roles of HCO_3^- transporters in keeping pH_c constant even if NHE is inhibited. However, it is unknown how other transporters act on regulation of pH_c under NHE-inhibited conditions [23, 41]. Therefore, in the present study we investigated the function of DIDS-sensitive HCO_3^- transporters such as AE, NDCBE, and $Na^+-HCO_3^-$ cotransporter (NBC) in the presence of EIPA. We confirmed that mRNAs of AE isoform 2 (AE2), NDCBE isoforms (slc4A8 and slc4A10), and NBC isoform 2 (NBC2) were expressed in MKN28 cells (data not shown). Therefore, we studied the effect of DIDS on the regulation of pH_c . DIDS alone did not alter pH_c , but the co-administration of DIDS and EIPA significantly reduced pH_c . These observations suggest that pH_c of MKN28 cells is regulated by not only NHE but also HCO_3^- transporters, and that HCO_3^- transporters compensate for pH_c changes caused by NHE inhibition via HCO_3^- import into the cytosolic space. Further, our present study indicated that EIPA decreased $[Cl]_c$, and the EIPA-induced decrease in $[Cl]_c$ was rescued by co-administration of DIDS and EIPA. This suggests that the compensation of pH_c changes caused by NHE inhibition via HCO_3^- import into the cytosolic space is accompanied by a release of Cl^- via DIDS-sensitive HCO_3^- transporters. Therefore, we suggest that AE and/or NDCBE (DIDS-sensitive Cl^-/HCO_3^- exchangers) but not Cl^- -independent HCO_3^- transporters such as NBC would compensate for pH_c changes caused by NHE inhibition via HCO_3^- import into the cytosolic space. AE-2 is a Cl^-/HCO_3^- exchanger. The extracellular Cl^- concentration ($[Cl]_e$) was 123 mM, and the $[Cl]_c$ was about 40 mM. On the other hand, the extracellular HCO_3^- concentration ($[HCO_3^-]_e$) was 25 mM, but we had no information on the cytosolic HCO_3^- concentration ($[HCO_3^-]_c$). In general, if P_{CO_2} in the cytosolic space would be identical to that in the extracellular space (i.e., 40 mm Hg), $[HCO_3^-]_c$ should be identical to $[HCO_3^-]_e$, since pH_c was also identical to the extracellular pH (pH_e) (7.4; Fig. 4). Under this condition, Cl^- should be imported into the cytosolic space from the extracellular space, and HCO_3^- should be released from the cytosolic space to the extracellular space. If so, AE would not compensate for NHE-inhibition induced decrease in pH_c ; i.e., AE would export HCO_3^- from the cytosolic space into the extracellular space by using the chemical potential of Cl^- ($[Cl]_e = 123$ mM; $[Cl]_c = 40$ mM). On the other hand, NDCBE imports HCO_3^- from the extracellular space to the cytosolic space using the electrochemical potential of Na^+ larger than the chemical potential of Cl^- . Based on electrochemical potentials of Na^+ , Cl^- , and HCO_3^- , the compensation of NHE-inhibition induced decrease in pH_c would be mediated by NDCBE but not by AE2.

It is obvious that various fundamental cellular functions including cell proliferation depend on pH_c . Therefore, various ion transporters such as Cl^-/HCO_3^- exchangers act together to keep pH_c constant (normal) associated with changes in other ionic environments such as

Fig. 8. Summary of action of NHE inhibitor on proliferation in MKN28 cells. 1) EIPA causes accumulation of H^+ in the intracellular space by inhibiting NHE. 2) NDCBE compensates for the NHE-caused increase in the concentration of H^+ in the cytosolic space, $[H^+]_c$ (a decrease in pH_c) by importing HCO_3^- coupled with release of Cl^- (a decrease in $[Cl^-]_c$), resulting in no pH_c change. 3) NDCBE-induced decrease in $[Cl^-]_c$ activates MAPKs (increases in phosphorylation of p38 and JNK). 4) Increases in MAPKs cause up-regulation of p21 expression. 5) Up-regulation of p21 expression decreases phosphorylated Rb (inactivation of Rb). 6) Inactivation of Rb induces G_0/G_1 arrest suppressing the proliferation of gastric cancer MKN28 cells.

$[Cl^-]_c$ as shown in the present study. On the other hand, Cl^- has been shown to play various roles in cellular functions in our previous reports [25, 26, 28, 31, 37, 42-48]. Previously, we reported low Cl^- condition inhibited proliferation of MKN28 cells via activated stress activated MAPKs (p38 and JNK) and increased p21 expression, and each inhibitor of MAPKs (p38 and JNK) abolished the effects [28]. Our present and previous studies [28, 31] clearly indicates that even if EIPA causes no alteration in pH_c , an EIPA-induced change in $[Cl^-]_c$ is a large enough signal to affect proliferation of cancer cells by inactivating Rb via up-regulation of p21 expression mediated through activation of MAPKs (p38 and JNK). Observations obtained from our present and previous studies [31] at least suggest that reduction of $[Cl^-]_c$ activates MAPKs, although we should further clarify the mechanism how reduction of $[Cl^-]_c$ increases phosphorylation of MAPKs; a possible mechanism is that cytosolic Cl^- regulates phosphorylation of MAPKs via a change in affinity of MAPKs to MAPK kinase (MAPKK) or MAPKK activity by directly binding to MAPKs or MAPKK like regulation of rhodopsin [49].

To further confirm that the target of EIPA is NHE, we applied siRNA on NHE1 and NHE2. siRNA against NHE1 and NHE2 diminished the mRNA expression of NHE1 and NHE2 to 10% of negative control (data not shown), but decreased the protein expression of NHE1 and NHE2 only to 50% of negative control (data not shown). Namely, we succeeded the knock-down of mRNA expression of NHE1 and NHE2 by siRNA; nevertheless, we could not knock down the protein expression of NHE1 and NHE2 using siRNA against NHE1 and NHE2. This means that we could not apply siRNA techniques to confirm the effect of NHE on proliferation of MKN28 cells.

If cancer cells develop much more activity and/or expression of ion transporting systems keeping pH_c constant (normal) even under high metabolic conditions producing a much larger amount of H^+ compared with normal cells, it is a better idea for us to develop some drugs targeting disturbance of $[Cl^-]_c$ homeostasis rather than that of pH_c using blockers of H^+ transporters for novel anti-cancer therapies.

In conclusion, NHE inhibition suppressed the proliferation of gastric cancer MKN28 cells by inactivating Rb via up-regulation of p21 expression mediated via activation of MAPKs (p38 and JNK) through NDCBE-induced reduction of $[Cl^-]_c$ without any change in pH_c (Fig. 8). This is the first study revealing that an NHE inhibitor suppresses the proliferation of cancer cells indirectly by regulating $[Cl^-]_c$. Our observations also suggest that the regulation of $[Cl^-]_c$ could be an important target for the development of novel cancer therapeutics.

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