Original Paper

Non-Lethal Levels of Oxidative Stress in Response to Short-Term Intermittent Hypoxia Enhance Ca^{2+} Handling in Neonatal Rat Cardiomyocytes

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Key Words

Intermittent hypoxia • Reactive oxygen species • Calcium regulation • Sarcoplasmic reticulum Ca^{2+} ATPase • Sodium calcium exchanger • Protein kinase C

Abstract

Background/Aims: Intermittent hypoxia (IH) may exert pre-conditioning-like cardioprotective effects and alter Ca^{2+} regulation; however, the exact mechanism of these effects remains unclear. Thus, we examined Ca^{2+} -handling mechanisms induced by IH in rat neonatal cardiomyocytes.

Methods: Cardiomyocytes were exposed to repetitive hypoxia-re-oxygenation cycles for 1–4 days. Mitochondrial reactive oxygen species (ROS) generation was determined by flow cytometry, and intracellular Ca^{2+} concentrations were measured using a live-cell fluorescence imaging system. Protein kinase C (PKC) isoforms and Ca^{2+} -handling proteins were analysed using immunofluorescence and western blotting. **Results:** After IH exposure for 4 days, the rate of Ca^{2+} extrusion from the cytosol to the extracellular milieu during 40-mM KCl-induced Ca^{2+} mobilization increased significantly, whereas ROS levels increased mildly. IH activated PKC isoforms, which translocated to the membrane from the cytosol, and $\text{Na}^+/\text{Ca}^{2+}$ exchanger-1, leading to enhanced Ca^{2+} efflux capacity. Simultaneously, IH increased sarcoplasmic reticulum (SR) Ca^{2+} -ATPase and ryanodine receptor 2 (RyR-2) activities and RyR-2 expression, resulting in improved Ca^{2+} uptake and release capacity of SR in cardiomyocytes. **Conclusions:** IH-induced mild elevations in ROS generation can enhance Ca^{2+} efflux from the cytosol to the extracellular milieu and Ca^{2+} -mediated SR regulation in cardiomyocytes, resulting in enhanced Ca^{2+} -handling ability.

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Introduction

Intermittent hypoxia (IH) occurs frequently under many physiological conditions, such as strenuous exercise and sojourns at high altitude, as well as pathophysiological conditions, such as asthma and obstructive sleep apnoea [1-3]. IH is characterized as cyclic episodes of hypoxia of short duration followed by normoxia [4], leading to increased generation of intracellular reactive oxygen species (ROS) during re-oxygenation following hypoxia [5]. Previously, excess ROS accumulation was itself considered injurious because it can cause lipid peroxidation, protein oxidation, DNA damage [6] and intracellular ion deregulation [7], damaging cellular physiological function. However, it is generally accepted at present that ROS may exert beneficial actions [8] because short or moderate durations of ROS generation can result in pre-conditioning protection of the heart [9] against ischemia/reperfusion (I/R) injury [1]. This protective effect is partially due to IH-induced increases in antioxidative capacity for removing excess ROS [10] and improved Ca^{2+} handling for attenuating cytosolic Ca^{2+} overload [11].

Ca^{2+} is an intracellular signalling molecule that regulates many different cellular functions. In cardiomyocytes, Ca^{2+} homeostasis plays a crucial role in maintaining the normal cycles of cardiac contraction/relaxation under physiological conditions [12]. During contraction, Ca^{2+} enters the myocyte through L-type Ca^{2+} channels and induces Ca^{2+} release from the sarcoplasmic reticulum (SR) through the ryanodine receptor (RyR). During relaxation, Ca^{2+} is transported into SR via SR Ca^{2+} -ATPase (SERCA) [13] and by Ca^{2+} extrusion via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) and other Ca^{2+} pumps [14]. Recently, there has been significant interest in improving the Ca^{2+} -handling ability for the prevention of heart disease using non-pharmacological interventions, including ischemic pre-conditioning, exercise and IH. Ischemic pre-conditioning can exert cardioprotective effects by decreasing I/R injury-induced Ca^{2+} overload [11, 15], as evidenced by the enhanced recovery of the myocardial contractile function and prevention of arrhythmias [16]. Regular moderate exercise training also results in beneficial heart adaptations by improving contractile capacity, which is associated with faster generation and decay of intracellular calcium transients [17]. In IH pre-conditioning, although the activity of RyR of SR and sarcolemmal NCX increased during I/R in cardiomyocytes isolated from rats exposed to chronic IH, these IH-induced beneficial effects were not observed in the pre-I/R status [18]. In contrast, IH with severe hypoxia yields deleterious effects, causing increases in myocardium infarction during I/R [19]. Thus, the ROS levels induced by IH may influence the injurious or beneficial cardiovascular effects because mitochondria respond to graded hypoxia by increasing ROS generation [20]. Moreover, under physiological conditions, the effects of IH-induced ROS generation on Ca^{2+} handling remain unclear. Therefore, the aims of this study were to examine the level of ROS generation induced by short-term IH exposure and the mechanisms by which the generated ROS affect alteration in Ca^{2+} regulation mediated by the Ca^{2+} -handling proteins NCX-1, SERCA-2 and RyR-2 under adaptation to IH in rat neonatal cardiomyocytes.

Materials and Methods

Chemicals and solutions

N-2-hydroxy-ethylpiperazine-N'-2-ethanesulphonic acid (HEPES)-buffered Tyrode solution consisted of 140 mM NaCl, 4.5 mM KCl, 1.0 mM MgCl_2 , 2.0 mM CaCl_2 , 11 mM glucose, 10 mM HEPES and 1.2 mM KH_2PO_4 , with pH adjusted to 7.4 at 37°C with NaOH. Ca^{2+} -free medium lacked CaCl_2 and contained 0.5 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, foetal bovine serum (FBS), horse serum (HS), Hank's balanced salt solution and penicillin were purchased from Gibco/Life Technologies, USA. Anti-protein kinase C (alpha, beta, gamma) [PKC (α, β, γ)], anti-RyR-2, anti-SERCA-2, anti-NCX-1 and anti- β -actin monoclonal antibodies were obtained from Chemicon (USA). All other chemicals were purchased from Sigma (USA).

Preparation of cultured ventricular myocytes

All procedures were performed in accordance with the Animal Care Guidelines of Tzu Chi University. Because male and female neonatal rats are not easily sexed visually, ventricles from multiple hearts are typically pooled, so neonatal rats cardiomyocytes preparations represent a mix cells from both males and females [21]. Two-day-old Sprague-Dawley rats were killed by cervical dislocation and decapitated, and ventricular myocyte cultures were prepared as described previously [22]. In brief, myocytes were dissociated from the ventricles through repeated digestion in 2 ml of enzyme solution (0.051% pancreatine and collagenase in Hank's buffer), followed by enzyme inactivation using an F-12 medium (80% F-12 nutrient mixture, 10% FBS, 10% HS and 1% penicillin). Non-myocytes were removed when the cells were pre-plated for 1 h at 37°C in an incubator (21% O_2 , 5% CO_2). Ventricular myocytes were plated on 35-mm collagen-coated cover slips in F-12 medium with 10 μM cytosine arabinoside. The medium was replaced daily. All experiments were performed on day 1 after cardiomyocyte plating.

Intermittent hypoxia exposures

Ventricular myocytes were incubated in Plexiglas box chambers (length 25 cm, width 30 cm, height 15 cm). The myocytes were exposed to normoxia (NOX; 21% O_2 , 5% CO_2 and balanced N_2) or IH (5% O_2 , 5% CO_2 and balanced N_2 for 30 min alternating with 30-min NOX), using a timed solenoid valve controlled for 1-4 days. Oxygen fractions in the chambers were continuously monitored by an oxygen detector. A micro dissolved oxygen electrode from Lazar Research Laboratories (DO-166MT-1) was used to detect fluctuation of oxygen concentrations in the medium and the chamber.

Measurement of intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$)

Cells were loaded with 5 μM Fluo-3 AM for 60 min at room temperature. Fluorescence was monitored with a fluorescent microscope (DMI3000B; Leica) using the excitation/emission wavelengths of 488/520 nm, respectively. Signal increases are presented as the peak/basal fluorescence ratio.

Measurement of membrane potentials

The membrane potential-sensitive dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($\text{DiBAC}_{4(3)}$) was used to measure membrane potentials as described previously [23]. Briefly, cardiomyocytes were incubated with HEPES-buffered Tyrode solution containing 100 nM $\text{DiBAC}_{4(3)}$ (30 min, 37°C). $\text{DiBAC}_{4(3)}$ is a bis-barbituric acid oxonol compound that partitions into the membrane as a function of membrane potential. Hyperpolarization causes extrusion of the dye and decreased fluorescence, whereas depolarization causes enhanced fluorescence. Fluorescence was monitored with a fluorescent microscope (DMI3000B; Leica) using the excitation/emission wavelengths of 490/516 nm, respectively. $\text{DiBAC}_{4(3)}$ was maintained at 100 nM in all solutions.

Mitochondrial ROS measurements

Cardiomyocytes were incubated with MitoSOX™ Red reagent 2.5 μM for 30 min before harvesting. After a PBS wash, fluorescence was measured on a FACS Calibur Flow Cytometer (Becton Dickinson Biosciences, USA) using excitation/emission wavelengths of 510/580 nm.

Immunofluorescence staining of ventricular myocytes

Cells were fixed with methanol at 4°C for 10 min. After incubation for 5 min in 5% non-fat milk, the cells were reacted with primary antibody for 60 min at 37°C, followed by secondary antibody (FITC-conjugated goat anti-rabbit IgG or anti-mouse IgG) for 60 min at 37°C. Images were obtained by confocal microscopy (TCS-SPII; Leica) with resolution of 512×512 pixels. FITC represented green fluorescence with excitation/emission wavelengths at 488/500-550 nm.

Analysis of nuclear areas

Cells were observed using laser scanning confocal microscopy (TCS-SPII; Leica). Images were analysed using Leica confocal software to calculate the sizes of nuclei stained with Hoechst 33342 fluorochrome blue fluorescence with excitation/emission wavelengths at 340/450-500 nm.

Flow cytometric analysis of cell death

Apoptosis/necrosis was determined by Annexin V-FITC Apoptosis Detection Kit (BioVision, Inc., Mountain View, CA) according to the manufacturer's recommendations. After 4-day IH or H₂O₂ treatment for 30 min at 37°C, cardiomyocytes were washed with NT, trypsinized, harvested, and stained with Annexin V-FITC and SYTOX green (BioVision, Inc., Mountain View, CA) in binding buffer for 10 min at room temperature. Fluorescence was measured on a FACSCalibur Flow Cytometer (Becton Dickinson Biosciences, USA). The excitation/emission wavelengths for Annexin V-FITC and SYTOX were 488/530 nm, respectively.

Western blots

Cells were lysed on ice with 200 µl of lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 5 mM MgCl₂, 0.5% NP-40, 1 mM PMSF, 1 g/ml pepstatin and 50 g/ml leupeptin) and centrifuged at 10,600 × *g* at 4°C for 10 min. The protein concentrations in the supernatants were quantified using a BSA Protein Assay Kit. Electrophoresis was performed using an SDS-PAGE Bis-Tris Electrophoresis System using 30 µg of reduced protein extract per lane. Resolved proteins were then transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk for 1 h at RT and probed with appropriate dilutions of primary antibodies against β-actin, PKC (α,β,γ) and NCX-1 at 4°C overnight. After the PVDF membranes were washed three times with Tris buffered saline with 0.05% Tween-20 (0.05% TTBS, 125 mM NaCl, 125 mM Tris-HCl, 0.05% Tween-20, pH 7.6) at RT, they were incubated with the appropriate secondary antibody (goat anti-mouse or anti-rabbit, 1:10,000) labelled with horseradish peroxidase for 1 h at RT and then washed three times with 0.05% TTBS. All proteins were detected using Western Lightning™ Chemiluminescence Reagent Plus (Amersham Biosciences, Arlington Heights, IL, USA). The signals were visualized by exposing the membranes to X-ray films (Kodak, USA). The resulting bands were captured with a scanner and quantified as arbitrary units (OD × band area) with ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA).

Real-time quantitative polymerase chain reaction (q-PCR)

RNA was extracted from cardiomyocytes using Trizol Reagent (Invitrogen, USA) and cDNA was synthesized using a Verso™ cDNA kit (Thermo, USA). Real-time qPCR was performed using an ABI 7300 Real Time PCR system (Life Technologies, USA) with 2× Maxima SYBR green qPCR Master Mix and ROX solution (Thermo, USA). The following primer pairs were used: NCX-1 [24] forward 5'-GTGTTTGTGCTCTTGGAACCTC-3' and reverse 5'-CGTTGCTTCCGGTGACATTG-3' and RyR-2 forward 5'-AGAGAAGGAAGTGGCAGGAA-3' and reverse 5'-CCAGTAACTCGCTGATTCTGTCT-3' and SERCA-2 forward 5'-ATGAACCTGAAATGGGCAAG-3' and reverse 5'-GGAACCTTGTACCAACAGCA-3' and Calsequestrin-2 [25] forward 5'-GGAGCATCAAAGACCCACCC-3' and reverse 5'-TTCTCCGCAAATGCCACAAT-3' and GAPDH forward 5'-TGCACCACCAACTGCTTAGC-3' and reverse 5'-GGCATGGACTGTGGTCATGAG-3'. Total RNA (3 µg) was used to perform the reverse transcription reaction. A 1:10 dilution of the synthesized cDNA with RNase-free water (total volume = 25 µl) was subsequently used for qPCR. The comparative C_t method (2^{-ΔΔC_t}) was used to quantify gene expression, where ΔΔC_t = ΔC_t (sample) - ΔC_t (reference).

Statistics

Statistical analyses were performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). For fluorescence measurements and Western blot analyses, all results are expressed as means and standard errors of the means (mean ± SEM). Statistical differences were compared using the t-test and one-way ANOVA with post-hoc test, taking a *p* value of < 0.05 as significant.

Results

IH profiles and IH-induced mitochondrial ROS generation in cardiomyocytes

IH profiles were recorded in the gas phase and medium in IH chambers, revealing the time lag in the development of hypoxia between the gas phase and medium. The nadir and peak oxygen fractions recorded in the gas phase were 5% during hypoxia and 20% during normoxia, respectively (Fig. 1A). The nadir and peak dissolved oxygen recorded in the culture medium were 9% during hypoxia and 18% during normoxia, respectively (Fig. 1B). In mitochondrial ROS measurements, the ROS levels were significantly increased by IH on

Fig. 1. Intermittent hypoxia (IH) induced mitochondrial reactive oxygen species (ROS) generation in cardiomyocytes. Normoxia (NOX) and IH profiles recorded in the gas phase (A) and medium (B) in Plexiglas box chambers. Mitochondrial ROS generation was determined by flow cytometry using the mitochondrial superoxide probe Mito-SOX Red (C). Quantification of the levels of mitochondrial ROS generation in cardiomyocytes exposed to NOX for 4 days (NOX4, $n = 5$), IH for 1–4 days (IH1–4, $n = 5$ –6 for each group), IH4 plus 1,10-phenanthroline (IH4+Phen, $n = 3$) and Mn (III) tetrakis(4-benzoic acid)porphyrin (IH4+MnTBAP, $n = 3$) (D). $**p < 0.01$ compared to NOX4. $##p < 0.01$ compared to IH4. Values are presented as means $\pm$ SEM.

days 2–4 compared with normoxia (all $p < 0.05$) (Fig. 1C and 1D). To further clarify whether the increased levels of mitochondrial ROS were induced by IH, 100 nM 1,10-phenanthroline (Phen), an Fe^{2+} chelator that reduces $\text{OH}^\cdot$ generation by inhibiting Fenton reactions, was added daily to the culture medium for 4 days (IH4+Phen). Significantly lower $\text{OH}^\cdot$ levels were observed in mitochondria for IH4+Phen than for IH4 ($p < 0.05$) (Fig. 1D), indicating that Phen could abolish increases in $\text{OH}^\cdot$ generation induced by IH4, leading to reduced mitochondrial ROS generation ($p < 0.05$) (Fig. 1C and 1D). Moreover, Phen was replaced by Mn (III) tetrakis(4-benzoic acid)porphyrin (MnTBAP) chloride, an SOD mimic and a superoxide scavenger, to confirm whether IH induced increased levels of mitochondrial ROS generation in cardiomyocytes. Consistent with the addition of Phen to IH4, 100 μM MnTBAP (IH4+MnTBAP) abolished mitochondrial ROS generation in 4-day IH-exposed cardiomyocytes ($p < 0.05$) (Fig. 1D). Therefore, our findings suggested that 4 days of IH exposure induced an increase in mitochondrial ROS generation in rat neonatal cardiomyocytes.

IH induced non-lethal ROS levels in cardiomyocytes

Cardiomyocyte death was quantified by condensation nuclei, a characteristic apoptotic feature, because condensed apoptotic nuclei have lower nuclear area values. Our results illustrated that the nuclear areas neither differed between the 1- to 4-day IH and NOX groups nor were there significant differences within any of the IH or NOX groups (Fig. 2A and 2B). To examine the levels of oxidative stress that caused nuclear condensation in cardiomyocytes, intracellular oxidative stress was induced in cardiomyocytes via exposure to various H_2O_2 concentrations for 1 h. The nuclear areas were significantly decreased by 30, 50 and 100 μM H_2O_2 (all $p < 0.05$) but not by 10 μM H_2O_2 compared with those in the control group ($p > 0.05$, Fig. 2B). To further confirm these results, we performed an Annexin V assay for calculating the percentage of viable cells (M1), apoptotic fractions (M2) and necrotic fractions (M3). The percentage of apoptotic and necrotic cells did not differ between the NOX4 and IH4 groups

Fig. 2. Intermittent hypoxia (IH) did not alter cell death in cardiomyocytes. The nuclear area was examined using Hoechst 33342 fluorochrome staining and imaged by confocal microscopy (A). The nuclear area (blue) in cardiomyocytes exposed to normoxia (NOX) and IH for 1–4 days and various concentrations of H_2O_2 ($n = 7$ –15 for each group) was quantified. Nuclear condensation was measured 4 h after washout of H_2O_2 (10, 30, 50 or 100 μM treatment for 1 h) (B). The percentage of viable cells (M1), apoptotic fractions (M2) and necrotic fractions (M3) were examined by flow cytometry using an Annexin V assay (C–E). The viable, apoptotic and necrotic fractions in cardiomyocytes in the NOX4 ($n = 12$), IH4 ($n = 15$) and NOX4+100 μM H_2O_2 ($n = 12$) groups were quantified (F). * $p < 0.05$ compared to control. Values are presented as means $\pm$ SEM.

(Fig. 2C, 2D and 2F, both $p > 0.05$). However, 100 μM H_2O_2 plus IH4 caused tremendous cell death in cardiomyocytes compared with NOX4 and IH4 (Fig. 2E and 2F, both $p < 0.05$). These findings were consistent with the results shown in Fig. 2A and 2B. Therefore, our findings suggested that 4 days of IH exposure induced non-lethal ROS levels in rat neonatal cardiomyocytes.

IH increased total PKC (α,β,γ) protein expression and markedly induced translocation of the PKC isoforms from the cytosol to the plasma membrane in cardiomyocytes

The expression levels of PKC (α,β,γ) in cardiomyocytes significantly increased in the IH2, IH3 and IH4 groups compared with the NOX4 group (all $p < 0.05$). However, significantly lower levels of the PKC isoforms were found in the IH4+Phen group than in the IH4 group. These findings indicated that Phen abolished the upregulation of PKC (α,β,γ) induced by IH ($p < 0.05$) (Fig. 3A and 3B). In addition, immunofluorescence images of PKC (α,β,γ) revealed that the PKC isoforms appeared to translocate to the plasma membrane in the IH4 group (Fig. 3C). Thus, cardiomyocytes were stained with β -catenin, a membrane marker, to determine whether the isoforms translocated from the cytosol to the plasma membrane. The merge image in Fig. 3D illustrates that staining for PKC (α,β,γ) overlaid that for β -catenin. In addition, the increased translocation of the PKC isoforms to the plasma membrane in the IH4 group was abolished by Phen and MnTBAP (Fig. 3C). Therefore, our results indicated that IH-induced increases in mitochondrial ROS generation increased the total levels of PKC (α,β,γ) and activated these PKC isoforms in rat neonatal cardiomyocytes.

Fig. 3. Intermittent hypoxia (IH)-induced increases in PKC (α,β,γ) expression and activation in cardiomyocytes. PKC (α,β,γ) protein expression in cardiomyocytes was examined by western blotting (A). The expression of total PKC (α,β,γ) protein in cardiomyocytes exposed to room air for 4 days (NOX4, $n = 7$), IH for 1–4 days (IH1–4, $n = 7$ for each group) and IH4 plus 1,10-phenanthroline (IH4+Phen, $n = 6$) was quantified (B). Representative images of PKC (α,β,γ) expression (green) as examined using immunofluorescence staining and imaged by confocal microscopy (C). Representative image of PKC (α,β,γ) translocation from the cytosol to the plasma membrane, as confirmed by staining for PKC isozymes (green) and β -catenin (red) [the plasma membrane (PM) is denoted by white arrows] (D). * $p < 0.05$ compared to NOX4; # $p < 0.05$ compared to IH4. Values are presented as means $\pm$ SEM.

IH affected the Ca^{2+} efflux capacity for alleviating KCl-induced Ca^{2+} accumulation in cardiomyocytes

A 40 mM KCl solution was used to assay alterations in $[\text{Ca}^{2+}]_i$ using the fluorescent Ca^{2+} indicator dye Fluo-3 AM and fluorescent microscope. The peak amplitude of $[\text{Ca}^{2+}]_i$ after KCl treatment did not differ between the IH and NOX groups on days 1–4, and no significant differences were found among any exposure days in the IH or NOX group (Fig. 4A and 4B). In addition, to determine whether ROS and PKC (α,β,γ) play a role in the effect of IH on KCl-mediated increases in $[\text{Ca}^{2+}]_i$, 100 nM Phen and 5 μM chelerythrine, a PKC inhibitor, were added daily in the IH4 groups (IH+Phen and IH+Che groups, respectively). However, the KCl-induced peaks in $[\text{Ca}^{2+}]_i$ did not differ between the IH4 and IH4+Phen groups or between the IH4 and IH4+Che groups.

To maintain Ca^{2+} homeostasis in response to KCl-induced increases in $[\text{Ca}^{2+}]_i$, cardiomyocytes activate two major mechanisms to decrease $[\text{Ca}^{2+}]_i$: high $[\text{Ca}^{2+}]_i$ -activated Ca^{2+} uptake by SR and Ca^{2+} efflux from the intracellular space to the extracellular space. The decay slopes of the Ca^{2+} indicator dye Fluo-3 AM were calculated at 0–30 s and 30–180 s after the initial increase in $[\text{Ca}^{2+}]_i$ induced by KCl to assess Ca^{2+} efflux capacity. Our results demonstrated that the rate constants of the $[\text{Ca}^{2+}]_i$ decay slopes at 0–30 s and 30–180 s after the initial induction by KCl were significantly higher in the IH group than in the NOX group on days 3–4 (both $p < 0.05$) and lower in the IH+Phen and IH+Che groups than in the IH4 group (both $p < 0.05$) (Fig. 4C and 4D). These results indicated that Phen and Che could abolish Ca^{2+} efflux induced by 4 days of IH exposure. In addition, the membrane potential-sensitive dye DiBAC₄₍₃₎ was used to confirm that KCl induced the same extent of plasma membrane depolarization in NOX4 and IH4 cardiomyocytes. We observed no differences in the plasma membrane potential between NOX4 and IH4 cardiomyocytes at depolarization (Fig. 4E and 4F), indicating that the enhancement of Ca^{2+} efflux induced by 3–4 days of IH exposure resulted from IH-induced ROS generation and PKC (α,β,γ) activation but not KCl-induced depolarization-dependent increases in $[\text{Ca}^{2+}]_i$. Therefore, our findings suggested that IH affected the enhancement of Ca^{2+} efflux in response to KCl-induced increases in Ca^{2+} levels in cardiomyocytes.

Fig. 4. Intermittent hypoxia (IH) enhanced the capacity of Ca^{2+} efflux mechanisms for alleviating KCl-induced increases in intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) in cardiomyocytes. Representative Ca^{2+} transients, examined by live cell imaging in fluorescence microscopy using the Fluo-3 AM dye, induced by exposure to 40 mM KCl in cardiomyocytes exposed to 4 days of normoxia (NOX4) or IH (IH4), IH4 plus 1,10-phenanthroline (IH4+Phen) or IH plus chelerythrine (IH4+Che) (A). Quantification of the peak $[\text{Ca}^{2+}]_i$ induced by 40 mM KCl (B) and the velocity of Ca^{2+} efflux as represented by the decay slopes at 0–30 s (C) and 30–180 s (D) following 40 mM KCl-induced increases in $[\text{Ca}^{2+}]_i$ in HEPES-buffered Tyrode's solution in cardiomyocytes exposed to normoxia for 1–4 days, IH for 1–4 days and IH4+Phen and IH4+Che ($n = 7$ –15 for each group). Representative changes in membrane potentials, examined by live cell

imaging in fluorescence microscopy using the DiBAC₄₍₃₎ dye, induced by 40 mM KCl (E). Quantification of membrane depolarization induced by 40 mM KCl in cardiomyocytes in the NOX4 ($n = 11$) and IH4 groups ($n = 12$) (F). * $p < 0.05$ compared to the NOX group on the same exposure day; # $p < 0.05$ compared to IH exposure for 4 days. Values are presented as means $\pm$ SEM.

IH induced NCX-1 activation in cardiomyocytes

To examine whether NCX played a role in IH-enhanced Ca^{2+} efflux, HEPES-buffered Tyrode's solution perfusion buffer was replaced by a Na^+ -free buffer or KBR7943 (an NCX transport inhibitor) to inhibit the function of NCX when cardiomyocytes were perfused with 40 mM KCl. Our results revealed no differences in the KCl-induced Ca^{2+} peaks between the IH4 and NOX4 groups in HEPES buffer as well as in Na^+ -free or KBR7943 buffer (Fig. 5A). Both decay slopes at 0–30 s and 30–180 s after the initial induction by KCl in the IH4 group were significantly higher than those in the NOX4 group perfused with HEPES buffer. However, the decay slopes did not differ between the IH4 and NOX4 groups perfused with Na^+ -free and KBR7943 buffers (Fig. 5B and 5C). These results indicated that NCX might play a role in the enhancement of Ca^{2+} efflux in cardiomyocytes induced by 4 days of IH exposure. Next, the mRNA and protein levels of NCX-1 were examined to confirm the role of NCX-1 in Ca^{2+} efflux in IH-exposed cardiomyocytes. Our results clarified that the mRNA and protein levels of NCX-1 were significantly increased in the IH4 group compared with the NOX4 group ($p < 0.05$). Concerning the protein levels, significantly lower NCX-1 levels were measured in the IH+Phen group ($p < 0.05$) (Fig. 5D and 5E). We further examined NCX-1 levels expressed on cardiomyocyte membranes using immunocytofluorescence. Our results indicated that NCX-1 levels on cardiomyocyte membranes (white arrows) were significantly higher in the IH4 group than in the NOX4 group (Fig. 5F). Thus, IH activated NCX-1, contributing to increases in the Ca^{2+} -handling ability to relieve the KCl-induced increases in $[\text{Ca}^{2+}]_i$ associated with IH-mediated increases in ROS generation in cardiomyocytes.

Fig. 5. Intermittent hypoxia (IH)-induced increases in NCX-1 activation contribute to increases in Ca^{2+} efflux capacity in cardiomyocytes. Quantification of peak intracellular calcium concentrations $[\text{Ca}^{2+}]_i$ examined by live cell imaging in fluorescence microscopy using the Fluo-3 AM dye, induced by exposure to 40 mM KCl (A) and the velocity of Ca^{2+} efflux as represented by the decay slopes at 0–30 s (B) and 30–180 s (C) following 40 mM KCl-induced increases in $[\text{Ca}^{2+}]_i$ in HEPES-buffered Tyrode's (control), Na^+ -free buffer and KBR7943 buffer in cardiomyocytes exposed to 4 days of normoxia (NOX4) or IH (IH4) ($n = 7$ –15 for each group). Quantification of NCX-1 mRNA expression in cardiomyocytes in the NOX4 ($n = 12$) and IH4 groups ($n = 12$) (D). Representative western blotting images and quantification of NCX-1 protein expression in cardiomyocytes in the NOX4 ($n = 5$), IH4 ($n = 5$) and IH4 plus 1,10-phenanthroline (IH4+Phen) groups ($n = 5$) (E). Representative images of NCX-1 expression (green) on the plasma membrane (white arrowheads) as determined by immunofluorescence and imaged by confocal microscopy (F). N: nucleus (blue). * $p < 0.05$ compared to NOX4; # $p < 0.05$ compared to IH4. Values are presented as means $\pm$ SEM.

IH induced enhancement of Ca^{2+} -mediated SR regulation in cardiomyocytes

In this study, Ca^{2+} -mediated SR regulation was examined using thapsigargin (TG) to block Ca^{2+} uptake by SR before cardiomyocytes were perfused with Na^+ -free buffer. The peak amplitude of $[\text{Ca}^{2+}]_i$ after Na^+ -free treatment did not differ between the NOX4 and IH4 groups, and no significant differences were found in TG-treated or untreated cardiomyocytes in the IH4 or NOX4 group. The rate constants of the $[\text{Ca}^{2+}]_i$ decay slopes at 0–120 s after the initial induction by Na^+ -free treatment were significantly higher in the IH4 group than in the NOX4 group ($p < 0.05$). This effect was abolished by TG treatment (Fig. 6A–C). Next, 20 mM caffeine, a RyR agonist [26], was used to induce the opening of RyRs on SR following cardiomyocyte perfusion with Ca^{2+} -free HEPES-buffered Tyrode's solution to remove

Fig. 6. Intermittent hypoxia (IH) enhanced the Ca^{2+} release capacity of SR in cardiomyocytes. Representative Ca^{2+} transients, examined by live cell imaging in fluorescence microscopy using the Fluo-3 AM dye, in cardiomyocytes treated with thapsigargin (TG) to block Ca^{2+} uptake by SR before perfusion with Na^+ -free buffer (A). Quantification of peak intracellular calcium transients induced by Na^+ -free buffer (B) and the velocity of Ca^{2+} uptake by SR as represented by the decay slopes at 0–120 s (C) following Na^+ -free buffer-induced increases in $[\text{Ca}^{2+}]_i$ in cardiomyocytes exposed to 4 days of normoxia (NOX4), NOX4 plus TG (NOX4+TG), 4 days of IH (IH4) and IH4 plus TG (IH4+TG) ($n = 8$ –10 for each group). Representative Ca^{2+} transients induced by 20 mM caffeine in cardiomyocytes perfused with Ca^{2+} -free 0.2 mM ethyleneglycol-bis

(beta-aminoethylether)-N, N'-tetraacetic acid (Ca^{2+} -free+EGTA) solution in the NOX4, IH4, IH4 plus 100 nM 1,10-phenanthroline (IH4+Phen) and IH4 plus 5 mM chelerythrine (IH4+Che) groups (D). Quantification of the Ca^{2+} storage capacity of SR as represented by the amplitude of the Ca^{2+} peak induced by 20 mM caffeine in the NOX4 ($n = 31$), IH4 ($n = 42$), IH4+Phen ($n = 25$) and IH4+Che groups ($n = 8$) (E). Quantification of ryanodine receptor 2 (RyR-2) (NOX4, $n = 15$; IH4, $n = 15$), calsequestrin-2 (CASQ-2) (NOX4, $n = 13$; IH4, $n = 13$) and sarco/endoplasmic reticulum Ca^{2+} -ATPase 2 (SERCA-2) (NOX4, $n = 12$; IH4, $n = 12$) mRNA expression in cardiomyocytes exposed in the NOX4 and IH4 groups (F). Representative images of RyR-2 fluorescence staining imaged by confocal microscopy (G) and quantification of RyR-2 protein expression levels as represented by immunofluorescence intensity in cardiomyocytes in the NOX4 ($n = 21$), IH4 ($n = 18$) and IH4+Phen groups ($n = 18$) (H). N: nucleus (blue). * $p < 0.05$ compared to NOX4; # $p < 0.05$ compared to IH4. Values are presented as means $\pm$ SEM.

cytosolic Ca^{2+} . Our results demonstrated that the caffeine-induced increase in the Ca^{2+} transient amplitude, representing the Ca^{2+} -handling ability of SR, was significantly higher in the IH4 group than that in the NOX4 group ($p < 0.05$). A significantly lower Ca^{2+} transient amplitude was noted in the IH+Phen group than in the IH4 group ($p < 0.05$), whereas no difference was observed between the IH4+Che and IH4 groups ($p > 0.05$) (Fig. 6D and 6E). Next, we examined the mRNA expression of RyR-2, calsequestrin-2 (CASQ-2) and SERCA-2

in SR in IH-induced cardiomyocytes. Figure 6F reveals that the mRNA expression levels of RyR-2 were significantly higher in the IH4 group than in the NOX4 group ($p < 0.05$); however, no difference was found in the mRNA expression levels of CASQ-2 and SERCA-2 between the two groups ($p > 0.05$). In addition, we used an immunocytofluorescence stain to further confirm the protein expression levels of RyR-2 in cardiomyocytes. Our results indicated that the protein expression levels of RyR-2 in SR were significantly increased in the IH4 group compared with the NOX4 group ($p < 0.05$) (Fig. 6G). However, significantly lower protein expression levels of RyR-2 were observed in the IH4+Phen group ($p < 0.05$) (Fig. 6H). Therefore, our results indicated that IH-induced increases in ROS generation played a role in the IH-induced augmentation of RyR-2 mRNA and protein expression and the activity of RyR-2 and SERCA-2, contributing to the increase in Ca^{2+} -mediated SR regulation in IH-exposed cardiomyocytes.

Discussion

A cell culture model of IH might provide further insight into the cellular mechanisms associated with Ca^{2+} regulation during adaptation to IH in cardiomyocytes. The phenotype of cultured neonatal cardiomyocytes is extremely stable, and their contractile profile during hypoxia-re-oxygenation is comparable to that of in situ hearts during I/R [27]. Previous findings indicated that hypoxic stress is not associated with any significant metabolic, structural or functional damage in cardiomyocytes [28]. In this study, spontaneous contractile activity remained stable up to 4 days in cultured neonatal cardiomyocytes exposed to NOX or IH. In addition, our major findings provide evidence that IH-induced non-lethal oxidative stress generation increased PKC (α, β, γ) expression, which resulted in increased NCX-1 expression and activity, leading to enhanced Ca^{2+} efflux capacity. Simultaneously, the IH-induced increases in SERCA and RyR-2 activities and RyR-2 expression resulted in improved Ca^{2+} storage and release capacity of SR in cardiomyocytes. These alterations worked to strengthen the Ca^{2+} -handling ability of cardiomyocytes.

There are at least two types of IH: acute intermittent hypoxia (AIH; several episodes over a short period) and chronic intermittent hypoxia (CIH; lasting several days or weeks). Both AIH and CIH essentially involve recurrent re-oxygenation episodes, leading to increased intracellular oxidative stress generation [29]. The burst of ROS generation observed during hypoxia-re-oxygenation is considered one of the key factors in cell injury. Mild ROS generation can also be protective in a pre-conditioning-like manner and induce stress responses, leading to survival [9]. However, increases in infarct size induced by I/R were found in the hearts of rats exposed to IH induced by exposure to 5% O_2 for 4 h [30] and 35 consecutive days (8 h/day) [1]. Of note, IH pre-conditioning with 10% O_2 for 4 h induces cardioprotection, as illustrated by a reduction in infarct size in isolated rat hearts [30]. These cardioprotective effects were also observed after chronic IH induced by exposure to 9.5–10% O_2 for 20 consecutive days (25–70 min/day) [31]. These effects were observed probably because of the correlation between significant increases in cellular ROS generation and the degree of hypoxia compared with the degree of normoxia [20]. In this study, IH induced by 1–4 days of exposure to 9% O_2 dissolved in the culture medium did not increase cell death in cardiomyocytes, possibly because 9% O_2 dissolved in medium did not cause a burst of ROS generation in cardiomyocytes. Thus, cardiomyocytes were treated with H_2O_2 (10, 30, 50 and 100 μM) to mimic different degrees of ROS-induced cardiomyocyte death. H_2O_2 -induced ROS generation, which significantly increases cell death in cardiomyocytes, was not noted at lower concentrations (approximately 10 μM) [20, 32] but was observed at higher concentrations (>50 μM) [33, 34]. Compared with the number of cardiomyocytes treated with 10 μM H_2O_2 , the number of cardiomyocytes was not decreased by 4 days of IH exposure, which suggests that 4 days of IH induces non-lethal oxidative stress in cardiomyocytes.

Oxidative stress during adaptation to IH plays an important role in the induction of endogenous PKC isozymes [8]. Furthermore, diacylglycerol and phospho-

lipase C phosphorylation induced by ROS increase PKC activation [8, 35, 36]. In both neonatal and adult ventricular myocytes, the conventional PKC isozymes (α , β , γ) can be activated by ROS [8, 35, 36], Ca^{2+} and lipid [37] through translocation of the isozymes from the cytosol to the plasma membrane [38]. PKC also plays important regulatory roles in the upregulation of cardiac NCX-1 [39], the stimulation of Ca^{2+} -pumping ATPases [40] and the function of cardiac SR Ca^{2+} release channels in heart tissue, resulting in an increase in the Ca^{2+} -handling ability [41, 42], which may help to prevent Ca^{2+} overload-induced cell death [32].

Of note, under physiological conditions, increases in myocardial force production and power output in cardiomyocytes are associated with a faster rise and decay of the intracellular calcium transients and improved myofilament sensitivity to calcium [17]. However, these capacity were not observed in adult cardiomyocytes isolated from rats exposed to IH for 7 days [18] or rats exposed to intermittent high-altitude hypoxia for 42 days [11]. In this study, to examine Ca^{2+} -handling ability in IH-exposed cardiomyocytes, we used high K^+ [43] to increase $[\text{Ca}^{2+}]_i$ to activate the Ca^{2+} efflux mechanism [44] and Ca^{2+} uptake by SR [45]. Our findings showed that IH did not alter voltage-gated Ca^{2+} channel opening, resulting in a similar degree of Ca^{2+} influx into cardiomyocytes as that induced by KCl. Conversely, to reduce cytosolic Ca^{2+} levels in cardiomyocytes, Ca^{2+} is removed via NCX and taken up into SR [45]. Thus, IH-induced ROS generation plays a role in increasing NCX activation by PKC (α , β , γ) and enhancing SERCA activity, helping to enhance the removal of cytosolic Ca^{2+} .

It is well known that CASQ2 is a high-capacity Ca^{2+} -binding protein expressed inside SR [46]. CASQ-2 binds RyR-2 to form the SR luminal RyR-2 Ca^{2+} release channel complex, which participates in regulating Ca^{2+} release from SR [47]. Further, SR is the primary component responsible for Ca^{2+} storage that plays a key role in the maintenance of intracellular Ca^{2+} homeostasis in cardiomyocytes [48]. In this study, the amplitude of caffeine-stimulated Ca^{2+} transients in IH-exposed cardiomyocytes for 4 days was significantly increased compared with that in cardiomyocytes under normoxia. RyR-2 expression was increased by IH. However, CSAQ-2 and SERCA-2 mRNA expression was unaltered in IH-exposed cardiomyocytes. These findings suggest that the increased caffeine-stimulated Ca^{2+} transients in cardiomyocytes result from increases in Ca^{2+} release by SR. Thus, our findings indicate that ROS generation induced by 4 days of IH exposure enhances RyR-2 activity and expression levels, resulting in increased Ca^{2+} release by SR in cardiomyocytes.

In summary, IH induced a mild increase in ROS generation that did not cause cardiomyocyte death. The non-lethal ROS level helped, in part, to increase PKC (α , β , γ) protein expression and translocation, resulting in increased NCX-1 expression and activity and leading to enhanced Ca^{2+} efflux from the cytosol to the extracellular milieu. In addition, Ca^{2+} uptake and release by SR were significantly enhanced by IH because of the increased SERCA and RyR-2 activities and RyR-2 expression levels induced by mildly increased ROS generation. In conclusion, this study suggests that IH can promote the Ca^{2+} -handling ability of rat neonatal cardiomyocytes by mildly increasing ROS generation, resulting in enhanced Ca^{2+} efflux from the cytosol to the extracellular milieu and increased Ca^{2+} release and storage by SR.

Limitations

Although the use of animal models to study cardiac diseases has become standard in cardiovascular research [49], it is difficult to analyse Ca^{2+} regulation in the whole hearts or animals. Recently, multiparametric electrophysiological imaging of the mammalian heart was developed to detect Ca^{2+} transients in vivo; however, this measurement is difficult to apply under IH. Conversely, freshly isolated adult or cultured neonatal rat cardiomyocytes have proven useful for analysing states of oxygen and volume restriction conditions that are known to stimulate anoxia and ischaemia at the cellular level, respectively [50]. However, in freshly isolated cardiomyocytes, membrane proteins, such as receptors and ion channels, are damaged during the enzymatic isolation procedure [51]. These impairments of membrane proteins might prevent the adaptation of cardiomyocytes to IH under physiological

conditions. For instance, IH-induced enhanced Ca^{2+} handling was observed under I/R but not at pre-I/R in adult cardiomyocytes isolated from rats exposed to IH for 7 [18] or 42 days [11]. In this study, although neonatal cardiomyocytes exposed to IH provided a convenient method for investigating the mechanisms of IH-induced cardiomyocyte injuries, other physiological events, such as cardiovascular circulation and hormone production, must be considered when our findings are applied to systematic physiology.

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