Original Paper

Expressional and Functional Characterization of Intracellular pH Regulators and Effects of Ethanol in Human Oral Epidermoid Carcinoma Cells

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Key Words

Human Oral Epidermoid Carcinoma (OEC-M1) Cells • Ethanol • Intracellular pH Transporters • BCECF • Na⁺-H⁺ Exchanger (NHE) • Na⁺-HCO₃⁻ Cotransporter (NBC)

Abstract

Background/Aims: To functionally characterize intracellular pH (pH_i) regulating mechanisms, such as Na⁺-H⁺ exchanger (NHE) and Na⁺-HCO₃⁻ co-transporter (NBC), and further examine effects of ethanol on the pH_i regulating mechanism in human oral epidermoid carcinoma (OEC-M1) cells. **Methods:** OEC-M1 cells were a gift from Tri-Service General Hospital. Changes of pH_i were detected by microspectrofluorimetry with a pH-sensitive fluorescent dye, BCECF. Isoforms of transporters were examined by Western blot technique. **Results:** i) the steady-state pH_i value shifted from alkaline (7.35~7.49) to acidic (7.0~7.03) following acid/base impacts; ii) in HEPES-buffer system, pH_i recovery following induced-acidification was totally blocked by either removing [Na]_o⁺ or adding HOE 694 (a NHE1 specific inhibitor), which demonstrates existence of NHE1; iii) in HCO₃⁻/CO₂-buffer system, the pH_i recovery following induced-acidification was entirely blocked by either removing [Na]_o⁺ or adding HOE 694 plus DIDS (a NBC specific inhibitor), which suggests existence of Na⁺- and HCO₃⁻-dependent acid-extruder, i.e. NBC; iv) the isoforms of the two acid extruders were NHE1, NBCn1, NBCe1 and NDCBE; v) ethanol (10-1000 mM) showed a biphasic and concentration-dependent effect on resting pH_i (i.e. increase then decrease) by changing the activity of NHE1 and NBC accordingly; vi) treatment with ethanol for 24 hr (≥ 300 mM) significantly inhibited the expression of

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NHE1, NBCn1 and NDCBE, while up-regulated NBCe1. **Conclusions:** Ethanol affects pH_i in a concentration-dependent manner by changing function and expression of NHE1 and NBC isoforms in OEC-M1 cells.

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Introduction

Oral cancer is the 11th most common malignancy in the world [1]. About 90% of oral cancers are subtyped to oral epidermoid carcinoma [2]. Eight biological capabilities have been comprised as the hallmarks of cancer that include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, changing energy metabolism, evading immune destruction, and activating invasion and metastasis [3]. Alterations in extracellular pH (pH_e) and intracellular pH (pH_i) trigger many pathological processes, including cancer [4]. The microenvironment within tumors is hypoxic, which overproduces acids mainly by glycolytic metabolism, resulting in very acidic pH_e condition [5]. Such low pH_e inside solid tumors is mainly attributable to the pH_i extruders, such as Na^+ - H^+ exchanger (NHE) and Na^+ - HCO_3^- exchanger (NBC) [6, 7]. Indeed, NHE activation has been found to enhance tumor cell migration and invasion by creating a distinct cell surface pH gradient and invadopodial-dependent extracellular matrix degradation in human endometrial cancer cells, human breast carcinoma and melanoma cells [8-12]. On the other hand, change of pH_i affects many mammalian cellular functions, such as ion channel permeability [13], cell volume [14], proliferation, apoptosis, differentiation and metastasis [15, 16]. The rise in pH_i through activation of the NHE that modulated by growth factors plays an important role in growth control in tumor tissues [17, 18]. Some of the Bcl-2 family proteins [19] and endonucleases [20] have been identified to be sensitive to apoptosis related early intracellular alkalinization [21]. Moreover, treatment with p90rsk inhibitor has been found to reduce the ethanol-induced increase in viability of cell and expression of NHE1 and Bcl-2 in hepatocellular carcinoma [22].

The pH_i is precisely regulated through the combined process of active transmembrane transporters and passive intracellular buffering power [15, 23, 24]. The active membrane transporters can be separated into two main groups: acid extruders and acid loaders. Acid extrusion regulators, such as NHE and NBC, can be triggered when cells are in acidic condition [15, 25, 26]. NHE is an electroneutral housekeeping transporter that mediates the exchange of $[Na^+]_o$ for $[H^+]_i$ [15, 25, 26]. Under HEPES-buffered media (HCO_3^- -free), the pH_i recovery from acidification through NHE can be inhibited by removal of $[Na^+]_o$ or by the addition of a specific and potent NHE1 inhibitor, such as Hoe 694 [26, 27]. However, in CO_2/HCO_3^- buffered Tyrode solution, NBC coexists with NHE to achieve acid-equivalent extrusion in many mammalian cells [24, 25, 27, 28]. This cotransporter of NBC is largely (56% to 91%) 4, 4-diisothiocyanatostilbene-2, 2-disulphonic acid (DIDS) sensitive, while HOE 694-resistant [23, 25, 27-29]. NBC is also inhibited by removal of external Na^+ [29, 30]. The isoforms of Na^+ -dependent bicarbonate transporters include at least five members of the slc4 family: 2 electrogenic Na^+ , HCO_3^- cotransporters (NBCe1/SLC4A4 and NBCe2/SLC4A5), 1 electroneutral Na^+ , HCO_3^- cotransporter (NBCn1/SLC4A7) and 2 Na^+ -dependent Cl^-/HCO_3^- exchangers (NCBE/SLC4A10 and NDCBE/SLC4A8) [15, 31]. In vascular smooth muscle cells of rat and mice mesenteric small arteries, one and only isoform of NBC has been identified, i.e. NBCn1 (SLC4A7; electroneutral) [15]. However, in our previous study, mRNA/protein levels of three different isoforms of NBC were detected, i.e. NBCn1, NBCe1 (SLC4A4; electrogenic) and NBCe2 (SLC4A5) in human renal artery smooth muscle cells [25]. However, no related knowledge of OEC-M1 cells has been reported.

Although the relationship between alcohol consumption and cardiovascular system function is ambiguous, the alcohol consumption has been long linked to cancer risk [32, 33]. Previous studies indicate that alcohol induced DNA damage and oxidative stress are associated with cancer risk, and promoted tumor metastasis [34-36]. According to epidemiological evidence, alcohol might play a causative role in developing oral cancer [37-

39]. The risk of oral cancer demonstrates strong dose-dependent of alcohol: from 1.75 to 6.01 with increased amounts of alcohol drinking from 25 g/day to 100 g/day, respectively [37, 39, 40]. Our previous research shows that ethanol (30-1000 mM) induced a biphasic and concentration-dependent change in resting pH_i in human myocardial tissue and umbilical cord blood stem cells by altering the activity of the two acid extruders, i.e. NHE and NBC [23, 41]. Moreover, it has been shown that ethanol treatment (10~30 mM) induced cell proliferation mainly by increasing the activities of ERK1 and p90rsk in HepG2 cells, and the maximum effects were shown at the concentration of 20 mM both [22]. However, the related knowledge of ethanol on pH_i regulators is none in OEC-M1 cells. In the light of the importance of pH_i regulation and the serious problem of alcohol abuse in the modern society, the aim of this study is to investigate the effect of ethanol on functional pH_i acid extruding mechanism in OEC-M1 cells.

Materials and Methods

Cell culture

Primary human oral epidermoid carcinoma (OEC-M1) cells (gift from Tri-Service General Hospital, Taipei, Taiwan) were cultured at 37°C with RPMI 1640 media contained serum (10%; Biological Industries, USA) and penicillin/streptomycin (1%). The culture media was renewed every 2-3 days, and routinely subcultured and further experiments were conducted when the cells reached 70-80% in growth density.

Western blotting

The procedure has been described in detail in our previous reports [25]. For immunoblotting analysis, the individual membranes were exposed to anti-SLC9A1 (NHE1, TA328914, 1:1000, OriGene Technologies), anti-SLC4A4 (NBCe1, TA332616, 1:1000, OriGene Technologies), anti-SLC4A7 (NBCn1, AP5655b, 1:1000, Abgent), anti-SLC4A8/10 (NDCBE, OAEC03098, 1:1000, Aviva Systems Biology), and anti- β -actin (GTX100118, 1:5000, GeneTex) at 4°C overnight.

Weak acid/ base pre-pulse technique

NH_4Cl pre-pulse techniques were used to induce acute intracellular acid loading, and the procedure has been described in detail in our previous reports [25, 26, 42]. This method can be explained by 4 phases as shown in the most left part of Fig. 1C: phase 1 (rapid alkalization), phase 2 (slow recovery), phase 3 (rapid acidification) and phase 4 (pH_i regulation). Thus, the acid extruder activity was measured as the slope of recovery from NH_4Cl (20 mM) induced intracellular acidification.

In situ calibration of intracellular pH fluorescent dye BCECF in OEC-M1 cells

To measure pH_i and characterize pH_i regulating mechanism, OEC-M1 cells were detected by microspectrofluorimetry with a pH-sensitive fluorescent dye, BCECF (2',7'-bis(2-carboxethyl)-5(6)-carboxy-fluorescein-acetoxymethyl; 6.25 μ g/ml, Molecular Probes), and the procedure has been described in detail in our previous reports [25, 28]. The result of *in situ* calibration curve by using microspectrofluorimetry technique with nigericin (10 μ M) has been shown in the very first part of Result section, and Fig. 1A, B. Note that nigericin acted as a potassium ionophore to equalize the pH_i to the pH_e .

To measure the chronic change of resting pH_i , cells in 24-well plates were loaded with 6.25 μ g/ml BCECF-AM in cultured medium at 37°C for 30 min, and the procedure has been described in detail by other groups [43, 44]. To keep the CO_2 partial pressure stable during the pH measurements in the reader, the well was covered with a plastic lid. The change of solution was done outside the reader by a pipette. In brief, we aspirated the loading medium and washed with standard HEPES-buffered solution for 2 times. Subsequently, the HEPES- or 5% CO_2/HCO_3^- -buffered solution contained with different concentrations of ethanol were added to the cell and cultured at 37°C for the designed hour. After the treatment, 24-well plates were moved to the Synergy 2Multi-Mode Reader (BioTek, USA) and the ratios of excitation 490 ± 10 nm and 440 ± 10 nm to emission 530 ± 10 nm were recorded every 10 s for 10 min. For the *in situ* calibration of 24-well plate, after BCECF-AM loading, 5 wells of the 24-well plate were washed twice with calibration solution of 5 different pH values (i.e. 5.5, 6.5, 7.0, 7.5 and 8.5) without nigericin, and then calibration solution

with nigericin (10 μ M) was added at 37°C for 10 min, respectively. Subsequently, the plate was ready for the fluorescence detection both for the *in situ* calibration on the 5 nigericin-contained wells (data of calibration was now shown) and the designed pH_i experiments on the rest 19 wells.

Chemicals and solutions

Standard HEPES-buffered Tyrode solution (air equilibrated) was composed of (in mM): 140 NaCl, 4.5 KCl, 1 MgCl₂, 2.5 CaCl₂, 11 glucose, 20 HEPES. In Na⁺ free HEPES solution, 140 NaCl were replaced with 140 NMDG. Standard bicarbonate-buffered Tyrode solution (equilibrated with 5% CO₂/23 mM HCO₃⁻) was the same as standard HEPES-buffered Tyrode solution, except that the concentration of NaCl was reduced to 117 mM and 20 mM HEPES were replaced with 23 mM NaHCO₃. In NH₄Cl pre-pulse solution, NH₄Cl was added directly to solution without osmotic compensation. All different solutions were adjusted to 7.4 with 4N NaOH, 4N HCl and KOH, respectively, at 37°C. 4, 49-diisothiocyanatostilbene-2, 29-disulphonic acid (DIDS) and HOE 694 (3-methylsulfonyl-4-piperidinobenzoyl, guanidine hydrochloride) were added to solutions shortly before use.

HOE 694 was kindly provided by Sanofi-Aventis (Germany). All other chemicals were purchased from Sigma (UK) and Merck (UK).

Statistical analysis

Statistical analysis was performed using Student's t-test and one-way ANOVA. The level of significance was set at **p* < 0.05 or ***p* < 0.01 verse to control. All data are expressed as means $\pm$ standard error of the mean (SEM).

Results

In situ calibration standard curve and equation

Fig. 1A showed the emission ratio observed on perfusion with six different pH calibration solutions (5.5~9.5) in OEC-M1 cells. The maximum and minimal emitted ratio (R_{max} and R_{min}) of 530 nm at 490 nm and 440 nm excitations were obtained from perfusion with pH 9.5 and 5.5 calibration solution, respectively. As shown in Fig. 1B, the mean apparent dissociation constant (pK_a) at 37°C could be derived from the standard nonlinear curve. The below equation [25, 26, 45] was used to convert the fluorescent ratio into pH_i:

$$\text{pH}_i = \text{pK}_a + \log[(R_{\text{max}} - R)/(R - R_{\text{min}})] + \log(F_{440\text{min}}/F_{440\text{max}})$$

The R is the ratio of the 530 nm fluorescence emission at 490 nm and 440 nm excitation, and F is the fluorescence at 490 nm and 440 nm excitation. The maximum and minimal values were obtained from the data curve and the pK_a (-log of dissociation constant) is 7.22. Therefore, our present study has provided the equation of standard calibration curve *in situ* for OEC-M1 cells as shown in Fig. 1B.

Resting and new steady-state intracellular pH value

Under the HEPES-buffered solution, the original resting pH_i value was alkaline (7.49 $\pm$ 0.05; n=40), while shifted to the new acidic steady-state value of pH_i (7.00 $\pm$ 0.09; n=40; *p*<0.05) after intracellular acid/base impact by applying NH₄Cl pre-pulse for 3 times or more, as shown in the Fig. 1C. Similar results were found under CO₂/HCO₃⁻-buffered solution, the pH_i value was alkaline at the beginning (7.35 $\pm$ 0.06; n=25) and shifted to steady-state but more acidic afterwards (7.03 $\pm$ 0.07; n= 25; *p*<0.05), as shown in the Fig. 1D.

Functional identification of intracellular acid extruders

As shown in the Fig. 2A and 2B, the pH_i recovery after NH₄Cl-induced intracellular acidification was entirely blocked by perfusion with Na⁺ free solution and application of 300 μ M HOE 694 (a specific inhibitor of NHE-1) in HEPES-buffered system, respectively. Histograms of Fig. 2C show the pH_i recovery slope (%) following induced intracellular acidification averaged for 6 experiments similar to those shown in A and B, respectively. It demonstrated

Fig. 1. In situ calibration of BCECF fluorescent dye, resting pH_i and kinetic steady-state pH_i in OEC-M1 cells. (A) The trace shows the BCECF fluorescence (510 nm emission at 490 nm and 440 nm excitations) that calibrated by superfusing with 6 different pH (5.5–9.5) of nigericin solutions in OEC-M1 cells. (B) The curve and equation show the relationship of BCECF fluorescence ratio and pH_i that averaged from 6 similar experiments as shown in A. (C, D) The above bars show the buffer system used, and the periods of application of NH_4Cl -pre-pulse are shown with bars above the trace. Note that the new steady-state pH_i is reached following NH_4Cl pre-pulse induced intracellular acidification.

that the mechanism of acid-extrusion in HEPES-buffered system is purely dependent on Na^+ - H^+ exchanger-1 (NHE1) in OEC-M1 cells.

Under 5% CO_2/HCO_3^- -buffered solution, as shown in Fig. 3, removal of Na^+ and application of HOE 694 (300 μM) pulse DIDS (400 μM) completely blocked pH_i recovery, as shown in the Fig 3A and the most right part of Fig. 3D, respectively. On the contrary, adding HOE 694 or DIDS alone only decreased pH_i recovery partially (29% and 25%, respectively), as shown in Figs 3B, 3C and 3D. Histogram in Fig. 3E shows the normalization of pH_i recovery rate of acid extrusion averaged for 6 experiments similar to those shown in Fig. 3A, B, C and D. Our present results indicated that the NHE and NBC functional co-exist in OEC-M1 cells.

The acute and chronic effect of ethanol on resting pH_i

To explore the acute and chronic effect of ethanol on resting pH_i in OEC-M1 cells, different concentrations of ethanol (10 to 1000 mM) were superfused under HEPES- (Fig. 4A and 4B) and 5% CO_2/HCO_3^- -buffered condition (Fig. 4C and 4D). Note that the resting pH_i was observed after 3 times' NH_4Cl pre-pulse (Fig. not shown). The present results showed that 10–30 mM ethanol didn't affect resting pH_i , while the higher concentrations of ethanol (>100 mM) changed resting pH_i significantly both in HEPES- and CO_2/HCO_3^- -buffered condition, as shown in Figs. 4A and 4C, respectively. To rule out the possibility of run-down of pH_i by time, the wash-out after perfusion of different concentrations of ethanol had been examined, as shown in Fig. 4B and 4D. Our present results showed that ethanol (10–1000 mM) showed a concentration-dependent inhibitory effect on resting pH_i (–0.01~–0.05 pH_i unit) in HEPES-buffered solution, while a concentration-dependent and biphasic

Fig. 2. Effect of Na^+ -free solution and HOE 694 on pH_i recovery subsequent induced intracellular acidification in HEPES-buffered condition. (A, B) The above bars show the buffer system used, and the bars above or below the trace show the application of NH_4Cl and treatment. (C) Histograms show the pH_i recovery slope (%) following induced intracellular acidification averaged for 6 experiments similar to those shown in A and B, respectively. The pH_i recovery rate was measured at $pH_i = 6.80 \pm 0.04$, $**p < 0.01$ verse to control.

Fig. 3. Effect of Na^+ -free solution, HOE 694, DIDS and HOE 694 plus DIDS on pH_i recovery subsequent induced intracellular acidification in 5% $\text{CO}_2/\text{HCO}_3^-$ -buffered condition. (A, B, C, D) The above bars show the buffer system used, and the bars above or below the trace show the application of NH_4Cl and treatment. The first part of trace A, B, C, D shows a typical pH_i recovery form an induced intracellular acidification in 5% $\text{CO}_2/\text{HCO}_3^-$ -buffered solution as control. The other part shows the pH_i recovery slope after application of Na^+ free, HOE 694, DIDS, and HOE 694 plus DIDS, respectively. (E) Histogram shows the normalization of pH_i recovery rate of acid extrusion that are averaged for 6 experiments similar to those shown in A, B, C, D. The pH_i recovery rate was measured at $\text{pH}_i = 6.80 \pm 0.04$, $**p < 0.01$ verse to control.

Fig. 4. Effect of acute and chronic treatment with ethanol (10 to 1000 mM) on resting pH_i value in HEPES- and $\text{CO}_2/\text{HCO}_3^-$ -buffered solution. (A, B, C, D) The above bars show the buffer system used, and the bars above or below the trace show the acute application of ethanol. The first of 3 min of trace shows the resting pH_i value as a control. (E) Histograms show the normalization of change of ethanol on resting pH_i averaged in 7 experiments similar to that shown in A and C, respectively. (F) Histograms show the normalization of change on resting pH_i after chronic application of ethanol (24 hr) either in HEPES- or 5% $\text{CO}_2/\text{HCO}_3^-$ -buffered condition, respectively. $**p < 0.01$ verse to control of HEPES-buffered system group, $ss < 0.01$ verse to control of 5% $\text{CO}_2/\text{HCO}_3^-$ -buffered system group, $###p < 0.01$ HEPES-buffered system group vs 5% $\text{CO}_2/\text{HCO}_3^-$ -buffered system group.

effect on resting pH_i (+0.01~-0.05 pH_i unit) in $\text{CO}_2/\text{HCO}_3^-$ -buffered condition, as shown in histograms of Fig. 4E. Moreover, in experiments of chronic effect of ethanol on pH_i (i.e. 24 hr), the changes are about up to five or even six times higher than that of acute treatment with ethanol, as shown in the histogram of Fig. 4F (HEPES-buffered: -0.03~-0.3 pH_i unit; $\text{CO}_2/\text{HCO}_3^-$ -buffered: + 0.07~-0.09 pH_i unit).

The effect of ethanol on the activity of NHE and NBC

To measure the effect of ethanol on the activity of NHE, the cells were superfused with various concentrations of ethanol (10-1000 mM) during the pH_i recovery following NH_4Cl -induced intracellular acidification in HEPES-buffered solution (Fig. 5). As shown in the Fig. 5A, lower concentrations of ethanol (10-30 mM) did not change pH_i recovery

in HEPES-buffered solution. In HEPES-buffered solution, adding higher concentrations of ethanol (100-1000 mM) inhibited the pH_i recovery in a concentration-dependent and reversible way, as shown in Fig. 5B-E. Note that the inhibition of NHE1 activity by 1000 mM of ethanol was dramatically but reversibly.

To elucidate the effect of ethanol on NBC activity, the cells were superfused with of different concentrations of ethanol (10-1000 mM) during the pH_i recovery following NH_4Cl -induced intracellular acidification in CO_2/HCO_3^- -buffered solution. As shown in the Fig. 6, lower concentrations of ethanol (10-30 mM) didn't change pH_i recovery (Fig. 6A), while higher concentrations of ethanol (100-300 mM) increased pH_i recovery. Note that adding the highest concentration of ethanol (1000 mM) dramatically inhibited pH_i recovery (~50%) under CO_2/HCO_3^- -buffered condition (Fig. 6D). Histogram in Fig. 6E shows the normalization of pH_i recovery rate of acid extrusion averaged in 6 experiments similar to that shown in Fig. 6A, 6B, 6C and 6D, respectively. Our present results provided direct evidence that higher concentrations of ethanol showed opposite effects on activity of NHE1 and NBC, i.e. decreasing and increasing, respectively, in OEC-M1 cells.

The effect of ethanol on protein expression of NHE and NBC

In order to check the impact of ethanol on the protein expression of NHE and NBC, the characterization of isoform of NHE and NBC was first examined. Our experiments showed that the isoforms of NHE and NBC are including NHE1 (SLC9A1), NBCe1 (SLC4A4), NBCn1 (SLC4A7) and NDCBE (SLC4A8), as shown in the left part of Fig. 7A. As shown in right parts of Fig. 7A, chronic exposure of ethanol with various concentrations for 24 hours caused a significantly decreasing effect on the protein expression of NHE1, NBCn1 and NDCBE, while a dramatically increasing effect on that of NBCe1 at concentration ≥ 300 mM. These results suggested that the decreasing of resting pH_i by ethanol in HEPES-buffered was due to the down-regulation of protein expression of NHE-1 and/or inhibition of functional activity. Moreover, note that 1000 mM of ethanol caused a diverse effect on the protein expression of NBCe1 (i.e. increasing to 2.82 fold) and that of NBCn1 and NDCBE (decreasing to 0.43 fold and 0.46 fold, respectively).

Discussion

The resting pH_i and new steady-state pH_i in OEC-M1 cells

Many cellular functions are sensitive to changes of pH_i . Recently, dysregulated pH is evolving as a hallmark of cancer tissues, i.e. high pH_i and low pH_o that enables cancer

Fig. 5. Effect of acute treatment of ethanol (10 to 1000 mM) on pH_i recovery after induced intracellular acidification in OEC-M1 cells superfused with HEPES-buffered solution. (A, B, C, D) The above bars show the buffer system used, and the bars above or below the trace show the acute application of ethanol and NH_4Cl . The first part of trace A, B, C, D shows a typical pH_i recovery form an induced intracellular acidification in HEPES-buffered solution as control. The other part shows the pH_i recovery slope after application of ethanol in different concentration. (E). Histogram shows the normalization of pH_i recovery rate of acid extrusion averaged in 6 experiments similar to that shown in A, B, C and D, respectively. The pH_i recovery rate was measured at $pH_i = 6.90 \pm 0.04$, ** $p < 0.01$ verse to control.

Fig. 6. Effect of acute treatment of ethanol (10 to 1000 mM) on pH_i recovery after induced intracellular acidification in OEC-M1 cells superfused with CO_2/HCO_3^- -buffered solution. (A, B, C, D) The above bars show the buffer system used, and the bars above or below the trace show the acute application of ethanol and NH_4Cl . The first part of trace A, B, C, D shows a typical pH_i recovery form an induced intracellular acidification in CO_2/HCO_3^- -buffered solution as control. The other part shows the pH_i recovery slope after application of alcohol in different concentration. (E). Histogram shows the normalization of pH_i recovery rate of acid extrusion averaged in 6 experiments similar to that shown in A, B, C, D, respectively. The pH_i recovery rate was measured at $pH_i = 6.90 \pm 0.04$, ** $p < 0.01$ verse to control.

Fig. 7. Effect of chronic treatment of ethanol (24 hr) on isoforms of acid extruders in OEC-M1 cells. (A) The western blot results show the changes of isoforms of NHE1, NBCe1, NBCn1, NDCBE and GAPDH expression (marked at the most left) after 24 hr treatment with ethanol (10 to 1000 mM; marked at the top) in OEC-M1 cells. (B, C, D, E) The histogram show the relative expression ratio of NHE1, NBCe1, NBCn1 and NDCBE to GAPDH that were averaged for 4 to 6 experiments similar to those shown in A, respectively. * $p < 0.05$ or ** $p < 0.01$ verse to control.

progression by promoting proliferation, the evasion of apoptosis, metabolic adaptation, migration and invasion [46]. Indeed, as expectation, we have found that the value of original resting pH_i of OEC-M1 is quite alkaline (7.49 and 7.35) under HEPES-buffered Tyrode and HCO_3^- -buffered Tyrode solution, respectively (Fig. 2). Such alkaline pH_i value was shifted to a new, steady-state but acidic pH_i value (7.0 and 7.03) after a few intracellular acid/base impacts of NH_4Cl pre-pulse, under HEPES-buffered Tyrode and HCO_3^- -buffered Tyrode solution, respectively (Fig. 2). Such acidic steady-state pH_i value is not only significantly lower than that of highly proliferated cells, i.e. embryonic stem cells and cancer cells (~7.4) [43, 46, 47], but also lower than that of normal animal and human mature cells (~7.2) [23-25, 28, 48]. As steady-state pH_i is a balance through the combined operation of passive intracellular buffering power and active transmembrane pH_i transporters [24-26], therefore, the shift of ~0.4 pH_i units from alkaline to acidic means a dramatic kinetic change in the pH_i regulating mechanism. Whether such shift from alkaline to acidic after the intracellular acid/base impacts implies the typical and flexible character of pH_i regulating mechanism of OEC-M1 cells awaits further study. For example, the acidification after acid/base impact(s) in OEC-M1 is probably due to the limitation of cytosolic buffering capacity, and easily be saturated. Alternatively, the activity of the acid loaders, such as AE and CHE, is another factor to decide the steady-state pH_i . Therefore, the characterization of active acid loaders

and passive cytosolic buffering capacity under different pathophysiological conditions wait to be examined in OEC-M1 cells. Indeed, oral cavity is subject to be exposed to various pH conditions. For example, in the inflammatory tissue, a lower value of pH_e (i.e. tissue acidosis) is frequently observed [49]. Also, during the process of abscess development, the oral cavity is exposed to lactic acid, the predominant by-product of caries-associated bacteria, and exhibits a low level of pH (~ 4.9) [50]. On the other hand, mineral trioxide aggregate (MTA), a potential root-end filling material, produces an initial pH_e of 10.2, which rises to 12.5 at 3 hours after mixing [51]. Therefore, the special character of shifting pH_i from alkaline to acidic after intracellular acid/base impacts might provide another new maneuver on developing related medicines to cure OEC-M1 cells.

Potential role of inhibitors/activators of isoforms of NHE and NBC in clinical setting

In mammalian cells, in addition to the ubiquitous two acid extruders, i.e. NHE and NBC, the vacuolar- H^+ -ATPase (V-ATPase) utilizes ATP to pump protons to extracellular environment. V-ATPase has been reported as an important pH_i regulator in many different types of cancers and positively correlated to cancer invasion and metastasis [52, 53]. In our present study, we provide straightforward and convincing *functional* evidence that NHE and NBC are functionally responsible for acid extrusion, following induced acidosis in OEC-M1 cells, as shown in Figs. 2-3. In other words, surprisingly, the V-ATPase has not been found to play a role in the functional pH_i recovery following induced intracellular acidification in OEC-M1 cells. This is a very unique character of OEC-M1 cells, and can provide a new strategy while apply possible treatment on oral cancer in clinic.

Furthermore, by using Western blot technique, apart from the housekeeping NHE1, we have provided the directly molecular evidence of existence of 3 NBC isoforms, i.e. NBCn1, NBCe1 and NDCBE, in the OEC-M1 cells (Fig. 7A). The result of 3 isoforms of NBC in OEC-M1 is different to that found in vascular cells of mouse, rat and human models. The difference is probably due to differences in species/organs. Moreover, some isoforms of NBC might play a vital role on the development of oral carcinogenesis. Indeed, it has been found that the enhancement of NBC activity is the main reason for an alkaline shift (~ 0.3 units of magnitude) in steady-state intracellular pH of human primary breast carcinomas compared to normal breast tissue [54]. Furthermore, upregulated NBCn1 during human breast carcinogenesis contributes to the characteristic acid distribution within human breast carcinomas [54]. Therefore, our present study has provided a new strategy of potential role of inhibitors/activators of isoforms of NHE and NBC in clinical setting. Moreover, further study on pinpointing the specific isoform(s) expression for OEC-M1/oral cancer tissues development and progression waits to be examined. For example, in the future, normal and cancer tissues from clinical patients can be collected to further identify the expression of isoforms of NHE and NBC by IHC. Moreover, the effects of knock down or overexpression of specific isoform will be checked to observe its effects on cellular growth, apoptosis, migration, invasion and metabolism of cancer cells.

The acute and chronic effect of ethanol on intracellular pH regulating mechanism

Our present study has, for the first time, provided straightforward evidence about acute (Fig. 3, 4 and 6) and chronic (i.e. 24 hr; Figs. 4 and 7) effects of various concentrations (i.e. 10-1000 mM) of ethanol on pH_i regulating mechanism. Note that the changes in pH_i upon a 24 h ethanol treatment are about up to five or even six times higher (Fig. 4F) than those observed immediately after alcohol exposure (Fig. 4E). The ethanol-induced changes on expression of acid loaders, i.e. NHE and NBC isoforms (Fig. 7), might be the reason to account for the higher extend on pH_i changes upon 24 h ethanol treatment. Our present study shows that ethanol concentration-dependently decreased NHE1 activity, while showed a concentration-dependent but biphasic effect, on NBCs activity, increase following decrease (Fig. 6). As NHE and NBC contributed together for acid extruding in CO_2/HCO_3^- -buffered solution (see Fig. 3), therefore, considering the ethanol induced inhibition on NHE activity, the slightly ethanol induced increases on pH_i recovery shown in Fig. 6 represent a larger scale of ethanol-induced

changes of NBC activity. For example, 1000 mM ethanol induced changes is smaller (~50%) under $\text{CO}_2/\text{HCO}_3^-$ -buffered condition than that in HEPES (~86%) (Fig. 6D).

Moreover, chronic exposure to higher concentrations of ethanol significantly inhibited the expression of NHE1, NBCn1 and NDCBE, while up-regulated NBCe1 (Fig. 7). Because an acidic pH condition induce growth arrest or cell death [55], thus change of any one of these H^+ -equivalent transporters may play a significant role in the progression of carcinogenesis of oral cancer. Recently, Kim et al. have reported that ethanol (10~30 mM) induces cell proliferation of HepG2 cells, activates NHE1 and anti-apoptotic Bcl-2 through enhancing the activities of ERK1 and p90rsk, and the maximum effects are shown at the concentration of 20 mM both [22]. Also, in our present study, the similar trend of ethanol (10~30 mM) on NHE1 expression has been found (Fig. 7). Indeed, p90rsk, hyperactive NHE1 even at the resting pH and the resulting cellular alkalinization have been reported to be directly related to uncontrolled proliferation in malignancy cells [22]. Thus, p90rsk that regulates cellular proliferation, as well as NHE1, may be an important molecule for therapeutic targeting in ethanol-induced carcinoma progression. Hence, our present study has provided a promising target for new treatment strategies to prevent or cure alcohol consumption-associated oral diseases and cancers.

Conclusion

In the present study, we have, for the first time, provided straightforward, functional and molecular evidence of coexistence of NHE1, NBCn1, NDCBE and NBCe1 for acid extruding mechanism in the OEC-M1 cells. Moreover, we have found that ethanol affects pH_i value by changing function and expression of NHE1 and NBC isoforms.

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Disclosure Statement

The authors declare that there is no Disclosure Statement regarding the publication of this paper.

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