J Med Sci 2015;35(5):208-217
DOI: 10.4103/1011-4564.167775

ORIGINAL ARTICLE

Physiological and Pharmacological Characterization of Transmembrane Acid Extruders in Cultured Human Umbilical Artery Smooth Muscle Cells

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Background: Intracellular pH (pH_i) is a pivotal factor for cellular functions and homeostasis. Apart from passive intracellular buffering capacity, active transmembrane transporters responsible for kinetic changes of pH_i impacts. Acid extrusion transporters such as Na^+/H^+ exchanger (NHE) and Na^+/HCO_3^- cotransporter (NBC) have been found to be activated when cells are in an acidic condition in different cell types. However, such far, the pH_i regulators have not been characterized in human umbilical artery smooth muscle cells (HUASMCs). **Materials and Methods:** We, therefore, investigated the mechanism of pH_i recovery from intracellular acidosis, induced by NH_4Cl-prepulse, using pH-sensitive fluorescence dye: 2',7'-bis(2-carboxethyl)-5(6)-carboxy-fluorescein in HUASMCs. Cultured HUASMCs were derived from the segments of the human umbilical artery that were obtained from women undergoing children delivery. **Results:** The resting pH_i is 7.23 ± 0.03 when cells in HEPES (nominally HCO_3^--free) buffered solution. The resting pH_i is higher as 7.27 ± 0.03 when cells in CO_2/HCO_3^--buffered solution. In HEPES-buffered solution, a pH_i recovery following induced intracellular acidosis could be inhibited completely by 30 µM HOE 694 (a specific NHE inhibitor) or by removing $[Na^+]_o$. In 5% CO_2/HCO_3^--buffered solution, 30 µM HOE 694 slowed the pH_i recovery from the induced intracellular acidosis only. On the contrary, HOE 694 adding together with 0.2 mM 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (a specific NBC inhibitor) or removal of $[Na^+]_o$ entirely blocked the acid extrusion. By using Western blot technique, we demonstrated that four different isoforms of NBC, that is, SLC4A8 (NBCBE), SLC4A7 (NBCn1), SLC4A5 (NBCe2) and SLC4A4 (NBCe1), co-exist in the HUASMCs. **Conclusions:** We demonstrate, for the 1st time, that apart from the housekeeping NHE1, another Na^+ couple HCO_3^--transporter, that is, NBC, functionally coexists to responsible for acid-extruding in HUASMCs.

Key words: Human umbilical artery smooth muscle cells, intracellular pH, Na^+-H^+ exchanger, Na^+-HCO_3^-—cotransporter, fluorescence dye-2',7'-bis(2-carboxethyl)-5(6)-carboxy-fluorescein–acetoxymethyl

INTRODUCTION

The human umbilical artery is a paired artery which transports deoxygenated blood from the fetus back to the placenta. The paired umbilical arteries connect with each other inside the placenta and subsequently branch into chorionic arteries or intraplacental fetal arteries.[1] In clinic, Doppler sonography of the umbilical artery allows very well to identify the endangered small-for-gestational-age foetuses on the one hand and to exclude foetal jeopardy with a normal pulsatility index on the other.[2] Different studies showed that increased proliferation and migration of vascular smooth muscle cells (VSMCs), which caused by influences of stimulatory cytokines and disturbances of intracellular pH (pH_i), are critical events in the pathophysiology of atherosclerosis and restenosis.[3-5]

Cellular homeostasis and functions are affected closely by a change of pH_i. For example, many cellular mechanisms including enzyme catalysts,[6] permeability of ion channels,[7] cell volume,[8] cell differentiation, growth, and apoptosis[9-12] have been demonstrated to sensitive to changes in pH_i. Same as in other cell types, disturbances on pH_i also play a critical role in contributing to the metabolic autoregulation of artery tone in VSMCs and participate in adjusting local blood flow to the metabolic demand.[13] Moreover, we have previously demonstrated that lipopolysaccharide, one of

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Received: July 02, 2015; Revised: August 03, 2015; Accepted: August 17, 2015
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the main inflammatory mediators, also increases cellular growth through by changing pH_i and activities of acid extruder(s) in a dose and time-dependent manner[12] in renal artery VSMCs. In mammalian cells, the pH_i is kept within a narrow range between 7.0 and 7.2 through the combined mechanism of active transmembrane transporters and passive intracellular buffering power.[12,14] The active membrane transporters can be divided into two main categories: Acid-extruding carriers and acid-loading carriers. Acid-extruding carriers such as an Na^+/H^+ exchanger (NHE) and Na^+/HCO_3^- cotransporter (NBC) can be activated when cells are in an acidic condition (pH_i <7.0).[9,12,15,16] On the contrary, when a cell is in an alkalized direction (pH_i >7.3), the acid loaders such as Cl^-/OH^- exchanger (CHE) and Cl^-/HCO_3^- exchanger (AE) will be triggered.[14,17]

It has been found that net acid extrusion from VSMCs in rat and mice mesenteric small arteries is mediated by the NHE1 and the Na^+-HCO_3^--cotransporter NBC.[9,18-20] Similar results have been demonstrated in our previous study in VSMCs of human renal artery.[12] NHE mediates the electroneutral exchange of extracellular Na^+ for intracellular H^+.[9,12,16,21] In HEPES-buffered media that is, HCO_3^--free condition, pH_i recovery following intracellular acidosis can be inhibited by the removal of extracellular Na^+ or by the addition of amiloride or Hoe 694 (3-methylsulfonyl-4-piperidinobenzoyl, guanidine hydrochloride), a compound that inhibits NHE activity through its high affinity and selectivity.[12,22] Under physiological-like condition, that is, in CO_2/HCO_3^- buffered Tyrode solution, Na^+-HCO_3^--dependent transporter, that is, NBC, has been found to be another acid extruder to accompany NHE to achieve acid equivalent extrusion following intracellular acidosis in many different cell types.[12,15,16,19] In the human atrial and ventricular myocyte, NBC accounts for about 30-40% of the total acid efflux[15,16] and is largely 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) sensitive, and it is amiloride- and HOE 694-resistant.[12,15,16,19] It is also inhibited by removal of external Na^+, whereas no inhibition of acid efflux following an intracellular acidosis has been found by removal of external K^+, decreasing external Ca^{2+} or, in mammalian cardiac cells, by removal of Cl^-.[23]

The disturbances on pH_i and activity of acid extruder(s) have been reported to be implicated in vascular atherosclerosis and hypertension, further underlining the regulation for normal cardiovascular function and pathological development of different diseases.[9,18,24,25] Therefore, more experimental evidence is required, however, to evaluate the cell biological effects of acid-base transport in vascular cells, especially in human tissues/cells. However, thus far there are not any related reports about active acid-extruding transports in human umbilical artery smooth muscle cells (HUASMCs). Therefore, the aim of this study is to characterize the functional acid extruders in HUASMCs, which were isolated/cultured from discarded human umbilical arteries obtained from pregnant women during the delivery procedure.

MATERIALS AND METHODS

Human umbilical artery smooth muscle cells

With the approval of the Institutional Review Board of Tri-Service General Hospital, National Defense Medical Center (TSGHIRB No. 1-101-05-065) and with prior written informed consent of pregnant women, umbilical arteries were collected from delivery-leftover umbilical cords during child delivery procedure at Tri-Service General Hospital, Taipei, Taiwan. Primary HUASMCs were isolated by the *ex-vivo* culture explant technique which has been described in detail in Fletcher *et al*.[26] In brief, the explanted cells were subsequently cultured in HAM's F12K medium containing 10% fetal bovine serum (GIBCO, Grand Island, NY, USA.), 100 U/ml penicillin, 100 mg/ml streptomycin, and 200 mM L-glutamate in a humidified incubator (at 37°C and 5% CO_2). The primary HUASMCs were used for experiments between 3 and 8 passages in this study. The cells were then superfused with oxygenated Tyrode solution, which was either 100% O_2 for HEPES-buffered Tyrode solution or 5% CO_2/95% O_2 for bicarbonate-containing Tyrode solution, at 37ºC, pH 7.40 ± 0.02 for experiments.

Immunocytochemistry

Immunocytochemistry method has been described in detail in our previous reports.[12] Briefly, HUASMCs were cultured on a 6-well plate (Macalaster Bicknell, New Haven, CT) for 2–3 days. Cells were subsequently washed 3 times in phosphate-buffered saline (PBS; Life Technologies, Grand Island, NY, USA). After washing, cells were fixed in 4% paraformaldehyde for 30 min at room temperature, then washed twice in PBS (Sigma-Aldrich, St. Louis, MO, USA) and blocked and permeabilized in PBS containing 0.3% triton and 5% normal goat serum for 60 min, and finally washed in PBS and incubated overnight with primary antibodies (e.g. smooth muscle alpha actin antibody; Pierce Biotechnology, Rockford, IL, USA) at 4°C. Before labeling with secondary antibodies (e.g. Goat anti-mouse IgG [H + L] cross adsorbed secondary antibody, DyLight™ 488 conjugate; Pierce Biotechnology, Rockford, IL, USA) for 1 h in the dark, HUASMCs were washed 4 times in PBS. After labeling, cells were washed with PBS and incubated with 4′,6-diamidino-2-phenylindole (DAPI) (1 µg/ml) (eBioscience, Forest City, CA, USA) for 40 min. Cells were washed twice more and mounted onto slides with Gel/Mount (Biomedia Corp., Forest City, CA, USA). Images were acquired with an OLYMPUS 200M (Japan) microscope system.

Western blot analysis of the SLC4 family of HCO_3^- transporters

Cell lysates were prepared using a 300 µl/well of six-well plates of RIPA/NP-40 lysis buffer (5 mM Tris pH 7.4, 30 mM NaCl, 1 mM PMSF, 1 µg/ml aprotinin). A 50 µg of total protein per sample was then subjected to 10% polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane and blocked for 2 h with 5% fat-free milk in Tris-buffered saline/0.1% Tween 20 (TBST). After three washes with TBST, the membranes were exposed to a 1:1000 dilution of a mouse antihuman antibody of SLC4 family of HCO_3^- transporters: NBCe1 (Millipore, Long Beach, CA, USA), NBCe2 (Abgent, San Diego, CA, USA), NBCn1 (Abgent, San Diego, CA, USA), and NDCBE (GeneTex, San Antonio, TX) at 4°C overnight. Following three washes with TBST, the membrane was exposed to a 1:15,000 dilution of goat anti-mouse IgG-HRP conjugate (Millipore, Long Beach, CA, USA) for 1 h and repeatedly washed with TBST. Chemiluminescence was detected using the SuperSignal Substrate (PIERCE, Rockford, IL, USA). Loading control was assessed by the detection of β-actin. SLC4 family of HCO_3^- transporters: SLC4A4 (NBCe1), SLC4A5 (NBCe2), SLC4A7 (NBCn1), SLC4A8 (NDCBE), and β-actin protein intensity were measured using the Analytical Imaging Station software version 2 (National institutes of health, Bethesda, MD, USA). Briefly, the specificity of the NBC antibodies used in this study are shown as following: NBCe1 (SLC4A4) is recognized by a major band of approximately 130 kDa and a major band of approximately 160 kDa in salamander kidney; NBCe2 (SLC4A5) is generated from rabbits immunized with a KLH conjugated synthetic peptide between 1073 and 1102 amino acids from the C-terminal region; NBCn1 (SLC4A7) is generated from rabbits immunized with a KLH conjugated synthetic peptide between 1193 and 1222 amino acids from the c-terminal region; NDCBE (SLC4A8) is generated from rabbits and recognized by a major band of 123 kDa.

Measurement and calibration of the intracellular pH

Measurement of the pH_i has been described in detail in our previous reports.[15,16] In brief, the pH_i in the HUASMCS was examined using the pH-sensitive, dual excitation dual-emission fluorescent dye, 2',7'-bis(2-carboxethyl)-5(6)-carboxy-fluorescein–acetoxymethyl (BCECF-AM) (molecular probes). The cells were loaded with BCECF-AM (5 µM) by incubating them for 30 min at room temperature and exciting them alternately with 490 and 440 nm wavelength light. The BCECF fluorescence emission ratio of the 510 nm emission at 490 nm and 440 nm excitation (490/440) was calibrated using the K^+-nigericin method.[20] Briefly, this method consisted of exposing a BCECF-loaded cell to the six nigericin calibration solutions (pH_o of 5.5, 6.5, 7.0, 7.5, 8.5, and 9.5) that clamps pH_i to the value of pH_o of the calibration solution. Figure 1a showed the emission ratio changes seen on perfusing human umbilical smooth muscle cells with calibration solutions with different 6 pH values (5.5 ~ 9.5) in the presence of 10 µM nigericin. The emitted ratio 510 nm emission at 490 nm and 440 nm excitations (R; $R = F_{490}/F_{440}$) was increased as the pH value of the superfusing solution was increased. R_{max} and R_{min} are, respectively, the maximum and minimum ratio values for the data curve. The fluorescence of BCECF at 490 nm to 440 nm is a function of pH_i and the overall sampling rate in the experiment was 0.5 Hz for the recorded fluorescent ratio (490 nm/440 nm). Using the linear regression fit of the data (shown in the Figure 1b) obtained from six calibration experiments similar to that shown in Figure 1a, the mean apparent dissociation constant (pKa) at 37°C was found to be 7.43 very close to the value determined by our previous study of the human heart, as well as the value determined by other investigators.[12,13,15] The following equation[27] was used to convert the fluorescent ratio into pH_i:

$$pH_i = pK_a + \log[(R_{max} - R)/(R - R_{min})] + \log(F_{440min}/F_{440max})$$

where R is the ratio of the 510 nm fluorescence at 440 nm and 490 nm excitation, R_{max} and R_{min} are, respectively, the maximum and minimum ratio values from the data curve and the pK_a (−log of dissociation constant) is 7.22. F_{490min}/F_{440min}

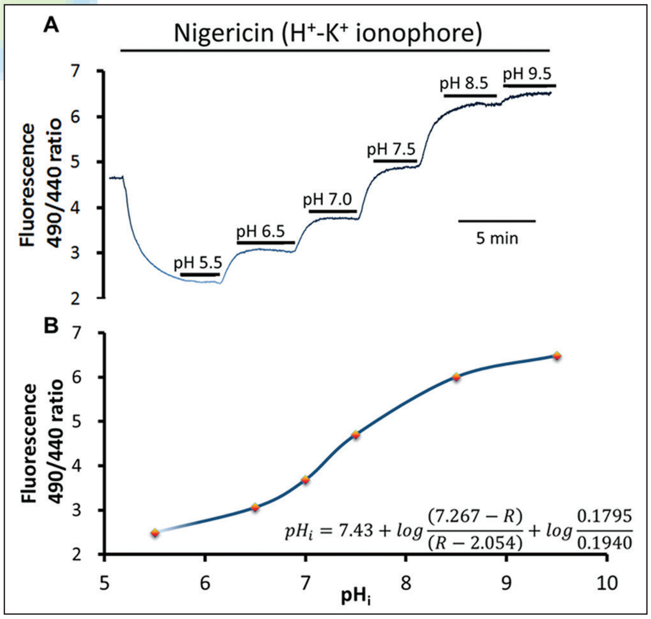

Figure 1: *In situ* calibration of intracellular pH in human umbilical artery smooth muscle cells. (a and b) *In situ* intracellular pH calibration curve in human umbilical artery smooth muscle cells. (a) The trace displays the 2',7'-bis(2-carboxethyl)-5(6)-carboxy-fluorescein fluorescence (510 nm emission at 440 nm and 490 nm excitations) in human umbilical artery smooth muscle cells (please see Materials and Methods for details). (b) The curve displays data combined from six similar experiments shown in a

and F_{490max}/F_{440max} is the ratio of fluorescence measured at 440 nm of R_{min} and R_{max}, respectively. In our study, the *in situ* calibration equation of HUASMCs is:

$pH_i = 7.43 + \log (7.267 - R/R - 2.054) + \log (0.179/0.194)$; where R is the emission ratio at 510 nm after excitation at 490 nm and 440 nm ($R = F_{490}/F_{440}$).

Experimental alteration of intracellular pH-weak acid/base prepulse technique

NH_4Cl prepulse technique, has been described in detail in our previous reports, was used in the present work to induce intracellular acidosis.[12,22] Briefly, NH_4Cl prepulses were achieved with (~10 min) extracellular exposures to 20 mM NH_4Cl and its characteristic of incomplete dissociation. Although both the charged and uncharged species of a weak base exist at the same time in solution, the uncharged species is lipid soluble and, therefore, able to permeate the lipid bilayer of the cell membrane very quickly. In contrast, the charged species permeates relatively slowly, through various membrane protein routes, such as K^+ channel. For example, the details of NH_4Cl prepulsing procedures can be explained in terms of four phases as shown in Figure 2a: Rapid entry (see phase 1 in left part of Figure 2a), slow recovery (see phase 2 in left part of Figure 2a), rapid exit (see phase 3 in left part of Figure 2a), and pH_i regulation (see phase 4 in left part of Figure 2a) that the sudden acidosis activates transmembrane acid extruder(s). Throughout the whole experiment, the change of pH_i induced by the tested drug was compared at the same testing pH_i after treating the drug, unless otherwise stated. The autofluorescence and background fluorescence were small (<5%) and haves been ignored in our study.

Chemicals and solutions

Standard HEPES-buffered tyrode solution (air equilibrated) contained (mM)

NaCl, 140; KCl, 4.5; $MgCl_2$, 1; $CaCl_2$ 2.5; glucose, 11; HEPES, 20; pH adjusted to 7.4 with 4N NaOH. Unless otherwise stated, pH adjustments of all HEPES-buffered solutions were performed at 37°C (these adjustments included those where ionic-substitutions were made, see below). Standard bicarbonate-buffered Tyrode solution (equilibrated with 5% CO_2/23 mM HCO_3^-) was the same as above, except that the sodium chloride concentration was reduced to 117 mM, and 23 mM $NaHCO_3$ was added instead of the HEPES (pH 7.40 at 37°C).

Ion-substituted solutions

In a Na^+-free, HEPES-buffered Tyrode solution, NaCl was replaced with 140 mM N-methyl-D-glucamine (NMDG)-Cl, and the pH was adjusted to 7.4 with HCl. The Na^+-free CO_2/HCO_3^--buffered Tyrode solution was the same as the CO_2/HCO_3^--buffered Tyrode solution above, except that 117 mM NaCl and 23 mM HCO_3^- were replaced by 117 mM NMDG and 23 mM NMDG, respectively, and pH was adjusted to 7.4 at 37°C with HCl under the condition of saturating with 5% CO_2/95% O_2. When 20 mM ammonium chloride was used, it was added directly as a solid to solution without osmotic compensation. DIDS and Hoe 694 (3-methylsulfonyl-4-piperidinobenzoyl, guanidine hydrochloride) were added, as solids, to solutions shortly before use.

Nigericin calibration solutions contained (mM)

KCl, 140; $MgCl_2$, 1; 10 µM nigericin; buffered with one of the following organic buffers: 20 mM 2-(N-morpholino) ethanesulfonic acid (MES, pH 5.5), 20 mM HEPES (pH 7.5) or 20 mM 3-(cyclohexylamino)-2-hydroxy-1-propane-sulphonic

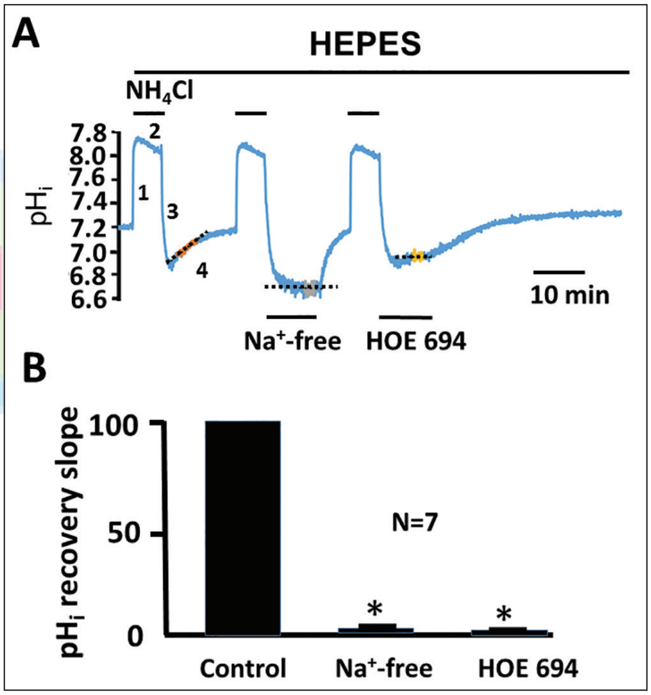

Figure 2: Effect of Na^+-free and 30 µM HOE 694 on pH_i recovery from induced acidosis (evidence of Na^+-H^+ exchanger) in human umbilical artery smooth muscle cells superfused with HEPES-buffered Tyrode solution. (a) The top bar shows buffer system used in the superfusate. The periods of the addition of NH_4Cl and tested drugs (Na^+-free solution, and 30 µM HOE 694, a NHE exchanger inhibitor, respectively) are indicated with bars above or below the trace. The left part of traces A displays a representative recovery of pH_i-recovery from an intracellular acidosis induced by a 7 min NH_4Cl (20 mM) prepulse in HEPES-buffered Tyrode solution (pH_o = 7.4, 37°C) in human umbilical artery smooth muscle cells. For details of the mechanism of the prepulse technique (please see the Materials and Methods section). The right parts of traces A represents experiments showing the effect of Na^+-free and 30 µM HOE 694 on pH_i recovery, respectively, in human umbilical artery smooth muscle cells. (b) Histograms, showing the pH_i recovery slope of acid extrusion after NH_4Cl-induced intracellular acidosis averaged for seven experiments (measured at $pH_i = 6.82 \pm 0.04$) similar to those shown in a. $^*P < 0.01$ versus control

acid (CAPSO, pH 9.5), and were adjusted (37°C) to the correct pH with 4N NaOH.

Hoe 694 was kindly provided by Hoechst Aktiengesellschaft (Germany). All other chemicals were from Sigma (UK) and Merck (UK).

Statistics

All data are expressed as the mean ± the standard error of the mean for *n* preparations. Statistical analysis was performed using one-way analysis of variance with Scheffe's posterior comparison. A *P* value smaller than 0.05 was regarded as significant.

RESULTS

The identification of isolated and cultured human umbilical artery smooth muscle cells derived from tissue

Our present study successfully isolated HUASMCs from human umbilical artery tissue using the so-called explant method. The HUASMCs were significantly migrated out from artery tissue at the 12th day, as shown in Figure 3a. Moreover, we used immunocytochemistry technique to identify and examine the purity of HUASMCs as shown in Figure 3b-d. In brief, HUASMCs were stained with α-SM-actin, the specific monoclonal antibody which serves as a smooth muscle differentiation marker [Figure 3b; green color] and DAPI, the nuclei counterstained marker [Figure 3c; blue color]. The cell pattern of Figure 3d, merging from Figure 3b and c, is nearly the same as that of Figure 3c and d. This clearly indicates that the cells are HUASMCs exclusively. Therefore, we have successfully derived a single HUASMCS from the tissue of human umbilical artery through explants technique.

The functional existence of A Na^+-H^+ exchanger

To check whether an active acid-extruding mechanism exists in the HUASMCs, we firstly performed the experiments in HEPES-buffered Tyrode's solution, that is, nominally free of CO_2/HCO_3^-. The steady-state pH_i value for the HUASMCs was found to be 7.23 ± 0.03 (*n* = 16) in HEPES-buffered Tyrode solution. The value of 7.23 in HUASMCs is similar to that reported in different mammalian cells, either animal or human models.[12-15]

As shown in the left part of Figure 2a, the pH_i recovered completely from intracellular acidosis following an NH_4Cl prepulse technique. This result demonstrated that there is an active mechanism of acid-extruding in the HUASMCs. Removing extracellular Na^+ from superfusate completely stopped the pH_i recovery from the induced intracellular acidosis, as shown in the middle part of Figure 2a. The first and second columns of the histogram [Figure 2b] shows the mean pH_i recovery slope (measured at pH_i = 6.82 ± 0.04) before and after Na^+ removal for seven experiments that are similar to those whose results are shown in Figure 2a. This clearly proves that, under HEPES, that is, nominally CO_2/HCO_3^--free, buffered solution, there is a Na^+-dependent, but CO_2/HCO_3^--independent, acid-extruding mechanism involved in the pH_i recovery following induced intracellular acidosis in the HUASMCs. To further examine if this Na^+-dependent acid extruder is the NHE, we added HOE 694, a specific NHE inhibitor, in the superfusate. As shown in the right part of Figure 2a, HOE 694 (30 μM) entirely blocked the pH_i recovery following the induced intracellular acidosis. The pH_i recovery rate (measured at pH_i = 6.82 ± 0.04) of seven similar experiments, like the result shown in Figure 2a, were pooled in the first (before HOE 694 addition) and third columns (after HOE 694 addition) of Figure 2b. Therefore, the present results provided clear physiological and pharmacological evidence that NHE functionally exists in HUASMCs.

The functional existence of A Na^+-HCO_3^- cotransporter

The steady-state pH_i value was found to be 7.27 ± 0.04 (*n* = 19) in CO_2/HCO_3^- buffered Tyrode solution in the HUASMCs. Note that the steady-state pH_i value in CO_2/HCO_3^- buffered Tyrode solution is slightly higher than that in a HEPES-buffered Tyrode solution, which is different to

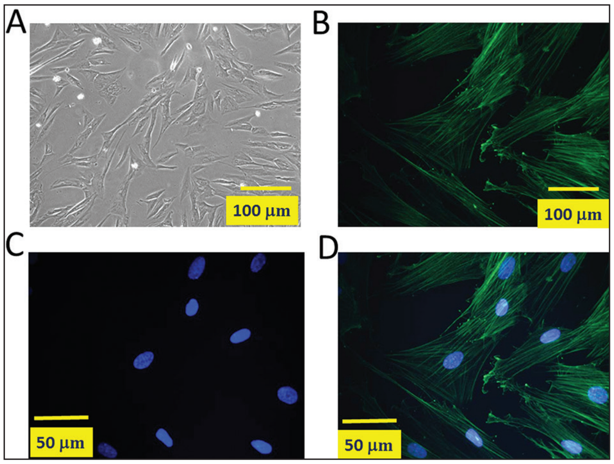

Figure 3: Purity and identification of cultured human umbilical arterial smooth muscle cells. (a) Phase-contrast micrograph of human umbilical arterial smooth muscle cells, isolated from human umbilical cord using explant culture technique. (b-d) Micrographs of immunofluorescence of human umbilical arterial smooth muscle cells. (b) Human umbilical arterial smooth muscle cells stained for the anti-smooth muscle alpha actin (green). (c) Human umbilical arterial smooth muscle cells counterstained with 4′,6-diamidino-2-phenylindole for nuclei (blue). (d) A merge micrograph that combines micrograph (b) and micrograph (c) (10 × 40)

that reported previously in different cell types including both animal and human models.[12-15]

The left part of the traces shown in Figures 4a, 5a, and 6a illustrate the pH_i recovery from an acid load induced in CO_2/HCO_3^- conditions. These conditions demonstrate the physical mechanism of acid-extruding following NH_4Cl-induced intracellular acidosis. To test if the HCO_3^--dependent mechanism is also Na^+-dependent, Na^+ was removed from the solutions in the subsequent experiments, as shown in the right panel of Figure 4a. Removing Na^+ from the 5% CO_2/HCO_3^- Tyrode solution completely blocked pH_i recovery following the induced intracellular acidosis, as shown in the right part of Figure 4a. The histogram in Figure 4b shows the pH_i recovery rate, which was estimated at pH_i 6.77 ± 0.07 after averaging for five experiments in HUASMCs. The present data suggests that this HCO_3^--dependent acid-extrusion mechanism is also Na^+-dependent. To further check whether this pH_i recovery HCO_3^--dependent acid-extrusion mechanism is purely NHE that is, found in HEPES-Tyrode superfusate above [i.e., Figure 2], 30 μM HOE 694 has been added. The pH_i recovery was partially inhibited, as expected, in the presence of HOE 694, as shown in the right part of Figure 5a. The second column of the histogram in Figure 5b shows the pH_i recovery slope following induced intracellular acidosis for five experiments (estimated at pH_i 6.81 ± 0.07) in HUASMCs similar to the result shown in Figure 5a. The significant difference between the first (control) and the second column (in addition of HOE 694) indicated that, apart from NHE, there is another HCO_3^--dependent acid-extrusion mechanism involved in the pH_i recovery in 5% CO_2/HCO_3^- Tyrode solution.

It has been demonstrated that the stilbene drug DIDS (0.2 mM) inhibits NBC effectively;[9,12,15,22] we therefore further tested if 0.2 mM DIDS could inhibit this HCO_3^--dependent, but HOE 694-independent, acid-extruding mechanism in the HUASMCs. As shown in the middle part of Figure 5a, the pH_i recovery was partially blocked in the presence of DIDS, similar to that of adding HOE 694. The second column of the histogram in Figure 5b shows the pH_i recovery slope after induced intracellular acidosis for seven similar experiments (estimated at $pH_i = 6.81 \pm 0.07$). Finally, a combination of 30 μM HOE 694 and 0.2 mM DIDS was tested and it was found to completely inhibit the pH_i recovery following intracellular acidosis in the 5% CO_2/HCO_3^- Tyrode solution, as shown in the right part of Figure 6a. The third histogram in Figure 5b shows the pH_i recovery rate, averaged for seven experiments (estimated at pH_i 6.84 ± 0.07). The present data provides pharmacological and physiological evidence that, apart from NHE, NBC functionally responsible for Na^+- and HCO_3^--dependent acid-extrusion mechanisms.

The isoforms of Na^+-HCO_3^- cotransporter (NBCn1, NBCe1, NBCe2 and NDCBE)

In order to further identify the isoform(s) of the functional NBC observed previously, the Western blot technique (see Materials and Methods for details) was used to test the response for the SLC4 family of HCO_3^- transporters: SLC4A4 (NBCe1), SLC4A5 (NBCe2), SLC4A7 (NBCn1), and SLC4A8 (NDCBE), respectively, in cultured HUASMCs. It is significant that four Na^+-coupled HCO_3^- co-transporters: NBCn1 (SLC4A7; electroneutral), NBCe1 (SLC4A4; electrogenic) and NBCe2 (SLC4A5), and NDCBE (SLC4A8) co-exist in the cultured HUASMCs, as shown in Figure 6. This is different to the case for animal models, because only NBCn1 (SLC4A7) mediates the Na^+-dependent bicarbonate transport that is, important for acid extrusion in the smooth

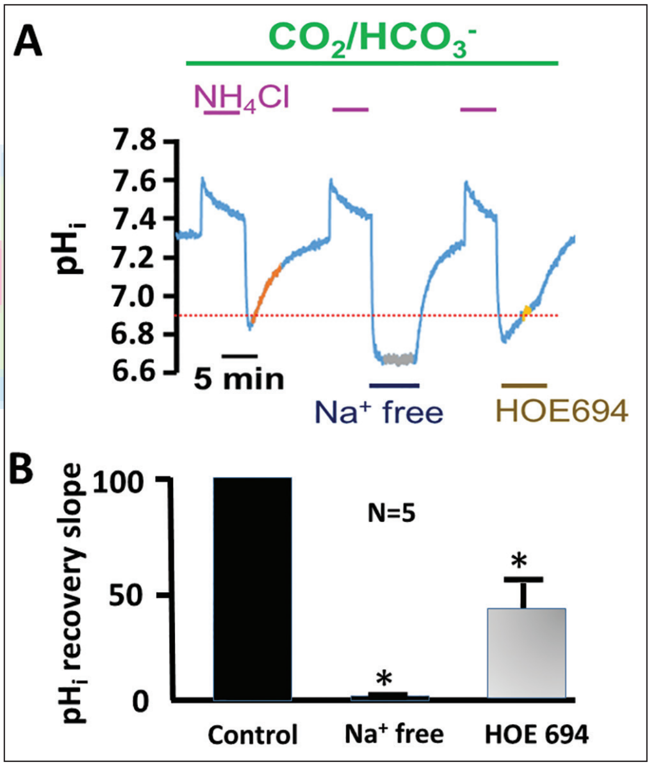

Figure 4: Effect of Na^+-free and 30 μM HOE 694 on pH_i recovery from induced acidosis in human umbilical artery smooth muscle cells superfused with 5% CO_2/HCO_3^- Tyrode solution. (a) The top bar shows the buffer system used in the superfusate. The periods of application of NH_4Cl and tested drugs (Na^+-free solution and 30 μM HOE 694) are shown with bars above or below the trace. The left part of traces A shows a typical pH_i recovery from an intracellular acidosis induced by a 10 min NH_4Cl (20 mM) prepulse in 5% CO_2/HCO_3^- Tyrode solution (pH_o = 7.4, 37°C) in human umbilical artery smooth muscle cells. The right part of traces A represents experiments showing the effect of the Na^+-free solution and 30 μM HOE 694 (a Na^+/H^+ exchanger inhibitor) on pH_i recovery, respectively, in human umbilical artery smooth muscle cells. (b) Histograms, showing the pH_i recovery slope of acid extrusion after NH_4Cl-induced intracellular acidosis averaged for five experiments similar to those shown in a. $^{**}P < 0.01$ versus control

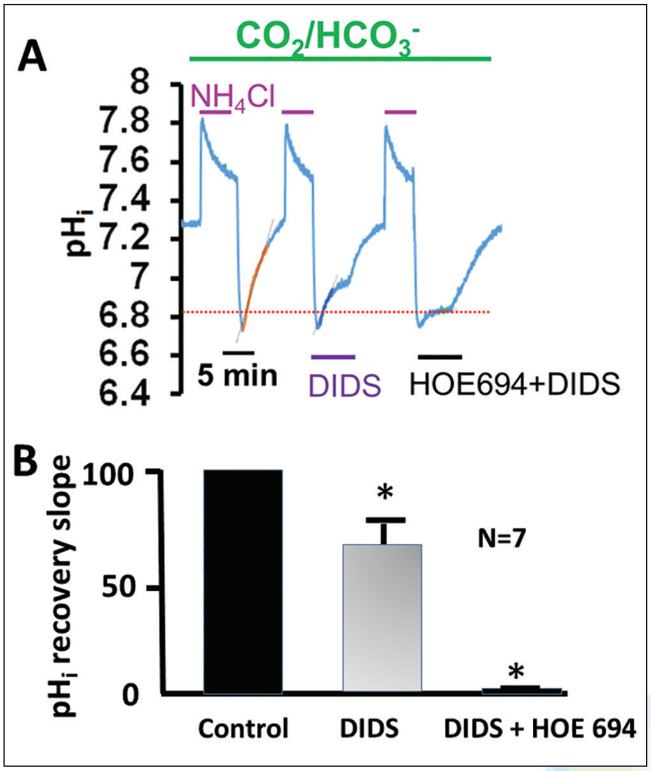

Figure 5: Effect of 30 μM HOE 694 and 0.2 mM 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid on pH_i recovery from induced acidosis in human umbilical artery smooth muscle cells superfused with 5% CO_2/HCO_3^- Tyrode solution. (a) The top bar shows the buffer system used in the superfusate. The periods of application of NH_4Cl and tested drugs (0.2 mM 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid and HOE 694 pulse 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid) are shown with bars above or below the trace. The left part of traces A shows a typical pH_i recovery from an intracellular acidosis induced by a 10 min NH_4Cl (20 mM) prepulse in 5% CO_2/HCO_3^- Tyrode solution (pH_o = 7.4, 37°C) in human umbilical artery smooth muscle cells. The right part of traces A represents experiments showing the effect of 0.2 mM 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (a NBC exchanger inhibitor), and HOE 694 (a Na^+/H^+ exchanger inhibitor) plus 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid on pH_i recovery, respectively, in human umbilical artery smooth muscle cells. (b) Histograms, showing the pH_i recovery slope of acid extrusion after NH_4Cl-induced intracellular acidosis averaged for seven experiments similar to those shown in a. $^{**}P < 0.01$ versus control

muscle cells of mice mesenteric, coronary, and cerebral small arteries.[19,20] This finding is similar to that we found in the human renal artery smooth muscle cells,[12] except the exist of NDCBE (SLC4A8) in HUASMCs.

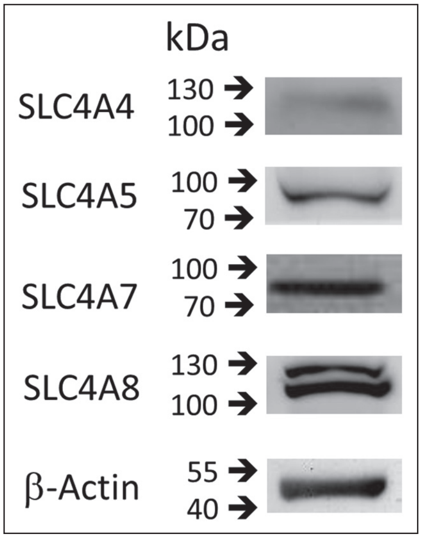

Figure 6: Western blot analysis of the SLC4 family of HCO_3^- transporters. The figure shows the result of Western blot analysis for β-actin, SLC4A8 (NBCBE), SLC4A7 (NBCn1), SLC4A5 (NBCe2), and SLC4A4 (NBCe1), from the bottom to the top, respectively, (n = 3)

The percentage of contribution of Na^+/H^+ exchanger and Na^+/HCO_3^- cotransporter under acid condition

To quantify the percentage of contribution of NHE and NBC at different pH values, the factor of passive intracellular buffering power has to be considered and calculated first. However, according to the result of Figures 4 and 5, the acid-extruding with HOE 694 or DIDS alone can represent the functional activity of NBC and NHE at that specific measured pH_i, respectively. In other words, the third column of Figure 4b and the second column of Figure 5b, therefore, provide the activity percentage of acid extrusion through NBC and NHE, respectively. It can be seen clearly that the percentage of contribution of NHE was larger than that of NBC (60% and 40%, respectively) at the acidic condition, that is, measuring pH_i around 6.80, in HUASMCs. Note that this is very similar to that report in different cell types of animal and human models (see Discussion for more details).

In conclusion, our present study has demonstrated for the 1st time, pharmacologically and physiologically, that NHE and NBC functionally co-exist for acid-extruding in HUASMCs.

DISCUSSIONS

The functional evidence of acid-extruding regulators-Na^+/H^+ exchanger 1 and Na^+/HCO_3^- cotransporter

By means of the microspectrofluorimetry technique, we have provided straightforward and convincing pharmacological and physiological evidence, for the 1st time, that NHE1 and NBC, are functionally responsible for acid extrusion following induced intracellular acidosis in HUASMCs. NHE's activity was HCO_3^--independent and Na^+ dependent (the middle part of Figure 2a).[12,16,22] This suggestion was confirmed by the finding that the acid-extruding mechanism could be entirely blocked by HOE 694 (the right part of Figure 2a), a highly-specific NHE1 inhibitor, in HEPES-buffered Tyrode solution.[22] The human genome encodes nine NHE isoforms (SLC9A1-9) genes, NHE 1-9 proteins that have different tissue and subcellular distributions.[24] However, the NHE1 protein has been identified as a protein which ubiquitously expresses in different tissues by molecular biology methods.[24,28,29] It has been shown that HOE 694 shows a high selectivity for cloned and expressed NHE1 that is, two or more orders of magnitude higher than for the other isoforms, such as NHE2 and NHE3.[22,30] Our present results revealed that the functioning NHE in the HUASMCs was also sensitive with low concentration of HOE 694 (30 μM) [Figure 2]. Therefore, our study suggests that NHE isoform is purely NHE1, instead of NHE2 and NHE3. Moreover, we excluded the possibility of the presence of other members of NHE4~9 in HUASMCs. Because it has been well known that NHE4 and NHE5 are essentially related insensitive to HOE694, and that NHE6~9 only exists in the membrane of intracellular organelles.[24] Therefore, using physiological and pharmacological maneuvers, our present study has provided direct evidence that the native NHE functioning during intracellular acidosis in the HUASMCs is the NHE1 isoform, instead of other members of NHE proteins.

Our present study also suggested that another acid-extruding mechanism whose activity was HCO_3^-- and Na^+-dependent [Figure 4] is NBC. This was supported by another additional results [Figures 4 and 5] in our present study, which showed that NBC was sensitive to DIDS, a NBC inhibitor, and insensitive to HOE 694.[12,19,22] It has been demonstrated that Na^+-dependent bicarbonate transport include five members of the SLC4 family, including two electrogenic NBC (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).[19,20] Recently, both in rat and mouse smooth muscle cells, the Aalkjaer group demonstrated that the NBC is NBCn1, that is, it is electroneutral.[19,20] They also found that disruption of the Na^+-HCO_3^--cotransporter NBCn1 (SLC4A7) inhibits nitric oxide (NO)-mediated vasorelaxation, smooth muscle Ca^{2+}-sensitivity and the development of hypertension in mice.[20] Indeed, our present study shows functionally that a Na^+ and HCO_3^- dependent acid-extruding mechanism is responsible for acid extrusion in the cultured HUASMCs [Figure 5]. Moreover, our present study demonstrates, for the 1st time, that four different isoforms of NBC: NBCn1 (SLC4A7; electroneutral), NBCe1 (SLC4A4; electrogenic), NBCe2 (SLC4A5), and NDCBE (SLC4A8) are detected in the protein level [Figure 6] in the cultured HUASMCs. In other words, the coexistence of four types NBC in this study is different to that found in mouse and rat models (c.f. Aalkjaer's group) and similar to what we have found in the human renal artery smooth cells. It indicates that the difference of isoforms is probably due to differences in species/organs. Information of the exact stoichiometry between HCO_3^- and Na^+ (coupling ratio) and the extent to which the NBC is electrogenic or electroneutral to the multiple NBC isoforms in the cultured HRASMCs will involve further study. In conclusion, our present study demonstrated functionally that there is a Na^+ and HCO_3^- dependent acid-extruding mechanism responsible for acid extrusion in the HUASMCs [Figures 2, 4 and 5].

The potential role of inhibitors of Na^+/H^+ exchanger 1 and Na^+/HCO_3^- cotransporters in clinic

In the HUASMCs, we found that the activity of NHE1 is larger than that of NBC in the pH_i value of acidosis (~6.8), as shown in Figure 5. In other words, NHE1 and NBC contribute the 65% and 35%, respectively, on acid extrusion during the conditions of server acidosis. Therefore, under conditions of intracellular acidosis such as ischaemia/reperfusion (i.e., pH_i <6.9), the NHE will be activated and responsible for nearly at least of 60% of the acid extrusion in the HUASMCs. This result is slightly similar to that derived from the human atrium,[15] and that animal vascular smooth cells.[9,13,19] Both in rat or mouse vascular smooth cells, it has been found that NHE1 is predominantly active at lower pH_i values and plays a major role for acid extrusion during the conditions of severe intracellular acidification[18], whereas NBC is active at both low and near-physiological pH_i values.[19,20] It has been demonstrated that NBCn1 modulates artery tone and blood pressure control through regulation of pH_i. Also, NBCn1 is crucial for pH_i regulation in endothelial cells and VSMCs and maintains NO production and Ca^{2+} sensitivity in VSMC.[19,20] Moreover, NBCn1 knockout mice are mildly hypertensive at rest, display attenuated blood pressure responses to NO-synthase and rho-kinase inhibition, and are resistant to developing hypertension during angiotensin-II infusion.[19,20]

According to the finding above, we predicted that the activity alteration either on NHE or of NBC will play a vital role on many physiological and pathological conditions, such as ischaemia-reperfusion, in HUASMCs, like that found by many other groups in other cell types.[10,11,25,31-34] Therefore, it is strongly believed that a development of new and specific NHE and/or NBC inhibitor is another indication for preventing pH-disturbance -induced injury such as ischaemia/reperfusion damage, apart from the knowing the basic physiological mechanism of pH_i regulating mechanism.

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