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Intracellular pH regulation in iPSCs-derived astrocytes from subjects with Chronic Mountain Sickness

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Abstract

Chronic Mountain Sickness (CMS) occurs in high altitude residence with major neurological symptoms such as migraine headaches, dizziness and cognitive deficits. Recent work demonstrated that highlanders have increased intracellular pH (pH_i) in their brain cells, perhaps for the sake of adaptation to hypoxemia and help to facilitate glycolysis, DNA synthesis, and cell cycle progression. Since there are well adapted (non-CMS) and maladapted (CMS) high altitude dwellers, it is not clear whether pH_i is differently regulated in these two high altitude populations. In this work, we obtained induced pluripotent stem cell (iPSC)-derived astrocytes from both CMS and non-CMS highlanders who live in the Peruvian Andes (>14000 ft) and studied pH_i regulation in these astrocytes using pH sensitive dye BCECF. Our results show that the steady-state pH_i (ss pH_i) is lower in CMS astrocytes compared with non-CMS astrocytes. In addition, the acid extrusion following an acid loading is faster and the pH_i dependence of H^+ flux rate becomes steeper in CMS astrocytes. Furthermore, the Na^+ dependency of ss pH_i is stronger in CMS astrocytes and the Na^+/H^+ exchanger (NHE) inhibitors blunted the acid extrusion in both CMS and non-CMS astrocytes. We conclude that a) NHE contributes to the ss pH_i stabilization and mediates active acid extrusion during the cytosolic acidosis in highlanders; b) acid extrusion becomes less pH_i sensitive in non-CMS (versus CMS) astrocytes which may prevent NHE from over-activated in the hypoxia-induced intracellular acidosis and render the non-CMS astrocytes more resistant to hypoxemia challenges.

Introduction

Chronic Mountain Sickness (CMS) or Monge's disease occurs in highlanders who consistently live at altitudes above 8000 ft. CMS is characterized by hypoxemia and polycythemia with increased hemoglobin and lowered oxygen saturation (Villafuerte and Corante, 2016). CMS affects more than 80 million people worldwide and currently there is

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no effective treatment available other than phlebotomy, which in itself is not satisfactory. The most frequent symptoms of CMS are neurological in nature, such as headache, dizziness, mental confusion and episode of coma (Penaloza and Arias-Stella, 2007). Studies have shown that the brain is one of the target organs of CMS (Reeves and Weil, 2001). Indeed, recent MRI studies have revealed pathological alterations, such as cerebral edema and multiple ischemic foci, in the brains of CMS patients (Bao et al., 2017), indicating that damage or malfunction of neurons and astrocytes does occur. The incidence of CMS among Andean highlanders is about 20% and the non-CMS highlanders are rather healthy and who can serve as a control population for the study of CMS pathophysiology.

Recent works have shown that intracellular pH (pH_i) is elevated in brain cells of highlanders residing in hypoxic environments (Shi et al., 2014). Similarly, chronic hypoxia increases pH_i in mouse brain cells (Mitsufuji et al., 1995). Since the alkalotic pH_i is favorable for augmenting glycolysis, increasing DNA synthesis and cell cycle progression, it is believed that the alkalization of brain cell pH_i may be one of the adaptation mechanisms for highlanders to survive the low O_2 environment. Previous work has shown that the shift to glycolytic metabolism is directly driven by the rise in pH_i in mouse fibroblast or astrocytes (Reshkin et al., 2000, Ruminot et al., 2011). Furthermore, acetazolamide (ACZ), a carbonic anhydrase inhibitor frequently used for attenuating the symptoms of acute mountain sickness, has also been shown to have beneficial effects in the treatment of CMS patients (Richalet et al., 2005). Interestingly, ACZ could increase pH_i in thalamic relay neurons in rat brain (Munsch and Pape, 1999). The alkaline effect of ACZ in brain could be one of the factors that leads to the beneficial effect to patients with altitude sickness. Although the exact mechanisms underlying ACZ-induced alkalosis is not well understood, evidence has shown that ACZ raises the pH_i by acting on the acid-base regulatory systems. For example, ACZ alkalinizes cells by enhancing H^+ extrusion via activating H^+-ATPase in ciliary epithelium (Shahidullah et al., 2014).

Elevation of pH_i in brain cells in highlanders could also result from the systemic alkalosis induced by the hyperventilation in response to hypoxia. However, several pieces of evidence have demonstrated that pH_i increase in hypoxic cells is not related to the systemic alkalosis. In mouse brain, for example, hypoxia induces significant intracellular alkalosis in brain cells even though mice undergo systemic acidosis (Mitsufuji et al., 1995). Chronic hypoxia elevates pH_i in pulmonary arterial smooth muscle cells by activating Na^+/H^+ exchanger on the cell membrane (Rios et al., 2005). Acute hypoxia also elevates pH_i in neurons and enhances acid extrusion in astrocytes in rodents (Yao et al 2001, Bevensee et al 2008). Indeed, high altitude natives in Andes show acidic CSF which is indicative of acidic extracellular pH in the brain of highlanders (Sorensen and Milledge, 1971). It is tempting to speculate that certain mechanisms promote acid extrusion in brain cells resulting in increased pH_i but decreased extracellular pH in Andean high altitude natives compared with lowlanders. Nevertheless, it is not clear if the alkalosis in brain cells of highlanders is due to the systemic alkalosis or whether it is intrinsic to cellular mechanisms, such as functional modifications in acid-base transporters on the cell membrane.

Symptoms frequently seen in CMS subjects are headache, dizziness, sleep disturbance, i.e., symptoms that are neurologic in nature. These clinical manifestations indicate a loss of

integrity in brain cells and MRI studies have demonstrated the existence of brain edema in CMS patients, most likely involving astrocytes (Bao et al., 2017). While astrocytes are the most numerous cell type in the brain and play a critical role in brain function and diseases, their role in CMS pathophysiology has not been well understood. It is tempting to hypothesize that pH_i in astrocytes from highlanders is elevated but pH_i in CMS astrocytes may not be as alkalinized as that in non-CMS ones since the maladaptation occurs in CMS patients. To determine whether the intrinsic acid-base regulatory mechanisms are changed in CMS astrocytes, we used reprograming techniques to obtain induced pluripotent stem cells (iPSCs) from skin biopsies of CMS or non-CMS high altitude dwellers in the Andes (>14000 ft). The iPSCs then were differentiated into astrocytes for the functional study of non-bicarbonate-dependent acid-base transporters, mainly Na^+/H^+ exchanger (NHE). Astrocyte-like cells generated using this protocol demonstrated characteristics of mammalian astrocytes which exhibited a typical astrocyte morphology, expressed astrocyte markers, such as GFAP and S100β, and demonstrated the capability of propagating calcium waves upon mechanical stimulation (Yao et al., 2016). These iPSCs-derived astrocytes not only have a steady-state pH_i value similar to those recorded from mouse or rat astrocytes (Bevensee et al., 1997, Hansen et al., 2015), but also express electrogenic sodium-bicarbonate cotransporter 1 (NBCe1) (Yao et al., 2016), an acid-base transporter that is highly expressed in astrocytes (Majumdar and Bevensee, 2010). In this work, we demonstrate that the ss pH_i is more acidic and the rate of H^+ flux (J_H)-pH_i relationship steeper (thus acid extrusion faster) in CMS astrocytes than in non-CMS astrocytes. Towards this end, the abnormality of pH_i regulatory mechanisms in brain cells may be an important target for the treatment of CMS.

Materials and Methods

Preparation of dermal fibroblasts from human skin biopsies

All subjects in the current study were adult males residing either at sea level in the United States or in the Andean mountain range, in Cerro de Pasco, Peru, at an elevation of more than 14000 ft. CMS patients met the diagnostic criteria for CMS used before, such as hematocrit, O_2 saturation and CMS score (> 12) (Zhao et al., 2015). Those highlanders with CMS scores less than 5 were considered as non-CMS subjects. Each subject signed an informed written consent under protocols approved by the University of California San Diego and the Universidad Peruana Cayetano Heredia. Biopsy samples (3 mm skin punch) were obtained from 2 sea level (SL) subjects residing in the United States, 3 highlanders with CMS and 3 highlanders without CMS residing in Cerro de Pasco, Peru. The skin biopsies were mechanically dissociated and plated for dermal fibroblast expansion in DMEM medium supplement with 20% fetal calf serum, 2.5% penicillin/streptomycin and 1% fungizone antibiotic (Life technologies, CA). Fibroblasts grew from explants for 2–3 weeks and then were passaged when they reached 80% confluence in the cell culture dish.

Reprogramming of fibroblast cells and generation of iPSCs and neuroprogeniter cells (NPCs)

Detailed protocols for generating iPSCs and NPCs were described in our previous study (Zhao et al., 2015). In brief, fibroblast cells were infected with retrovirus vectors containing

OCT4, SOX2, KLF4 and c-MYC human cDNAs (Salk Institute Gene Transfer, Targeting and Therapeutics Core, La Jolla, CA). The infected fibroblast cells were then plated onto the irradiated mouse embryonic fibroblast feeder cells incubated with human embryonic stem cell medium containing 20% knockout serum replacement, 1% non-essential amino acids, 0.2% beta-mercaptoethanol, and 30 ng/ml FGF2. Three weeks later, the iPSC colonies were manually picked and maintained in mTeSR™ medium (StemCell Technologies, Canada). Three clones were picked from iPSCs derived from each individual and one clone from each subject was used for further differentiation and characterizations. To obtain NPCs, iPSCs were triturated into single cells and embryoid bodies (EBs) were formed using AggreWell plate (Stem Cell Technologies, Canada) with N2 medium containing 0.5× N2, 0.5× B27, 1% penicillin/streptomycin in DMEM/F12 medium plus 5 μM ROCK inhibitor Y-27632 (Tocris Bioscience, MN) to promote cell survival, 1 μM dorsomorphin and 10 μM SB431542 (Tocris Bioscience, MN) for dual inhibition of SMAD signaling. A day later, EBs were transferred to an ultra low attachment petri dish for a 24 hr suspension culture. The next day, EBs were seeded on a matrigel-coated plate using N2 medium for 7–10 days. Rosette-bearing EBs were manually picked and dissociated into individual cells. These dissociated cells were then plated into a poly-l-ornithine/laminin-coated plate to generate a monolayer of NPC culture using N2 medium plus 20 ng/μl FGF2. NPC markers including Sox 2 (1:100, Stemgent, MA) and Nestin (1:60, R&D systems, MN) were confirmed positive in these cells using immunocytochemistry.

Generation of astrocytes from NPCs

Astrocytes were differentiated from the NPCs following a protocol detailed previously (Yao et al., 2016). Briefly, a confluent 100 mm diameter NPC plate was scraped forming neurospheres in a six-well plate by keeping at constant shaking (95 rpm). Media was changed on the day after cells were suspended once the neurospheres were well formed using NPC media containing FGF. After efficient formation of spheres around 48 hrs post scrapping, rock inhibitor was added to a final concentration of 5 μM for 48 hrs concomitant with the removal of FGF from the media in the next media change. Cells were kept in constant shaking with neuronal inducing media for a week. Next, astrocyte growth media (Lonza, Allendale, NJ) was added to the spheres for two weeks still under 95 rpm's. Following two weeks on astrocyte media, spheres were plated in laminin coated plates and the astrocytes grew out of the sphere spreading on the plate to form a monolayer cell formation. After the first passage, cells surrounding the neurospheres were dissociated enzymatically using accutase and plated. The neurospheres were removed manually by vacuum suction using a Pasteur pipet and the result was a confluent and homogeneous plate of GFAP and S100β positive astrocytes.

Experimental conditions

Hepes Solutions: the HEPES-buffered solution contained (in mM): 125 NaCl, 3 KCl, 1.2 $CaCl_2$, 1.2 $MgSO_4$, 1.25 NaH_2PO_4, 30 HEPES, and 10 glucose. This solution was titrated to pH 7.35 at 36°C with NaOH. For Na^+-free Hepes, Na^+ was removed from the above solution by replacing NaCl and NaH_2PO_4 with *N*-methyl-d-glucamine (NMDG) and KH_2PO_4, respectively. In the NH_4^+/NH_3 solution, 20 mM NaCl was replaced with the same concentration of NH_4Cl in the Hepes solution. Nigericin calibration solution contained (in

mM): 105 KCl, 50 NMDG, 5 $MgSO_4$, 10 glucose, and 30 HEPES. 2′,7′-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF-AM) was obtained from Molecular Probes Inc. (Eugene, Oregon, USA). Nigericin, cariporide and ethylisopropyl-amiloride (EIPA) were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA).

Intracellular pH measurement

A coverslip with cultured astrocytes was mounted on a thermostatically controlled holding chamber and incubated with 2 μM BCECF for 20 min at room temperature. The chamber was then secured on the stage of the microscope and supplied with ACSF bubbled with 5% CO_2 balanced with air. The perfusate was controlled at 36°C for each experiment. Intracellular fluorescence was determined in individual cells using fluorescence microscopy and digital image processing. A 175 W Xenon lamp and an ultrahigh speed wavelength switcher (Lambda DG-4, Sutter Instrument, Novato, CA) provided alternate 440/495 nm fluorescence excitation. The emission from BCECF-loaded cells was detected at wavelengths of 535 nm using a F-Fluar 40X/1.3 NA oil immersion objective (Zeiss Axiovert 200M microscope, Zeiss, Yena, Germany) and the attached 12 bit CCD camera. The light source, wavelength switcher, microscope and the camera were controlled by a computer. The ratio images were sampled every 15 seconds. Data were recorded and analyzed with MetaFlour imaging-processing software (Universal Imaging Corporation, Downington, PA). Ratios ($R_{495/440}$) were obtained from these two fluorescence emission intensities and pH_i calibration was conducted with the single-point nigericin method (Boyarsky et al., 1988).

Statistical Analysis

Data of SL group were pooled from 2 SL subjects and data of CMS or non-CMS group were pooled from 3 CMS subjects or 3 non-CMS subjects, respectively. Two to three experiments were performed using astrocytes obtained from each subject. The data structure was considered in the multilevel mixed model and spanned three levels of random grouping variables: the measurements of pH_i from each individual cells (level-1), individual subjects (level-2), and subject groups (level-3). Statistical analysis was done by using PROC MIXED procedure in SAS software (Version 9.4, SAS Institute, Cary NC). The data were tested for normality using a Shapiro–Wilk test and data were assessed for homogeneity of variance. Only if the data passed both tests were they subjected to further analyses. ‘n’ denotes the number of cells used for the experiment throughout. pH data were analyzed using OriginPro (OriginLab, Northampton, MA) or GraphPad Prizm software (GraphPad, San Diego, CA). Differences among multiple groups were determined by a two-way ANOVA followed by Bonferroni’s post hoc test. The Bonferroni post hoc tests were selected as appropriate for our sample size and to correct for multiple comparisons. Statistical significance was reached when p value was ≤ 0.05.

Results

ss pH_i in SL, CMS and non-CMS astrocytes in the nominal absence of CO_2/HCO_3^-

To study the contribution of non-bicarbonate-dependent mechanisms to pH_i regulation in astrocytes derived from human iPSCs, we used a Hepes-buffered solution to eliminate the activity of bicarbonate-dependent acid-base transporters in the experiments throughout.

Steady-state pH_i (ss pH_i) was measured from an average of 5 min recordings in each cell and the results obtained from SL, CMS and non-CMS astrocytes are shown in Fig. 1. Similar to our previous work shown in mice (Yao et al., 1999), astrocytes derived from human iPSCs also have a broad range of ss pH_i. Fig. 1A illustrates the frequency distribution of ss pH_i values for astrocytes from all three groups. The ss pH_i for CMS and non-CMS astrocytes ranged from 6.8 to 7.3, and 6.75 to 7.65, respectively, indicating that the ss pH_i distribution of the CMS astrocytes was narrower and shifted toward the acidic direction than the non-CMS astrocytes. The distribution of ss pH_i in SL astrocytes ranged from 6.5 to 7.1 and were skewed in the acidic direction, compared with that in astrocytes derived from both highlander groups. The mean ss pH_i for CMS and non-CMS astrocytes were 7.05 ± 0.03 (n = 36) and 7.18 ± 0.03 (n = 65), respectively, and CMS astrocytes had significantly lower ss pH_i than non-CMS astrocytes do ($p \leq 0.05$, Fig. 1B). The mean ss pH_i in SL astrocytes was 6.79 ± 0.04 (n=66), and this was significantly lower than ss pH_i of both CMS and non-CMS astrocytes ($p \leq 0.01$ for both comparisons, Fig. 1B).

Na^+ dependency of ss pH_i in the nominal absence of CO_2/HCO_3^-

In the nominal absence of CO_2/HCO_3^-, the major contributor of ss pH_i regulation can be the Na^+/H^+ exchanger (NHE). Therefore, in this experiment, we examined the changes of ss pH_i in astrocytes following the removal of Na^+ from the Hepes solution among astrocytes derived from SL, CMS and non-CMS subjects. As shown in Fig. 2A, Line 1, in SL astrocytes, a 10-min Na^+ removal led to a pH_i drop of 0.18 pH unit followed by a pH_i rise of 0.49 pH_i unit upon the addition of Na^+ back to the perfusate. Line 2 shows that, in non-CMS astrocytes, Na^+-removal elicited a 0.18 pH unit drop followed by a pH_i rise of 0.37 pH unit when perfusate was switched back to Na^+-containing solution. Line 3 demonstrates that Na^+-removal caused a bigger pH_i drop (0.22 pH unit) in CMS astrocytes and switching perfusate back to Na^+-containing solution led to a 0.42 pH unit rise in pH_i. Compared with the maximum pH_i drop in non-CMS astrocytes during the Na^+ removal, the maximum pH_i drop in CMS astrocytes was significantly greater (Figs. 2A & B, n=63 for CMS, n=66 for non-CMS, $p \leq 0.0001$). pH_i drop in SL astrocytes is similar to that in non-CMS astrocytes ($p > 0.05$) but significantly smaller than that in CMS astrocytes (Figs. 2A & B, n=63 for CMS, n=100 for SL, $p \leq 0.0001$). In addition, compared with astrocytes derived from highlanders, SL astrocytes (n=100) have a significantly bigger rise in pH_i when bath solution was switched from Na^+-free back to Na^+-containing Hepes ($p \leq 0.0001$ versus CMS, n=63; $p \leq 0.0001$ versus non-CMS, n=66, Fig. 2C). A significantly bigger rise in pH_i was also found in CMS astrocytes (n=63) when compared with non-CMS astrocytes ($p \leq 0.0001$, n=66). These data suggest that the activity of NHE contributes to the ss pH_i regulation in all three groups of astrocytes and NHE may play a bigger role in CMS astrocytes than in those derived from non-CMS or SL subjects.

Intrinsic buffering capacity in astrocytes derived from SL, CMS and non-CMS astrocytes

In order to compare the intrinsic intracellular buffering capacity (β_I) among SL, CMS and non-CMS astrocytes and compute the net efflux of H^+ from the rate of pH_i recovery from an acid load, we measured the β_I in astrocytes derived from all three subject groups. The β_I was determined for the SL, CMS and non-CMS astrocytes as described previously (Bevensee et al., 1997). An example of a typical β_I measuring experiment, performed on SL astrocytes, is

illustrated in Fig. 3A. The experiments were performed in Na^+- and HCO_3^--free media to prevent active acid extrusion in cells. Astrocytes were progressively perfused with decreasing concentrations of NH_3/NH_4^+-containing solution, and the change in pH_i was measured for each change in $[NH_4^+]_i$. β_I was found to vary with pH_i in a linear fashion (Fig. 3B). The best-fit line to each data set yielded a slope and a y-intercept for each group. As shown in Fig. 3B, a similar linear relationship (between β_I and pH_i) was observed for both the CMS and the non-CMS astrocytes. In CMS astrocytes, the slope and y-interception are -21.5 ± 2.4 mM/pH^2 and 157.9 ± 15.7 mM/pH, respectively, which are slightly smaller than -16.2 ± 3.4 mM/pH^2 and 123.8 ± 23.7 mM/pH in non-CMS astrocytes. The SL astrocytes had a slope of -18.08 ± 4.41 mM/pH^2 and a y-intercept of 144.16 ± 28.4 mM/pH, which had a significantly higher β_I than that in CMS or non-CMS astrocytes at each indicated pH_i value. For example, at $pH_i = 6.5$, the β_I was 27.1 ± 4.9 mM/pH in SL astrocytes (n=14), which was significantly larger than 17.4 ± 6.8 mM/pH in CMS (n=15, $p \leq 0.0001$) or 16.8 ± 4.1 mM/pH in non-CMS (n=18, $p < 0.0001$). There was no statistical difference between β_I of CMS and non-CMS astrocytes at pH_i less than 6.9. However, at pH_i 7.1, the β_I in CMS astrocytes was 6.6 ± 1.6 mM/pH (n=5), which was significantly lower than 9.2 ± 0.8 mM/pH (n=4, $p \leq 0.05$) in non-CMS astrocytes (Fig. 3B).

Acid extrusion in astrocytes in the nominal absence of CO_2/HCO_3^-

To compare the pH_i regulation mechanisms among astrocytes derived from SL, CMS, and non-CMS subjects, we acid-loaded cells using NH_4Cl pre-pulse technique (Boron and De Weer, 1976) and allowed the pH_i to recover from the low pH_i to the baseline pH_i in Hepes buffer. Fig 4A shows the traces of pH_i recovery of representative recordings from astrocytes derived from different experimental groups. After a stable baseline was obtained, the astrocytes were perfused with Hepes contained 20 mM NH_4Cl which elicited an alkaline pH_i followed by a significant acidification in astrocytes upon the withdrawal of NH_4Cl from the perfusate. The pH_i of astrocytes was then allowed to recover from acid loading in Hepes buffer to monitor the contribution of non-bicarbonate-dependent acid base transporters to the acid extrusion process. The overlapping traces (Fig. 4A) show that the slope of the recovery trace is flatter in the non-CMS astrocytes compared with that in the CMS astrocytes, while both CMS and non-CMS astrocytes have slower pH recovery profile compared with that of SL astrocytes Fig. 4A). The net efflux of H^+ (J_H) was computed from the rate of pH_i recovery (dpH_i/dt) and the β_I of each astrocyte. Fig. 4B shows that astrocytes derived from each group generally exhibit a pH_i-dependent H^+ efflux in Hepes buffer. The lower the pH_i goes, the bigger the J_H. However, the J_H-pH_i curve is flatter for non-CMS astrocytes than that for CMS or SL astrocytes (Fig. 4B). Statistical analysis shows that, at a pH_i range of 6.0 to 6.6, the J_H in CMS (n=18, 24 and 18 for pH 6.1, 6.3 and 6.5, respectively) was significantly larger than that in non-CMS astrocytes (n=16, 45 and 45 for pH 6.1, 6.3 and 6.5, respectively, $p \leq 0.0001$ for each comparison). Compared with both highlander groups, the J_H in SL astrocytes was significantly higher at a pH_i range from 6.2 to 7.2 (at least 19 cells were used for the calculation for each pH_i, $p \leq 0.0001$ for each comparison), although the significant difference was not reached in between SL and CMS astrocytes at pH_i 6.1 or 7.1 (Fig. 4B).

Effect of NHE1 inhibition on H^+ flux in astrocytes derived from SL, CMS and non-CMS subjects

To examine if the NHE1 contributes to the acid extrusion following an acid loading in SL, CMS and non-CMS astrocytes, we studied the effect of Na^+-free Hepes and the selective NHE1 blocker, EIPA or cariporide on H^+ extrusion following an acid load. Right after acid loading by NH_4Cl, astrocytes were perfused with Na^+-free Hepes, or Hepes supplemented with EIPA (20 μM) or cariporide (5 μM) so that the pH_i of these astrocytes was allowed to recover in an environment with the Na^+-dependent- or NHE1-dependent-mechanisms blocked. The representative traces obtained from SL astrocytes were overlapping (Fig. 5A) and show that, compared with non-treated astrocytes, the slopes of the pH_i recovery traces become smaller after the treatment with Na^+-free Hepes, EIPA or cariporide, although the effect of EIPA and cariporide were not as robust as that of Na^+-free Hepes. This is also true in astrocytes derived from two highlander groups (traces are not shown). The bar graph in Fig. 5B shows that, with the treatment of Na^+-free Hepes, EIPA or cariporide, the H^+ efflux is significantly decreased at the pH_i range from 6.2–6.4 in astrocytes from all three experimental groups (Fig. 5B), suggesting that Na^+-dependent mechanisms, mainly NHE1, play a role in the pH_i regulation of these astrocytes in the nominal absence of CO_2/HCO_3^-.

Discussion

In this study, we have made the following observations: a) compared with non-CMS astrocytes, CMS astrocytes have an acidic ss pH_i and an enhanced H^+ efflux following acid loading; b) removing Na^+ from the bath solution or adding NHE blocker EIPA or cariporide blunted the pH_i recovery from an acid load in all SL, CMS and non-CMS astrocytes; and c) the relationship between H^+ flux rate and pH_i was steeper in CMS astrocytes compared with that in non-CMS astrocytes. We conclude that NHE plays a role in pH_i regulation in highlanders from Andes and the reduced pH_i sensitivity of H^+ extrusion in non-CMS astrocytes could be one of the adaptation mechanisms to prevent neural cells from malfunction resulting in neurological deficits as seen in CMS patients.

Previous work has shown that in healthy highlanders, the ss pH_i of their brain cells is increased compared with that in sea level individuals (Shi et al., 2014). This alkalosis in ss pH_i is believed to be an adaptation mechanism taking place in highlanders since, generally, a rise in pH_i correlates with cell activation and a fall parallels a reduction in metabolism in mammalian cells. Indeed, an alkaline pH_i was also detected in brain cells in mice under chronic hypoxia (Mitsufuji et al., 1995). Our current data show that, ss pH_i is acidic in CMS compared with non-CMS astrocytes, but both are alkalotic compared with ss pH_i in sea level human subjects (6.87) and rodent (6.83 from rat, 6.82 from mouse) (Bevensee et al., 1997, Hansen et al., 2015). It is possible that the long term residents at high altitude have adjusted their cellular pH to cope with the harsh environment, especially hypoxemia. The finding that the pH_i in CMS astrocytes was not alkalinized to the same degree as that in the non-CMS astrocytes may be an indication of maladaptation in CMS cells. Our data support the notion that brain cells, at least astrocytes, adapt to high altitude environment by altering their acid-base regulatory systems.

Acid base transporters on the cell membrane actively keep pH_i stable by loading or extruding H^+ and its equivalents into or out of cells. Our current work has demonstrated that NHE is an important acid extruder in the absence of CO_2/HCO_3^- in SL, CMS and non-CMS astrocytes. In addition, our data from H^+ flux analysis and the Na^+-dependency test demonstrate that NHE is not quiescent at the ss pH_i level, indicating that NHE participates in the maintenance of ss pH_i in astrocytes derived from both sea level subjects and highlanders. The bigger pH_i drop in CMS cells, induced by the removal of extracellular Na^+, suggests that enhanced activity of a Na^+-dependent acid extruder, such as NHE, exists in CMS astrocytes for stabilizing ss pH_i. In addition, as previous work has demonstrated, the pH_i overshoot in astrocytes upon reperfusion after an ischemic challenge is associated with a burst of Na^+-H^+ exchange activity stimulated by the sudden increase of extracellular Na^+ (Chesler, 2005). Indeed, the significantly larger alkaline shift of pH_i in CMS after the addition of Na^+ (at the end of Na^+-removal, Fig. 2C) may also indicate that NHE activity in CMS is stronger than that in non-CMS astrocytes, although other mechanisms, such as an activated H^+ pump, may contribute to this transient pH_i overshoot as well.

The lowered ss pH_i in CMS astrocytes usually indicates a reduced acid extrusion or an enhanced acid loading in the pH_i regulatory system in these cells. The unchanged J_H in CMS (vs. non-CMS) astrocytes at ss pH_i level leads us to believe that the acidic ss pH_i in CMS is likely due to the enhanced activity and/or expression of (an) acid loader(s) in CMS astrocytes which causes the shift of the ss pH_i set point to the left relative to that in non-CMS astrocytes (Ruffin et al., 2014). Therefore, a possible next step is to examine the activities of non-bicarbonate-dependent acid loaders in both CMS and non-CMS astrocytes. Furthermore, SL astrocytes have a significantly bigger J_H than highlander ones but their ss pH_i is also lower than that in astrocytes derived from highlanders, suggesting that (a) more robust acid loader(s) operating in SL astrocytes, leading to a leftward shift in the ss pH_i set point in SL astrocytes.

Our data demonstrate that the slope of the J_H - pH_i relationship may be steeper in CMS compared with that in non-CMS astrocytes in the lower pH_i range, suggesting that NHE activity is stronger in CMS cells. The difference in J_H between CMS and non-CMS is likely due to the modification in NHE (and possibly other bicarbonate-independent acid extruders, such as H^+ pump) activity. Would an attenuated acid extruding activity in non-CMS astrocytes adapts better to a hypoxic stress than CMS cells? While our current data do not provide a categorical answer, we believe that a lower NHE activity would prevent non-CMS astrocytes from hypoxia-induced Na^+ overloading, hence reducing the activation of Na^+/Ca^{2+} exchanger and the excessive Ca^{2+} accumulation, protecting astrocytes against hypoxic damage (Chesler, 2005).

Although our current work is focused on the study of pH_i regulation in astrocytes affected by high altitude environment, the acid-base regulatory systems in CMS neurons may also be modified as previously reported under hypoxia in mice (Yao et al., 2001, Yao et al., 2003). Therefore, the communication between neuron-neuron, neuron-glia and most importantly, the performance of neural networks formed by these elements, could be affected by the change of pH_i regulation in these cells. Indeed, evidence has been accumulating that the alteration of pH_i regulation in astrocytes or neurons could significantly affect the

functionality, excitability and connectivity of neural networks in CNS and lead to pathological changes in the brain (Sinning and Hubner, 2013). It has been hypothesized that astrocytes wrap around the synapse and form a 'tripartite' structure with neurons to regulate the synaptic transmission (Sinning and Hubner, 2013). The acid-base transporters on the astrocytes and/or neuronal cell membrane can modulate the synaptic transmission by altering the pH_i and the pH levels in the synaptic cleft which in turn affects the function of ion channels and synaptic receptors (Hsu et al., 2000, Gu et al., 2001). Several pieces of evidence have demonstrated that the enhanced NHE activity at the site of synapse can increase the H^+ extrusion from astrocytes or neurons and acidify the milieu in the synaptic cleft which attenuates postsynaptic currents mediated by NMDA or AMPA receptors but potentiates the current mediated by $GABA_A$ receptors (Dietrich and Morad, 2010). Hence, the increased NHE activity in CMS astrocytes found in this work may decrease the excitability of CNS neural networks in CMS patients which could lead to the pathological symptoms such as confusion and coma as seen in CMS patients.

In summary, astrocytes derived from highlanders have alkaline pH_i compared with those derived from sea level subjects. The ss pH_i of CMS astrocytes does not shift to the same alkaline level as non-CMS ones do, manifesting an altered pH_i regulatory mechanism involved. The lower pH_i sensitivity of NHE-mediated acid extrusion found in non-CMS astrocytes may suggest an adaptation mechanism that assists the highlanders to survive the high altitude environment.

Acknowledgments

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Abbreviations

CMS	chronic mountain sickness
non-CMS	healthy highlanders
SL	sea level control
hiPSCs	human induced pluripotent stem cells
NPCs	neural progenitor cells
BCECF	2′,7′-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein
EIPA	5-(N-ethyl-N-isopropyl)amiloride
NHE	sodium hydrogen exchanger
ACZ	acetazolamide

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Highlights

- Intracellular pH (pH_i) is alkaline in astrocytes derived from high altitude dwellers compared with those from sea level subjects
- The alkaline-shift of pH_i is less marked in astrocytes derived from subjects with Chronic Mountain Sickness (CMS)
- Acid extrusion rate is decreased in astrocytes from highlander not affected by CMS (non-CMS)
- The Na^+/H^+ exchanger mediates the pH_i recovery from an acid loading in both CMS and non-CMS astrocytes
- The decreased pH_i sensitivity of acid extrusion in non-CMS astrocytes suggests an adaptation mechanism in highlanders

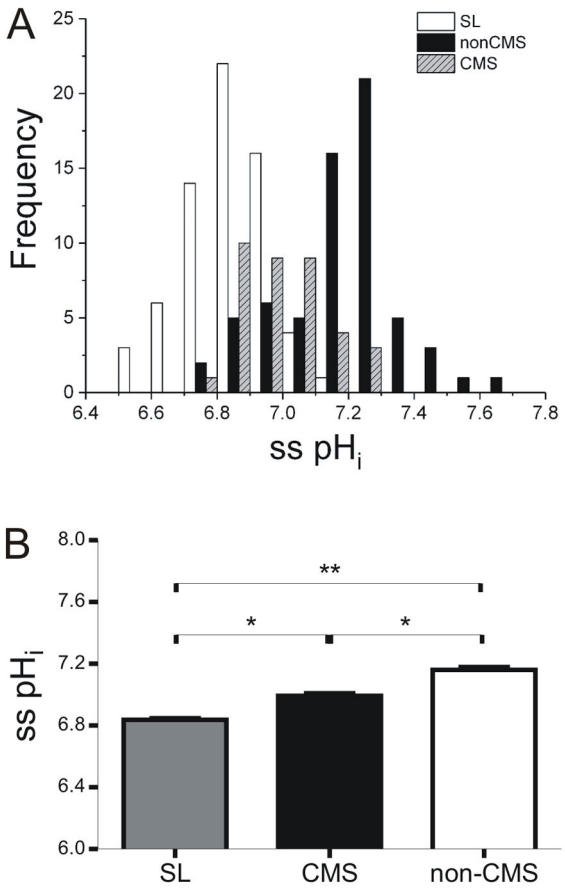

Fig. 1.

Comparisons of ss pH_i among astrocytes derived from SL, CMS and non-CMS subjects in the nominal absence of CO_2/HCO_3^-. A) histograms showing the distribution of ss pH_i in astrocytes with a bin width of 0.033 pH unit. B) bar graph showing the comparisons of ss pH_i among astrocytes derived from each experimental group. *: $p \leq 0.05$, **: $p \leq 0.01$ between each comparison.

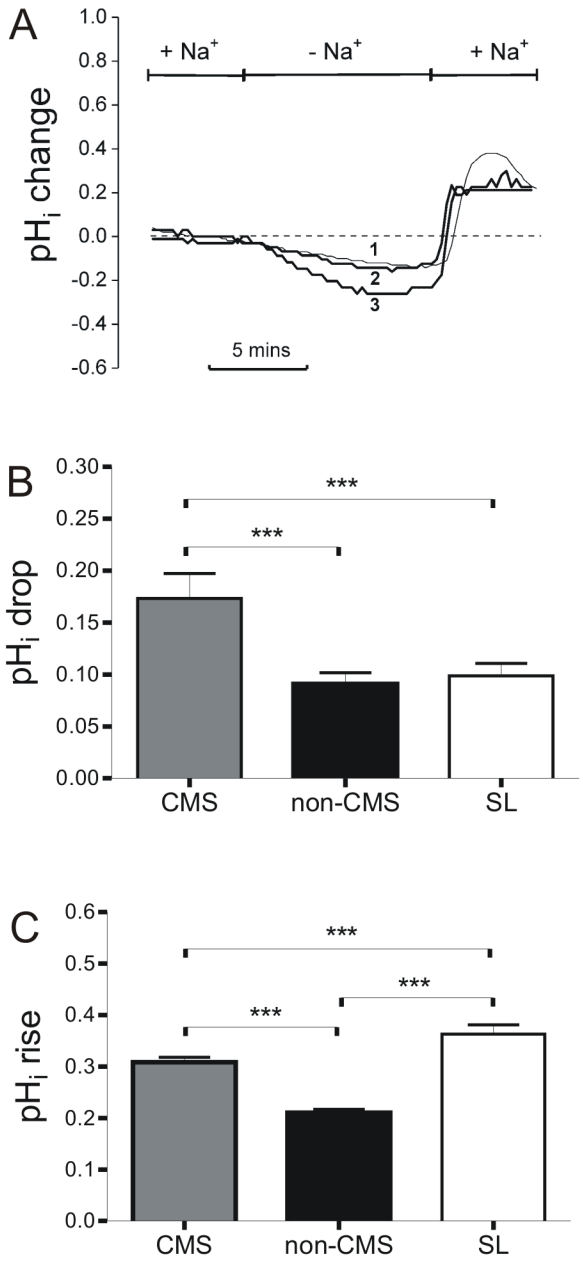

Fig. 2.

Na^+-dependency of ss pH_i in astrocytes derived from SL, CMS and non-CMS groups. A, representative traces recorded from SL (1), non-CMS (2) and CMS (3) astrocytes showing the responses of ss pH_i to a 10-mins Na^+-withdrawal from the extracellular solution. ss pH_i fell and remained at a low level until the external solution was switched back to standard, Na^+-containing Hepes buffer. B, bar graph showing the comparisons of maximum pH_i drop during a 10-mins Na^+ withdrawal in astrocytes from each experimental group. ***: $p \leq 0.0001$ versus CMS group. C, bar graph showing the comparisons of pH_i increase in astrocytes upon switching back to Na^+-containing Hepes. ***: $p \leq 0.0001$ between each comparison as indicated.

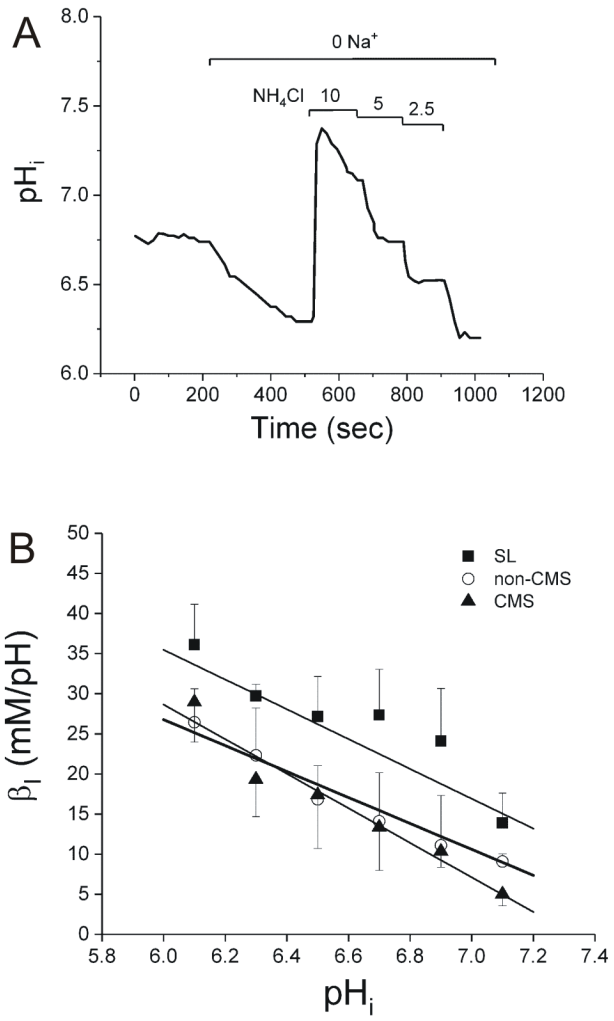

Fig. 3.

Comparisons of intrinsic buffering power in astrocytes derived from SL, CMS and non-CMS groups. A, a representative trace recorded from a SL astrocyte showing the pH_i changes in response to a graded reduction of extracellular NH_3/NH_4^+ concentration in the absence of extracellular Na^+. B, Relation of intrinsic intracellular buffering power (β_I) as a function of pH_i in astrocytes derived from SL, CMS and non-CMS groups. Data are summarized from two to three experiments in each experimental groups. Each β_I was calculated within pH_i intervals of 0.2.

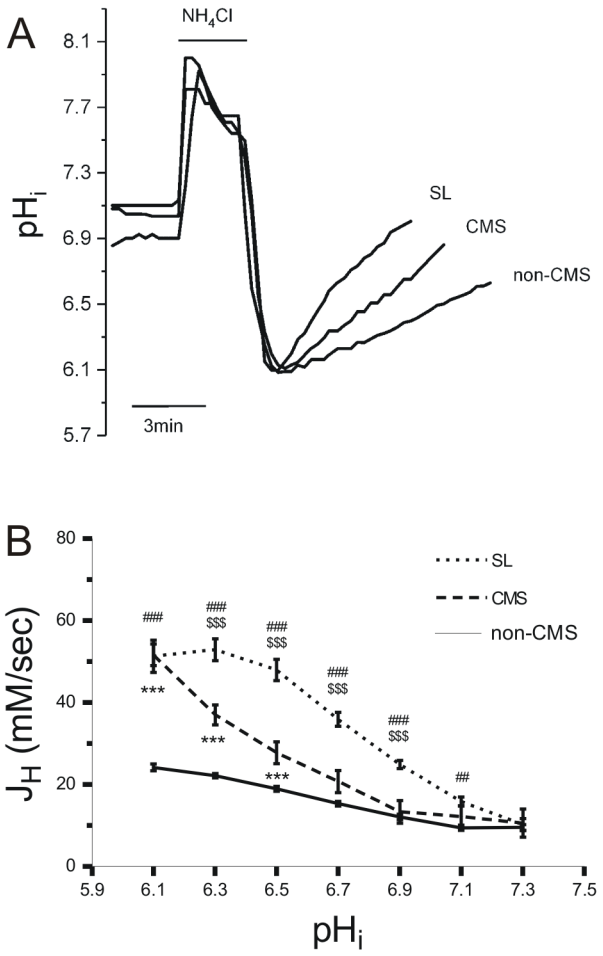

Fig. 4.

Comparisons of acid extrusion following an acid loading among astrocytes derived from SL, CMS and non-CMS subjects. A, representative traces showing the pH_i recovery from an acid loading in astrocytes derived from different groups as indicated at the end of each trace. B, J_H-pH_i relationship in astrocytes derived from different groups. The J_H at each pH_i was calculated from a 0.2 pH interval and two-way ANOVA was use to examine the significance of each difference. Data are shown as mean ± SD. ***: p≤0.0001 versus non-CMS groups. ###: p≤0.0001, ##: p≤0.01 versus non-CMS groups. $$$: p≤0.0001 versus CMS group.

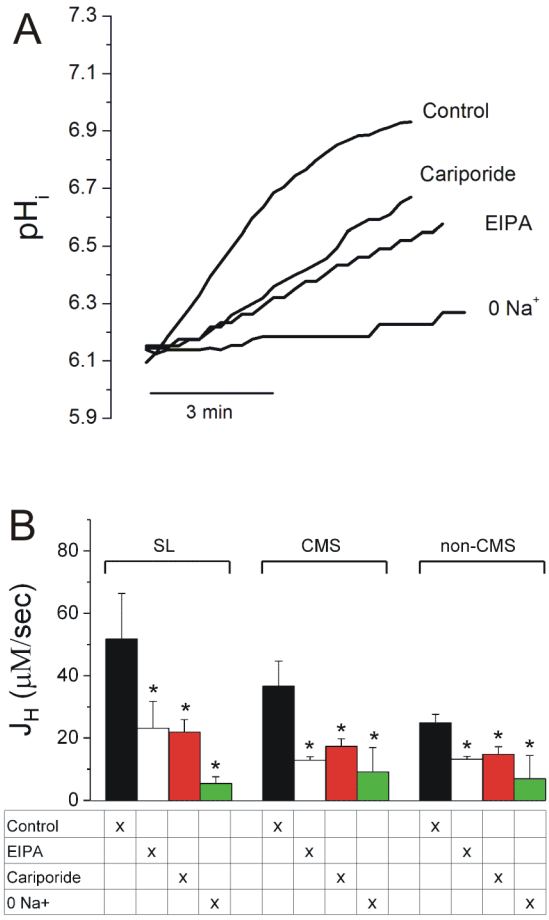

Fig. 5.

NHE plays a role in acid extrusions in astrocytes derived from SL, CMS and non-CMS subjects. A, representative traces of pH_i recovery from an acid load in SL astrocytes under different experimental conditions as indicated at the end of each trace. For clarity, the initial rise in pH_i produced by the NH_4Cl pulse is not shown in the overlapped traces. B, bar graph showing the comparisons of H^+ flux rate at pH_i range of 6.2–6.4 between with and without the treatment of EIPA, cariporide or Na^+-free Hepes in SL, CMS and non-CMS astrocytes. Data were pooled from subjects in each group (Two to three separate experiments were performed in each subject) and are shown as mean ± SD. *: $p \leq 0.05$ versus untreated control astrocytes in each experimental group as indicated on top of the bar graph. Each 'x' in the table under the graph denotes the treatment applied to the experiment represented by the bar in the graph in that column.