Original Paper

Intermittent Hypoxia Inhibits $\text{Na}^+\text{-H}^+$ Exchange-Mediated Acid Extrusion Via Intracellular Na^+ Accumulation in Cardiomyocytes

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Key Words

Intermittent hypoxia • Intracellular pH • Intracellular Na^+ • $\text{Na}^+\text{-H}^+$ exchange • Reactive oxygen species

Abstract

Background/Aims: Intermittent hypoxia (IH) has been shown to exert preconditioning-like cardioprotective effects. It also has been reported that IH preserves intracellular pH (pHi) during ischemia and protects cardiomyocytes against ischemic reperfusion injury. However, the exact mechanism is still unclear. **Methods:** In this study, we used proton indicator BCECF-AM to analyze the rate of pHi recovery from acidosis in the IH model of rat neonatal cardiomyocytes. Neonatal cardiomyocytes were first treated with repetitive hypoxia-normoxia cycles for 1-4 days. Cells were then acid loaded with NH_4Cl , and the rate of pHi recovery from acidosis was measured. **Results:** We found that the pHi recovery rate from acidosis was much slower in the IH group than in the room air (RA) group. When we treated cardiomyocytes with $\text{Na}^+\text{-H}^+$ exchange (NHE) inhibitors (Amiloride and HOE642) or Na^+ -free Tyrode solution during the recovery, there was no difference between RA and IH groups. We also found intracellular Na^+ concentration ($[\text{Na}^+]_i$) significantly increased after IH exposure for 4 days. However, the phenomenon could be abolished by pretreatment with ROS inhibitors (SOD and phenanthroline), intracellular calcium chelator or $\text{Na}^+\text{-Ca}^{2+}$ exchange (NCX) inhibitor. Furthermore, the pHi recovery rate from acidosis became faster in the IH group than in the RA group when inhibition of NCX activity. **Conclusions:** These results suggest that IH would induce the elevation of ROS production. ROS then activates Ca^{2+} -efflux mode of NCX and results in intracellular Na^+ accumulation. The rise of $[\text{Na}^+]_i$ further inhibits the activity of NHE-mediated acid extrusion and retards the rate of pHi recovery from acidosis during IH.

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Introduction

Intermittent hypoxia (IH), or periodic exposure to hypoxia interrupted by return to normoxia or less hypoxic conditions, occurs in several circumstances (e.g., asthma and obstructive sleep apnea). IH leads to an increase in intracellular reactive oxygen species (ROS) generation during reoxygenation following hypoxia [1]. Previously, excess ROS production was considered harmful because it may cause lipid peroxidation, protein oxidation, DNA damage, and intracellular ion deregulation [2, 3]. However, it is now generally accepted that ROS may exert beneficial effects because short or moderate durations of ROS generation confer a preconditioning cardioprotective effect against ischemia/reperfusion injury [4, 5].

Intracellular pH (pHi) is an important modulator of cardiac excitation and contraction and a potent trigger of electrical arrhythmia [6]. Thus, pHi regulation is an important process to preserve the physiological function of cardiomyocytes. An increased generation of protons extruded from the cell via Na⁺-H⁺ exchange (NHE) during ischemia results in an increased intracellular Na⁺ concentration ([Na⁺]_i) [7, 8]. This rise in [Na⁺]_i, coupled with the depolarized plasma membrane, results in a reversal of the Na⁺-Ca²⁺ exchange (NCX) to bring Ca²⁺ into the cardiomyocyte. This phenomenon causes ion disturbance (intracellular Ca²⁺ overloading) and results in cell death. More importantly, several studies have shown that IH may confer rat cardiomyocytes protection against ischemic acidosis and retard the pHi recovery rate during reperfusion, which might be beneficial in maintaining the cell function [9, 10]. However, the exact mechanism of an IH-induced cardioprotective effect remains unclear. Our previous study has shown that IH induces a mild increase in ROS generation not resulting in cardiomyocyte death. This non-lethal ROS level resulted in an increased NCX-1 expression and activity and led to enhanced Ca²⁺ efflux from the cells [11]. In this study, we further examined the rate of pHi recovery from acidosis in the IH model of rat neonatal cardiomyocytes. Our results showed that the production of IH-induced ROS could inhibit the activity of NHE-mediated acid extrusion via intracellular Na⁺ accumulation, which slowed the rate of pHi recovery from acidosis during IH.

Materials and Methods

Preparation of neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes were prepared and cultured as previously described [12]. In brief, 2-days-old Sprague-Dawley rats (males and females) were sacrificed by cervical dislocation and decapitated. The ventricles were minced, and tissue fragments were dissociated by enzyme digestion (0.051% pancreatine and collagenase in Hank's buffer), followed by enzyme inactivation using an F-12 medium (80% F-12 nutrient mixture, 10% fetal bovine serum, 10% horse serum, and 1% penicillin). Non-cardiomyocytes were removed when the cells were pre-plated for 1 hr at 37°C in an incubator (21% O₂, 5% CO₂). Cardiomyocytes were plated on 35-mm collagen-coated cover slips in F-12 medium with 10 μM cytosine arabinoside, and the medium was daily replaced. All procedures were performed in accordance with the Animal Care Guidelines of the Tzu Chi University.

Chemicals and solutions

N-2-hydroxy-ethylpiperazine-N'-2-ethanesulphonic acid (HEPES) buffered Tyrode solution comprised 140 mM NaCl, 4.5 mM KCl, 1.2 mM MgCl₂, 2.0 mM CaCl₂, 11 mM glucose, and 10 mM HEPES, with pH adjusted to 7.4 at 37°C with NaOH. Ca²⁺-free medium lacked CaCl₂ and contained 0.2 mM ethyleneglycol-bis (beta-aminoethylether)-N, N'-tetraacetic acid. For Na⁺-free medium, Na⁺ ions were isotonicly replaced with N-methyl-D-glucamine. All fluorescent indicators were purchased from Molecular Probes (Eugene, OR, USA). F-12 medium, fetal bovine serum, horse serum, Hank's balanced salt solution, and penicillin were purchased from Gibco/Life Technologies (USA). All other chemicals were purchased from Sigma (USA).

IH exposures

Neonatal rat cardiomyocytes were placed in plexiglas box chambers (25-cm length, 30-cm width, 15-cm height). The cardiomyocytes were exposed to normoxia/room air (RA; 21% O₂, 5% CO₂, and balanced N₂)

or IH (5% O₂, 5% CO₂, and balanced N₂ for 30 min alternated with 30-min RA), using a timed solenoid valve controlled for 1–4 days (Fig. 1A). Oxygen fractions in the chambers were continuously monitored using an oxygen detector. A micro-dissolved oxygen electrode from Lazar Research Laboratories (DO-166MT-1) was used to detect the fluctuations of oxygen concentrations in the medium and the chamber. Fig. 1B and 1C shows the RA and IH profiles recorded in the gas phase and medium, respectively, in the plexiglas chambers. The steady-state dissolved oxygen concentration in the medium was 16.3% and 9.8% within the RA and IH groups, respectively (Fig 1C).

Measurement of steady-state intracellular pH (pHi) by flow cytometric analysis

After 4 days of RA or IH treatment, cardiomyocytes were washed with Tyrode solution. The cardiomyocytes were loaded with a proton indicator 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF, 2 μM) for 30 min at 37°C. Subsequently, cells were trypsinized and harvested, and the pellet was resuspended in phosphate buffered saline. The positive control was resuspended in pH 4.5 high K⁺ buffer with nigericin. Fluorescence was measured on a FACSCalibur flow cytometer (Becton-Dickinson, BD Biosciences) with a 488-nm argon laser. For each sample, 10000 events were acquired. All analyses were performed using the CellQuest Pro software.

Measurement of dynamic pHi

The cardiomyocytes were loaded with a proton indicator BCECF (2 μM) for 15 min using dual excitation ratiometric image analysis on a fluorescence microscope (DMI3000B; Leica). Cells were excited at 440 and 490 nm with an emission of 535 nm.

The standard ammonium prepulse in HEPES-buffered Tyrode solution (40 mM NH₄Cl, 90 mM NaCl, 4.5 mM KCl, 1.0 mM MgCl₂, 2 mM CaCl₂, 11 mM glucose, and 10 mM HEPES) to decrease pHi was used [13]. In brief, NH₃ rapidly diffuses into the cell, where it primarily combines with H⁺ ions, resulting in a rise in pHi. This is followed by a slower passive entry of NH₄⁺ (i.e., via K⁺ channels) and slow reacidification (Fig. 2A) of the pHi. The removal of NH₄Cl then causes a pronounced intracellular acidification due to the accumulated NH₄⁺ ions diffusing out of the cell as NH₃ and leaving H⁺ behind.

The rate of pHi recovery from acidosis was defined as proton efflux rate (J_H), which was calculated as dpHi/dt × βi during the first 30 s. The dpHi/dt at each pHi (ranging from pH 5.9 to 6.5) was obtained from an exponential fit of the recovery phase. The βi (intrinsic intracellular buffering power) is described as the intracellular buffering capacity of the cardiomyocytes and was measured by exposing cells to varying concentrations of NH₄Cl in Na⁺-free HEPES-buffered Tyrode solution. pHi was allowed to stabilize in Na⁺-free solution before application of NH₄Cl. βi was calculated using the following equation: [NH₄⁺]_i = [NH₄⁺]_o × 10^{Δ(pHo - pHi)} and βi = Δ[NH₄⁺]_i/ΔpHi and is referred to as the midpoint values of measured changes in pHi. βi at different levels of pHi were estimated from the least squares regression lines βi vs. pHi plots [14, 15].

Measurement of intracellular Na⁺ concentration ([Na⁺]_i)

The [Na]_i of cardiomyocytes was measured by *in vivo* calibration, and was performed as described in the previous study [16]. In brief, the cells were loaded with 2.5 μM Sodium Green (a fluorescent sodium indicator) and 0.05% Pluronic F-127 for 1 hr at room temperature. Pluronic F-127 is a non-ionic surfactant which improves the uptake of several hydrophobic dyes. The loaded cells were washed thrice with Tyrode solution and trypsinized. Fluorescence was then measured on a Gallios flow cytometer (Beckman Coulter, USA) using excitation/emission wavelengths of 488/520 nm.

Calibration solutions containing increasing concentrations of Na⁺ (0–140 mM) were prepared by mixing different proportions of a high Na⁺ solution (115 mM sodium gluconate, 25 mM NaCl, 1 mM EGTA, 10 mM HEPES, and 11 mM glucose) and a high K⁺ solution (115 mM potassium gluconate, 25 mM KCl, 1 mM EGTA, 10 mM HEPES, 11 mM glucose), both containing the Na⁺ ionophore cocktail, gramicidin D (2 μM), monensin (40 μM), and strophanthidine (100 μM), and adjusting the pH to 7.4 at 37°C. R_{max} (140 mM Na⁺) and R_{min} (Na⁺/serum free) represent the maximum and minimum ratio values for the data curve, respectively. Using a Hanes plot of the data from 12 calibration experiments, the mean apparent dissociation constant (K_{app}) at 37°C was found to be 24.7 mM, close to the value determined in other studies [16, 17]. The following equation was used to convert the fluorescent ratio into [Na]_i: 24.7 × [(R - R_{min})/(R_{max} - R)].

Measurement of intracellular ROS

Intracellular levels of $\text{O}_2^{\cdot-}$ and H_2O_2 were detected using hydroethidine (HE) or 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA), respectively. Both HE and CM-H2DCFDA are cell permeable and become highly fluorescent upon oxidation by $\text{O}_2^{\cdot-}$ or H_2O_2 , respectively. Cells were loaded for 30 min at room temperature with 10 μM HE or 5 μM CM-H2DCFDA. The excitation/emission wavelengths for HE and CM-H2DCFDA were 488/610 nm and 488/540 nm, respectively. Signal increases are presented as the peak/basal fluorescence ratio (F/F_0).

Western blotting analysis

Equal amounts of protein lysates were loaded and electrophoresed via SDS-PAGE and transfected onto a PVDF membrane. Membranes were probed with primary antibodies recognizing NHE1 and actin. Each primary antibody binding was detected with a secondary antibody and visualized using the enhanced chemiluminescence (ECL) method.

Statistics

All results obtained from individual cells are expressed as mean $\pm$ SEM for the stated number of animal preparations (n), each tested in duplicates. Statistical differences were compared using Student's *t* test, and *p*-value of <0.05 was considered statistically significant.

Results

IH does not change the basal steady-state pH_i in neonatal cardiomyocytes.

The mean basal pH_i value of neonatal cardiomyocytes in the HEPES-buffered Tyrode solution was 7.21 ± 0.02 (n = 47). We first used flow cytometric analysis to measure the basal steady-state pH_i in neonatal cardiomyocytes after IH exposure for 1–4 days (Fig. 1D). The results showed that no difference in the basal pH_i between the RA and IH groups. Thus, IH did not change the basal pH_i of neonatal cardiomyocytes.

IH significantly slows the rate of pH_i recovery from acidosis in cardiomyocytes.

We used the standard ammonium prepulse method to examine the effect of IH on pH_i recovery from acidosis in neonatal cardiomyocytes (Fig. 2). The results showed that the magnitude of pH_i change (ΔpH) induced by the ammonium prepulse was the same in all groups (RA and IH, Fig. 2B). However, the rate of pH_i recovery from acidosis (i.e., proton efflux rate, J_{H^+}) became much slower in the IH group after IH exposure for 4 days (i.e., IH4D group) than in the RA group (Fig. 2B).

Fig. 1. Intermittent hypoxia (IH) dose not change the basal intracellular pH (pH_i) in the cardiomyocytes. (A) The cardiomyocytes were exposed to normoxia/room air (RA: 21% O_2) or intermittent hypoxia (IH: 5% O_2 for 30min alternating with 30min RA) using a timed solenoid valve controlled for 1–4 days. (B and C) The Figs show the RA and IH profiles recorded in the gas O_2 (B) and medium O_2 (C) in the Plexiglas box chambers. The steady-state oxygen concentration in the gas phase was 22% and 5.5% in the RA and IH groups, respectively. The steady-state dissolved oxygen concentration in the medium was 16.3% and 9.8% in the RA and IH groups, respectively. (D) The basal pH_i in the neonatal cardiomyocytes was measured by the flow cytometric analysis. There was no difference in the basal pH_i between the RA and IH groups. Quantification of the levels of the basal pH_i in the cardiomyocytes exposed to RA for 4 days (RA4D, n = 10) and IH for 4 days (IH4D, n = 10). pH4.5 was designed as positive control. ***p* < 0.01 compared to RA4D.

Fig. 2. IH significantly slows the rate of pHi recovery from acidosis in the cardiomyocytes. (A) The standard ammonium prepulse (40 mM NH_4Cl) in HEPES-buffered Tyrode solution was used to decrease pHi [13]. In brief, the NH_3 rapidly diffuses into the cell, where most of it combines with H^+ ions, resulting in a rise in pHi. This is followed by a slower passive entry of NH_4^+ (eg, via K^+ channels) and slow “reacidification” of the pHi. Removal of NH_4Cl then causes a large intracellular acidification, since all the accumulated NH_4^+ ions diffuse out of the cell as NH_3 , leaving behind H^+ . The rate of pHi recovery from acidosis was the same after IH exposure for 1–3 days (IH1D–3D), but became significantly slower after IH exposure for 4 days (IH4D). The maximal degree of intracellular acidification was about pH 6.4 in all groups. (B) The magnitude of pHi change (delta pH) induced by the ammonium prepulse were the same in all groups after IH exposure for 1–4 days. (C) The quantified rate of pHi recovery from acidosis (i.e., proton efflux rate, J_{H}) was the same after IH exposure for 1–3 days (IH1D–3D), but became much slower after IH exposure for 4 days (18.2 ± 0.7 and 12.6 ± 0.8 mM/min in the RA4D group and IH4D group, respectively, $n = 10$, $**p < 0.01$).

Sodium-proton exchange (NHE) plays a major role in pHi regulation in cardiomyocytes during IH

Both NHE and the sodium bicarbonate cotransporter (NBC) play important roles in pHi regulation in cardiomyocytes. When cardiomyocytes were treated with Na^+ -free Tyrode solution or NHE inhibitors (Amiloride and HOE642) during recovery, the rates of pHi recovery from acidosis became very slow and were almost equal within the RA and IH groups (Fig. 3). In contrast, when the NBC activity was inhibited by DIDS during recovery, the rates of pHi recovery from acidosis were not altered in either the RA or the IH groups (Fig. 3). These results strongly suggest that NHE, but not NBC, plays a major role in pHi recovery from acidosis in cardiomyocytes during IH.

IH could slow the rate of pHi recovery from acidosis via ROS production but not activation of protein kinase C (PKC)

Our previous study showed that IH induced a mild increase in ROS generation that did not cause cardiomyocyte death [11]. We measured the amount of ROS in cardiomyocytes by fluorescent probes HE and DCFDA (Fig. 4). The results showed that the levels of ROS were increased after IH exposure for 4 days (Fig. 4A and 4B). However, this increase could be abolished by SOD (superoxide scavenger) and phenanthroline (a Fenton's reaction generated hydroxyl radical blocker). Interestingly, when cardiomyocytes were pretreated with SOD and phenanthroline to inhibit ROS production, the rates of pHi recovery from acidosis became the equal in the RA and IH groups (Fig. 4C–E). In contrast, the recovery rate was still slower in the IH group versus the RA group when we pretreated cardiomyocytes with the PKC inhibitor chelerythrine. These results demonstrate that IH could slow the rate of pHi recovery from acidosis via ROS production but not the activation of PKC.

IH could significantly inhibit the activity of NHE-mediated acid extrusion even though NHE protein expression is upregulated

We have shown that NHE plays a major role in pHi regulation of the cardiomyocytes. IH could also significantly attenuate the activity of NHE-mediated acid extrusion in

Fig. 3. Sodium-proton exchange (NHE) plays a major role in the pHi recovery from acidosis. (A) When inhibition of NHE activity by Na⁺-free Tyrode solution, Amiloride or HOE642 during the recovery, the rates of pHi recovery from acidosis became very slow and the same in both RA and IH groups. In contrast, when inhibition of sodium-bicarbonate cotransporter activity by DIDS during the recovery, the rate of pHi recovery from acidosis was not altered in both RA and IH groups. The maximal degree of intracellular acidification was pH 5.98 ± 0.02 , 5.92 ± 0.03 , 5.95 ± 0.02 and 6.28 ± 0.01 in the Na⁺-free, Amiloride, HOE642 and DIDS groups, respectively. (B) The magnitude of pHi change (Δ pH) induced by the ammonium prepulse were the same in all groups after IH exposure for 4 days ($n = 8-12$ in each group). (C) The quantified rates of pHi recovery from acidosis (J_{H}) became very slow and the same in both RA and IH groups when treated with Na⁺-free Tyrode solution, Amiloride or HOE642 during the recovery. In contrast, the rate (J_{H}) was not altered in both RA and IH groups when treated with DIDS during the recovery. ($n = 8-12$ in each group. # $p < 0.01$ compared to RA4D in Control group. ** $p < 0.01$ compared to RA4D in each group.)

Fig. 4. IH could slow the rate of pHi recovery from acidosis via production of reactive oxygen species (ROS) but not activation of protein kinase C (PKC). (A and B) The amounts of ROS in the cardiomyocytes were measured by fluorescent probes HE (A) and DCFDA (B). The amounts of ROS were increased after IH exposure for 4 days (IH4D, $n = 8$) (* $p < 0.05$ compared to RA4D). Interestingly, the effect could be abolished by pretreatment with SOD (IH4D+SOD, $n = 8$) and phenanthroline (IH4D+Phe, $n = 8$) (# $p < 0.05$ compared to IH4D in Control). (C) When cardiomyocytes were pretreated with SOD (pre-SOD group) and phenanthroline (pre-Phe group) to inhibit ROS production, the rates of pHi recovery from acidosis became the same in RA and IH groups. In contrast, the recovery rate remained slower in the IH group than in the RA group when pretreated cardiomyocytes with chelerythrine (Che group). The maximal degree of intracellular acidification was pH 6.21 ± 0.03 , 6.28 ± 0.03 , and 6.32 ± 0.02 in the pre-SOD, pre-Phe and Che groups, respectively. (D) The magnitude of pHi change (Δ pH) induced by the ammonium prepulse were the same in all groups after IH exposure for 4 days ($n = 8-10$ in each group). (E) The quantified rates of pHi recovery from acidosis (J_{H}) showed the same in RA and IH groups for SOD (pre-SOD, $n = 8$) and Phe (pre-Phe, $n = 10$) pretreatment. But the recovery rate (J_{H}) remained slower in the IH group than in the RA group for Che pretreatment (pre-Che, $n = 8$). ** $p < 0.01$ compared to RA4D in each group.

cardiomyocytes. We examined the effect of IH on the NHE1 protein expression by Western blotting analysis (Fig. 5A). However, the amount of NHE1 protein expression was increased in the IH group compared to the RA group, indicating compensatory elevation of NHE1 protein expression after IH exposure for 4 days.

In addition, we used the Na^+ sensitive fluorescent indicator Sodium Green to measure the intracellular Na^+ concentration ($[\text{Na}^+]_i$) via flow cytometry (Fig. 5B). The results showed that $[\text{Na}^+]_i$ was significantly increased (~ 14 mM) after IH exposure for 4 days. However, the increase of $[\text{Na}^+]_i$ could be completely abolished by pretreatment with ROS inhibitors (SOD, 5 U and phenanthroline, 100 nM). Our previous study has shown that IH would activate a Ca^{2+} efflux (forward) mode of Na^+ - Ca^{2+} exchange (NCX), via mild ROS production, to carry extracellular Na^+ into the cell [11]. This would result in intracellular Na^+ accumulation. In this study, after inhibition of forward mode NCX activity by high-dose KBR-7943 (KBR, 30 μM) [18, 19], the rise of $[\text{Na}^+]_i$ was abolished after IH exposure for 4 days (Fig. 5B). Also, when cardiomyocytes were pretreated with the intracellular calcium chelator BAPTA-AM (2 μM), $[\text{Na}^+]_i$ was not increased but remained almost the same in the RA4D and IH4D groups (7.42 ± 2.06 and 7.08 ± 3.88 mM, respectively, $n = 8-10$, Fig. 5B). This indicated that the rise of $[\text{Na}^+]_i$ after IH exposure for 4 days is dependent on intracellular calcium. Furthermore,

Fig. 5. IH could significantly inhibit the activity of NHE-mediated acid extrusion although NHE protein expression is up-regulated. (A) The amount of NHE1 protein expression was measured by Western blotting analysis. The NHE1 protein expression showed a significantly increase in the IH group than in the RA group (1.51 ± 0.18 vs. 1 ± 0.09 , $n = 10$, $*p < 0.05$). (B) Intracellular Na^+ concentration ($[\text{Na}^+]_i$) was measured by Na^+ -sensitive fluorescent indicator Sodium Green with flow cytometry. The $[\text{Na}^+]_i$ was significantly increased after IH exposure for 4 days (23.35 ± 2.11 and 9.78 ± 2.15 mM in the IH4D and RA4D group, respectively, $n = 10$, $*p < 0.05$). Interestingly, the rise of $[\text{Na}^+]_i$ in the IH4D group can be abolished by pretreated with ROS inhibitors (SOD, 5 U and phenanthroline, 100 nM) or NCX inhibitor (high-dose KBR, 30 μM) (9.21 ± 2.41 , 8.58 ± 2.11 and 4.59 ± 2.25 mM in the IH4D+SOD, IH4D+Phe and IH4D+KBR group, respectively, $n = 8-10$, $*p < 0.05$ compared with IH4D). Also, when cardiomyocytes were pretreated with intracellular calcium chelator BAPTA-AM (2 μM), the $[\text{Na}^+]_i$ was noted elevated and almost the same in the RA4D and IH4D group (7.42 ± 2.06 and 7.08 ± 3.88 mM, respectively, $n = 8-10$). Furthermore, inhibition of Na^+ - K^+ ATPase by pretreated with Ouabain (Oua, 30 μM) would increase $[\text{Na}^+]_i$. However, the $[\text{Na}^+]_i$ was still more significantly elevated after IH exposure for 4 days (36.71 ± 2.26 and 24.87 ± 1.44 mM in the IH4D and RA4D group, respectively, $n = 10$, $*p < 0.05$). (C and D) When inhibition of NCX activity by Na^+ - and Ca^{2+} -free Tyrode solution or high-dose KBR (30 μM), the rate of pH_i recovery from acidosis (J_{H}) became faster in the IH group than in the RA group. The maximal degree of intracellular acidification was pH 6.54 ± 0.03 and 6.62 ± 0.02 in the Na^+ / Ca^{2+} free and KBR groups, respectively. The similar results were obtained in the summary data of J_{H} (Na^+ - and Ca^{2+} -free Tyrode treatment: 4.59 ± 0.34 and 6.12 ± 0.49 mM/min in the RA4D and IH4D group, respectively, $n = 10$, $*p < 0.05$; KBR treatment: 3.71 ± 0.26 and 4.97 ± 0.19 mM/min in the RA4D and IH4D group, respectively, $n = 10$, $*p < 0.05$).

with the inhibition of Na⁺-K⁺ ATPase by pretreatment with Ouabain (30 μM), [Na⁺]_i was still more significantly elevated after IH exposure for 4 d (36.71 ± 2.26 and 24.87 ± 1.44 mM in the IH4D and RA4D groups, respectively, n = 10, *p < 0.05). This indicates that the IH-induced rise of [Na⁺]_i is not due to the inhibition of Na⁺-K⁺ ATPase. All of these results strongly suggest that IH increases ROS production and then increases [Na⁺]_i via the activation of the Ca²⁺ efflux (forward) mode of NCX. Subsequently, the rise of [Na⁺]_i attenuates the activity of NHE-mediated acid extrusion in cardiomyocytes during IH.

We further used Na⁺- and Ca²⁺-free Tyrode solution or NCX inhibitor (KBR, 30 μM) to prevent the intracellular Na⁺ accumulation via NCX in ammonium prepulse after IH exposure for 4 days (Fig. 5C and 5D). Interestingly, the rate of pHi recovery from acidosis became faster in the IH group than in the RA group. These results were consistent with the compensatory elevation of NHE1 protein expression after IH exposure for 4 days (Fig. 5A).

Discussion

The principal findings of this study are as follows: IH (exposure to 9%–10% O₂ dissolved in culture medium for 4 days) increases ROS production, which then activates Ca²⁺ efflux (forward) mode of NCX and results in intracellular Na⁺ accumulation. The increase of [Na⁺]_i further inhibits the activity of NHE-mediated acid extrusion and decreases the rate of pHi recovery from acidosis during IH. The phenotype of cultured neonatal cardiomyocytes is extremely stable, and their contractile profile during hypoxia-reoxygenation is comparable to that of *in situ* heart during ischemia/reperfusion [20]. Previous findings have demonstrated that hypoxic stress is not associated with any significant metabolic, structural or functional damage in the cardiomyocytes [21]. In this study, spontaneous contractile activity remained stable up to 4 days in the cultured neonatal cardiomyocytes exposed to RA or IH. The cell culture model of IH might provide insight into the mechanism associated with pHi regulation during adaptation to IH in cardiomyocytes.

IH inhibits the activity of NHE-mediated acid extrusion in cardiomyocytes

Our results show that IH does not change the basal pHi in neonatal cardiomyocytes, consistent with the previous study [11]. We used Na⁺- and Ca²⁺-free Tyrode solution or NCX inhibitor (high-dose KBR, 30 μM) [18, 19] to prevent intracellular Na⁺ accumulation via NCX in ammonium prepulse after IH exposure for 4 days (Figs 5C and 5D). Interestingly, the rate of pHi recovery from acidosis became faster in the IH group than in the RA group. These data demonstrated that during exposure to IH, cardiomyocytes upregulate the expression of NHE1 protein to increase the process of acid extrusion and simultaneously activate NCX (due to elevated ROS production), thereby leading to intracellular Na⁺ accumulation, which inhibits the activity of NHE1 protein and, thus, decreases acid extrusion. In summary, IH could significantly inhibit the activity of NHE-mediated acid extrusion, although NHE1 protein expression is compensatorily upregulated.

The cardioprotective effect of IH is dependent on the degree of hypoxia

There are two types of IH: acute (several cycles over a short period) and chronic (lasting several days or weeks). Both acute and chronic IH essentially involve recurrent reoxygenation cycles, leading to an increased production of intracellular oxidative stress [1]. The burst of ROS production occurring during hypoxia-reoxygenation cycles is considered a key factor in cell damage. In contrast, mild ROS production can also be protective in a preconditioning-like manner and induce stress responses, leading to cell survival [4]. Some previous studies have demonstrated increases in the infarct size induced by ischemia/reperfusion in isolated rat hearts exposed to IH induced by exposure to 5% O₂ for 4 hr [9] for 35 consecutive days (8 hr/day) [22]. In contrast, IH preconditioning with 10% O₂ for 4 hr induces cardioprotection, as illustrated by a reduction in the infarct size in isolated rat hearts [9]. The cardioprotective effect was also observed after chronic IH induced by exposure to 9.5%–10% O₂ for 20

consecutive days (25–70 min/day) [10]. These different effects of IH were probably observed because of the correlation between significant increases in cellular ROS generation and the degree of hypoxia compared with the normoxia. This is why, in this study, we examined the cardioprotective effect of IH induced by 1–4 days of exposure to 9%–10% O_2 dissolved in the culture medium (Fig. 1).

Exogenous 100 μM H_2O_2 , but not 10 μM H_2O_2 , have been shown to stimulate NHE activity via the activation of extracellular signal-regulated kinase and PKC in adult rat ventricular myocytes [23]. A previous study has reported that the high production of mitochondrial ROS could activate NHE and NBC via the stimulation of the ROS-sensitive MAPK cascade [24]. Furthermore, Eigel et al. have reported that ROS is required for the rapid reactivation of the reverse mode of NCX in hypoxic reoxygenated guinea pig ventricular myocytes [25]. In their hypoxia-reoxygenation experimental model, they used an anoxia environment (approximately 0% O_2) during the hypoxia period [26, 27]. This induces a much larger amount of ROS generation than that within our IH model (9%–10% O_2 dissolved in medium). Our previous study has demonstrated that IH (exposure to 9%–10% O_2) for 1–4 days did not increase cell death possibly because hypoxia with 9%–10% O_2 would only induce non-lethal oxidative stress in cardiomyocytes [11]. In this study, we further demonstrated that a mild increase of IH (exposure to 9%–10% O_2) induced ROS that could inhibit the activity of NHE-mediated acid extrusion via intracellular Na^+ accumulation. Thus, the effects of ROS on NHE and NCX activity appear to be complex and may vary with cell types, amounts of ROS, and experimental models.

Intracellular Na^+ could control acid extrusion of cardiomyocytes

The transmembrane Na^+ gradient is essential for both the excitability of cardiomyocytes and the regulation of cytoplasmic concentrations of Ca^{2+} and protons. Na^+ influx into a resting cell occurs by several routes, including Na^+ channels, NCX, NHE, NBC, $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter, and $\text{Na}^+/\text{Mg}^{2+}$ exchange. The heart is not normally at rest, but rather regularly beats. This activity increases the amount of Na^+ entering via Na^+ channels and also via NCX (i.e., Na^+ enters the cell in exchange for the Ca^{2+} that enters via the L-type Ca^{2+} channels) [28]. However, throughout the cardiac cycle, there is a net Na^+ influx via NCX. Conversely, NHE uses the energy provided by Na^+ ions entering the cell to pump H^+ out (i.e., NHE-mediated acid extrusion). The turnover rate of NHE and, therefore, the activity of acid extrusion will be dependent on $[\text{Na}^+]_i$ [28]. $[\text{Na}^+]_i$ can be dysregulated in cardiac disease, which contribute to cardiac pathology associated with these diseases. For example, $[\text{Na}^+]_i$ has been shown to rise during ischemia or simulated ischemia [29–33], which contributes to ischemia/reperfusion injury. Both our previous [11] and present studies also demonstrated that IH would activate NCX via the mild ROS production, resulting in a rise of $[\text{Na}^+]_i$.

There is functional coupling among NHE, NCX, intracellular Na^+ , and Ca^{2+}

NCX is a plasma membrane transporter that moves Ca^{2+} in or out of the cell, depending on the intracellular concentrations of Na^+ and Ca^{2+} as well as the membrane potential [34]. Because NCX is the main pathway for Ca^{2+} extrusion from excitable cells, in the steady state, Ca^{2+} efflux through the exchange must equal the Ca^{2+} influx into the cell, which is largely through the L-type Ca^{2+} channel. Therefore, any maneuver that increases Ca^{2+} entry into the cell via the L-type Ca^{2+} channel (e.g. β -adrenergic stimulation) will result in an increased entry of Na^+ via NCX. Na^+ influx is a linear function of $[\text{Ca}^{2+}]_i$, and, therefore, higher $[\text{Ca}^{2+}]_i$ leads to greater Na^+ influx [28]. Moreover, our present study showed that IH activates Ca^{2+} efflux (forward) mode of NCX via mild ROS production and results in intracellular Na^+ accumulation. The rise of $[\text{Na}^+]_i$ further inhibits the activity of NHE-mediated acid extrusion.

Although NCX generally operates in the forward mode to pump Ca^{2+} out of the cell, depending on the electrochemical gradient, it can also produce net Ca^{2+} entry coupled with Na^+ efflux (i.e., the reverse mode). Net reverse mode can only occur at potentials positive to the reversal potential of NCX. In turn, the reversal potential depends on $[\text{Na}^+]_i$ and the concentration of intracellular Ca^{2+} [28, 34]. Under basal conditions, NHE is relatively

quiescent, the Na^+/K^+ ATPase utilizes ATP to extrude Na^+ , and the bidirectional NCX works predominantly in forward (Ca^{2+} efflux) mode. During ischemia, NHE becomes activated in response to intracellular acidosis and possibly by other NHE-stimulatory factors. The resulting influx of Na^+ , occurring in the presence of ischemia-induced attenuation of Na^+/K^+ ATPase activity, causes the intracellular accumulation of Na^+ . Such a rise in $[\text{Na}^+]_i$ during ischemia alters the reversal potential of NCX in a manner that inhibits its operation in the forward mode but favors its operation in the reverse (Ca^{2+} influx) mode, thus producing intracellular Ca^{2+} overload during both ischemia and subsequent reperfusion [6-8]. Thus, the IH-induced inhibition of the activity of NHE-mediated acid extrusion would afford a preconditioning-like cardioprotective effect during ischemia and reperfusion by inhibiting this sequence at an early stage.

Conclusion

The increase of IH-induced ROS can inhibit the activity of NHE-mediated acid extrusion via intracellular Na^+ accumulation. The effect would slow the rate of intracellular pH recovery from acidosis during IH.

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Disclosure Statement

No conflict of interests exists.

References

- McGuire M, Zhang Y, White DP, Ling L: Chronic intermittent hypoxia enhances ventilatory long-term facilitation in awake rats. *J Appl Physiol* (1985) 2003;95:1499-1508.
- Kolar F, Ostadal B: Molecular mechanisms of cardiac protection by adaptation to chronic hypoxia. *Physiol Res* 2004;53:S3-13.
- Williams AL, Chen L, Scharf SM: Effects of allopurinol on cardiac function and oxidant stress in chronic intermittent hypoxia. *Sleep Breath* 2010;14:51-57.
- Becker LB: New concepts in reactive oxygen species and cardiovascular reperfusion physiology. *Cardiovasc Res* 2004;61:461-470.
- Kolar F, Jezkova J, Balkova P, Breh J, Neckar J, Novak F, Novakova O, Tomasova H, Srbova M, Ost'adal B, Wilhelm J, Herget J: Role of oxidative stress in PKC-delta upregulation and cardioprotection induced by chronic intermittent hypoxia. *Am J Physiol Heart Circ Physiol* 2007;292:H224-230.
- Vaughan-Jones RD, Spitzer KW, Swietach P: Intracellular pH regulation in heart. *J Mol Cell Cardiol* 2009;46:318-331.
- Avkiran M, Marber MS: Na^+/H^+ exchange inhibitors for cardioprotective therapy: progress, problems and prospects. *J Am Coll Cardiol* 2002;39:747-753.
- Fliegel L: Molecular biology of the myocardial Na^+/H^+ exchanger. *J Mol Cell Cardiol* 2008;44:228-237.
- Beguin PC, Joyeux-Faure M, Godin-Ribuot D, Levy P, Ribaut C: Acute intermittent hypoxia improves rat myocardium tolerance to ischemia. *J Appl Physiol* 2005;99:1064-1069.
- Zong P, Setty S, Sun W, Martinez R, Tune JD, Ehrenburg IV, Tkatchouk EN, Mallet RT, Downey HF: Intermittent hypoxic training protects canine myocardium from infarction. *Exp Biol Med* (Maywood) 2004;229:806-812.
- Chen TI, Hsu YC, Lien CF, Lin JH, Chiu HW, Yang KT: Non-lethal levels of oxidative stress in response to short-term intermittent hypoxia enhance Ca^{2+} handling in neonatal rat cardiomyocytes. *Cell Physiol Biochem* 2014;33:513-527.

- 12 Yang KT, Chang WL, Yang PC, Chien CL, Lai MS, Su MJ, Wu ML: Activation of the transient receptor potential M2 channel and poly(ADP-ribose) polymerase is involved in oxidative stress-induced cardiomyocyte death. *Cell Death Differ* 2006;13:1815-1826.
- 13 Wu ML, Tsai ML, Tseng YZ: DIDS-sensitive pH_i regulation in single rat cardiac myocytes in nominally HCO₃⁻-free conditions. *Circ Res* 1994;75:123-132.
- 14 Zaniboni M, Swietach P, Rossini A, Yamamoto T, Spitzer KW, Vaughan-Jones RD: Intracellular proton mobility and buffering power in cardiac ventricular myocytes from rat, rabbit, and guinea pig. *Am J Physiol Heart Circ Physiol* 2003;285:H1236-1246.
- 15 Yeves AM, Garciarena CD, Nolly MB, Chiappe de Cingolani GE, Cingolani HE, Ennis IL: Decreased activity of the Na⁺/H⁺ exchanger by phosphodiesterase 5A inhibition is attributed to an increase in protein phosphatase activity. *Hypertension* 2010;56:690-695.
- 16 Amorino GP, Fox MH: Intracellular Na⁺ measurements using sodium green tetraacetate with flow cytometry. *Cytometry* 1995;21:248-256.
- 17 Chen WH, Chu KC, Wu SJ, Wu JC, Shui HA, Wu ML: Early metabolic inhibition-induced intracellular sodium and calcium increase in rat cerebellar granule cells. *J Physiol* 1999;515 (Pt 1):133-146.
- 18 Iwamoto T, Watano T, Shigekawa M: A novel isothiourea derivative selectively inhibits the reverse mode of Na⁺/Ca²⁺ exchange in cells expressing NCX1. *J Biol Chem* 1996;271:22391-22397.
- 19 Amran MS, Homma N, Hashimoto K: Pharmacology of KB-R7943: a Na⁺-Ca²⁺ exchange inhibitor. *Cardiovasc Drug Rev* 2003;21:255-276.
- 20 Yamashita N, Nishida M, Hoshida S, Kuzuya T, Hori M, Taniguchi N, Kamada T, Tada M: Induction of manganese superoxide dismutase in rat cardiac myocytes increases tolerance to hypoxia 24 hours after preconditioning. *J Clin Invest* 1994;94:2193-2199.
- 21 Goldberg M, Zhang HL, Steinberg SF: Hypoxia alters the subcellular distribution of protein kinase C isoforms in neonatal rat ventricular myocytes. *J Clin Invest* 1997;99:55-61.
- 22 Joyeux-Faure M, Stanke-Labesque F, Lefebvre B, Beguin P, Godin-Ribuot D, Ribuot C, Launois SH, Bessard G, Levy P: Chronic intermittent hypoxia increases infarction in the isolated rat heart. *J Appl Physiol* (1985) 2005;98:1691-1696.
- 23 Snabaitis AK, Hearse DJ, Avkiran M: Regulation of sarcolemmal Na⁺/H⁺ exchange by hydrogen peroxide in adult rat ventricular myocytes. *Cardiovasc Res* 2002;53:470-480.
- 24 De Giusti VC, Caldiz CI, Ennis IL, Perez NG, Cingolani HE, Aiello EA: Mitochondrial reactive oxygen species (ROS) as signaling molecules of intravascular pathways triggered by the cardiac rennin-angiotensin II-aldosterone system (RAAS). *Front Physiol* 2013;4:126.
- 25 Eigel BN, Gursahani H, Hadley RW: ROS are required for rapid reactivation of Na⁺/Ca²⁺ exchanger in hypoxic reoxygenated guinea pig ventricular myocytes. *Am J Physiol Heart Circ Physiol* 2004;286:H955-H963.
- 26 Eigel BN, Hadley RW: Contribution of the Na⁺ channel and Na⁺/H⁺ exchanger to the anoxic rise of [Na⁺] in ventricular myocytes. *Am J Physiol Heart Circ Physiol* 1999;277:H1817-H1822.
- 27 Eigel BN, Hadley RW: Antisense inhibition of Na⁺/Ca²⁺ exchange during anoxia/reoxygenation in ventricular myocytes. *Am J Physiol Heart Circ Physiol* 2001;281:H2184-H2190.
- 28 Murphy E, Eisner DA: Regulation of intracellular and mitochondrial sodium in health and disease. *Circ Res* 2009;104:292-303.
- 29 Pike MM, Kitakaze M, Marban E: 23Na-NMR measurements of intracellular sodium in intact perfused ferret hearts during ischemia and reperfusion. *Am J Physiol* 1990;259:H1767-1773.
- 30 Murphy E, Perlman M, London RE, Steenbergen C: Amiloride delays the ischemia-induced rise in cytosolic free calcium. *Circ Res* 1991;68:1250-1258.
- 31 Butwell NB, Ramasamy R, Lazar I, Sherry AD, Malloy CR: Effect of lidocaine on contracture, intracellular sodium, and pH in ischemic rat hearts. *Am J Physiol* 1993;264:H1884-1889.
- 32 Donoso P, Mill JG, O'Neill SC, Eisner DA: Fluorescence measurements of cytoplasmic and mitochondrial sodium concentration in rat ventricular myocytes. *J Physiol* 1992;448:493-509.
- 33 Haigney MC, Lakatta EG, Stern MD, Silverman HS: Sodium channel blockade reduces hypoxic sodium loading and sodium-dependent calcium loading. *Circulation* 1994;90:391-399.
- 34 Blaustein MP, Lederer WJ: Sodium/calcium exchange: its physiological implications. *Physiol Rev* 1999;79:763-854.