Inhibition of Reverse-Mode Sodium-Calcium Exchanger Activity and Apoptosis by Levosimendan in Human Cardiomyocyte Progenitor Cell-Derived Cardiomyocytes after Anoxia and Reoxygenation

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Abstract

Levosimendan, a known calcium sensitizer with positive inotropic and vasodilating properties, might also be cardioprotective during ischemia-reperfusion (I/R) insult. Its effects on calcium homeostasis and apoptosis in I/R injury remain unclear. Na+/Ca2+ exchanger (NCX) is a critical mediator of calcium homeostasis in cardiomyocytes, with reverse-mode NCX activity responsible for intracellular calcium overload and apoptosis of cardiomyocytes during I/R. We probed effects and underlying mechanisms of levosimendan on apoptosis and NCX activity in cultured human cardiomyocyte progenitor cells (CPC)-derived cardiomyocytes undergoing anoxia-reoxygenation (A/R), simulating I/R in vivo. Administration of levosimendan decreased apoptosis of CPC-derived cardiomyocytes induced by A/R. The increase in reverse-mode NCX activity after A/R was curtailed by levosimendan, and NCX1 was translocated away from the cell membrane. Concomitantly, endoplasmic reticulum (ER) stress response induced by A/R was attenuated in CPC-derived cardiomyocytes treated with NCX-targeted siRNA or levosimendan, with no synergistic effect between treatments. Results indicated levosimendan inhibited reverse-mode NCX activity to protect CPC-derived cardiomyocytes from A/R-induced ER stress and cell death.

Introduction

Ischemia-reperfusion (I/R) myocardial injury is a common clinical occurrence: e.g., in aortic de-clamping after cardioplegic arrest in cardiac surgery or prompt therapeutic restoration of blood supply in acute myocardial infarction. Mechanisms mediating reperfusion injury include altered metabolism and Ca2+ handling, oxidative stress, microvascular dysfunction, and inflammation [1]. Tremendous effort has been devoted to strategy for ameliorating myocardial reperfusion injury [2], as it carries definite implications in cardiomyocyte survival and myocardial function [3].

Cardinal feature of higher cytosolic Ca2+ concentration ([Ca2+]i) in cells subject to I/R plays a pivotal role in subsequent cellular damage: e.g., hypercontracture as a direct result of excessive [Ca2+]i. Along with other physiological derangements, excessive [Ca2+]i causes arrhythmia, myocardial stunning, and cell death by necrosis or apoptosis. In cardiomyocytes, excitation-contraction coupling is mediated by cyclic changes of [Ca2+]i, which in turn is regulated by several Ca2+ channels. The Na+/Ca2+ exchanger (NCX) on plasma membrane, 10–15 times more efficient than Ca2+-ATPase, is the major channel for Ca2+ extrusion during diastole normally [4]. Depending on membrane potential and Na+ or Ca2+ gradients across plasma membrane, NCX functions in either forward mode (extracellular Na+ in exchange for intracellular Ca2+) as Ca2+ efflux mechanism in physiological conditions, or reverse mode (extracellular Ca2+ in exchange for intracellular Na+). NCX working in reverse mode is responsible for elevated [Ca2+]i, that occurs during ischemia and early reperfusion [5,6,7]. With cessation of blood flow, ATP is depleted when supply of oxygen and energy source are discontinued abruptly. The Na+/K+ pump, main Na+ extrusion pathway, comes to a halt, leading to accumulation of Na+ in cytosol. Higher intracellular Na+ concentration in turn reverses direction of action of plasma membrane NCX, causing Ca2+ influx. In early reperfusion, as extracellular H+ is washed away by re-established blood flow, Na+ influx through Na+/H+ exchanger aggravates Na+ and again through the action of reverse mode NCX, Ca2+ overload.

Ischemia induces accumulation of misfolded proteins in endoplasmic reticulum (ER) as well as endoplasmic reticulum stress (ERS) response [8]. Expression of glucose-regulated protein 78 (GRP78), an ER chaperone, increases in such response and can serve as a marker of ERS [8]. Intense or persistent ERS can initiate apoptosis, either by cleavage of pro-caspase 12 or ER-mitochondrial crosstalk, with subsequent activation of down-
stream caspase cascade [8]. Levosimendan, a Ca\textsuperscript{2+}-sensitizing inotropic [9], may help preserve cardiomyocytes in setting of I/R [10–17]. In animal experiments, levosimendan-conferred protection from I/R-induced apoptosis has been attributed to its activation of mitochondrial ATP-dependent K\textsuperscript{+} channels.[10–12] The present study evaluated effects of levosimendan on simulated I-R injury and apoptosis in human cardiomyocyte progenitor cell (CPC)-derived cardiomyocytes in vitro, plus involvement of NCX activity and ERS-induced apoptosis pathway in the mechanism. Among isoforms of NCX, ubiquitous NCX1 is highly expressed in the heart [4]. Our data was the first to suggest inhibition of NCX1 activity by levosimendan protecting CPC-derived cardiomyocytes against I/R-induced apoptosis.

**Materials and Methods**

**Human Cardiomyocyte Progenitor Cell (CPC)-derived Cardiomyocytes Culture**

This research was approved by China Medical University Hospital Institutional Review Board. Written informed consent was obtained from each patient’s guardian or caretaker. Human cardiomyocyte progenitor cells were isolated from right atrial auricle specimens of individuals undergoing cardiac surgery, which has been a frequently adopted method for isolation of human cardiac progenitor cells [18,19]. There were 10 patients with equal distribution in both genders, all less than 18 years of age, with various congenital heart diseases: ventricular septal defect, atrial septal defect, endocardial cushion defect and tetralogy of Fallot. Tissue specimens were washed, minced and maintained in growth medium composed of 1:3 Medium 200 (supplemented with LSGS) and M199 containing 1×MEM nonessential amino acid, 1×penicillin/streptomycin, 10 ng/ml bFGF, and 10% fetal bovine serum. Growing cells were harvested and subcultured on reaching 90% confluence and could be passaged for up to six generations. CPCs were subsequently cultured into CPC-derived cardiomyocytes in differentiation medium (Iscove’s Modified Dulbecco’s Medium containing equal volume of F12, 2 mM L-glutamine, 2% horse serum, 1×MEM nonessential amino acid, 2 ug/ml transferrin, 2 ug/ml insulin, and 1×penicillin/streptomycin). These CPC-derived cells were checked for expression of cardiomyocyte-specific α-sarcomeric actinin [19] and used for experiments. Electrophysiological characteristics of cardiomyocytes, including action potential duration and peak L-Type calcium current, were also measured [20].

**Ischemia-reperfusion Simulation in vitro: Anoxia-reoxygenation (A/R)**

To simulate I/R, CPC-derived cardiomyocytes were incubated in a hypoxic chamber (Biophoenix) filled with 95% N\textsubscript{2} and 5% CO\textsubscript{2} for 2 or 14 hours in culture medium deprived of glucose and serum, with glucose-free DMEM (Gibco-BRC) supplemented with 100 U/ml penicillin and 100 ug/ml streptomycin. This anoxic period was followed by reoxygenation by transferring cells to a normoxic incubator in 95% air/5% CO\textsubscript{2} for 2 hours in normal culture medium. Levosimendan 2 μM was added at the beginning of reoxygenation for the experimental group, experimental procedures illustrated in Figure 1.

**Antibodies**

The method to generate antibodies against NCX1 was described in our published report [4]. Antibodies against NCX1 were generated, recognizing full-length NCX1 overexpressed in HEK293T cells (with no detectable endogenous NCX) and endogenous mouse cardiac NCX1 [4]. Specificity of antibodies was established by demonstrating that addition of another antigen, peptide sequence of NCX1 intracellular loop, competes out bands formed by NCX1 and antibodies. Alexa Fluor 488 phaloin and Alexa Fluor 550 were purchased from Invitrogen. Donkey anti-mouse IgG-FITC was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-GRP78 (1:2000, Abcam, Cambridge, MA), anti-caspase-12 (1:2000, Abcam, Cambridge, MA) and anti-β-actin (1:30000, Sigma, St.Louis, MO) were obtained.

**Measurement of Reverse-mode NCX Activity**

Three pieces of 12 mm cover-glass were put in a 3.5 cm dish. Human CPC-derived CMs were cultured on gelatin-coated 12 mm cover-glass (1×10\textsuperscript{6} cells/dish), incubated with 10 μM fura-2 acetoxymethyl ester (AM) at 37°C for 2 h, then loaded with Na\textsuperscript{+} by immersion in Ca\textsuperscript{2+}-free Na\textsuperscript{+} loading buffer (145 mM NaCl, 5 mM KCl, 1 mM MgCl\textsubscript{2}, 100 μM ouabain, and 10 mM HEPES, pH 7.4) for 10 min at 37°C. Isolated CPC-derived cardiomyocytes were selected to measure reverse-mode NCX1 activity by abruptly replacing Ca\textsuperscript{2+}-free buffer with Na\textsuperscript{+}-free buffer (145 mM N-methylglucamine, 5 mM KCl, 1 mM MgCl\textsubscript{2}, 2 mM CaCl\textsubscript{2}, 1 mM ouabain, and 10 mM HEPES, pH 7.4). Fura-2 fluorescence ratio obtained by 340 nm and 380 nm excitation (F\textsubscript{340}/F\textsubscript{380}) was monitored by inverted microscope (IX-70, Olympus Co., Tokyo, Japan) using a 40x oil immersion objective (1Apo 40x oil/1.35; Olympus Corporation, Tokyo, Japan). Amplitude of [Ca\textsuperscript{2+}], rise upon replacement of buffers was then calculated as a measurement of reverse-mode NCX activity [4].

**Fluorescence-activated Cell Sorting (FACS) Analysis**

Apoptotic cells were quantified by propidium iodide (PI) and DiOC\textsubscript{6} staining with FACS analysis [21–23]. Cells plated in 60-mm Petridishes at 8×10\textsuperscript{4} cells/plate in solution containing 50 mg/ mL PI and 20 nM DI0C\textsubscript{6} (Molecular Probes, Sunnyvale, CA) were analyzed by FACS (FL-3 channel). Ratio of apoptotic cells was detected by DiOC\textsubscript{6} fluorescence, results obtained for both experimental and control groups.

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**Figure 1. Schematic diagram for experimental procedures of anoxia-reoxygenation. CM: cardiomyocyte; hCPC: human cardiomyocyte progenitor cell; Levo: levosimendan; Re: reoxygenation. doi:10.1371/journal.pone.0085909.g001**

**Table 1.** Levosimendan and Human Cardiomyocytes after Anoxia

| Condition          | Control | Anoxia 2h | Anoxia 14h |
|--------------------|---------|-----------|------------|
| hCPC-derived CM    | control | Re + Levo | Re + Levo  |
|                    |         | control   | Re         |
|                    |         | control   | Re + Levo  |
|                    |         | control   | Re + Levo  |
Immunocytostaining and Fluorescence Microscopy
Human CPC-derived CMs grown on cover slips were rinsed three times with PBS and fixed with 3.7% formaldehyde in PBS for 20 min at room temperature. Samples stained with appropriate primary antibodies were incubated with FITC-conjugated goat anti-mouse antiserum at 4°C overnight, cover slips mounted with Vectashield (Vector Laboratories, Burlingame, CA) and viewed under inverted confocal microscope (Zeiss Axiovert 200 M, Carl Zeiss, Jena, Germany) with 63×/1.4 Plan-Apochromat objective.

Results
Levosimendan Protects Cultured CPC-derived Cardiomyocytes Against Anoxia-reoxygenation (A/R)-induced Apoptosis
Effect of levosimendan on apoptosis of CPC-derived cardiomyocytes subjected to A/R was tested, with hydrogen peroxide at 10 mM as positive control. Adding levosimendan at reoxygenation reduced apoptotic CPC-derived cardiomyocytes from 27.6±2.4% to 8.2±2.1% (P<0.05) (Figure 2).

Increased Reverse Mode NCX Activity After A/R Abolished by Levosimendan
To identify CPC-derived cardiomyocytes, sarcomeric specific α-actinin were detected and cells co-stained with fluorescently labeled phalloidin (Figure 3A). Expression of sodium-calcium exchanger in CPC-derived cardiomyocytes was examined by immunocytochemistry with immunofluorescent microscope (Figure 3B). Under normoxic condition, administering levsimendan does not affect reverse-mode NCX activity in CPC-derived cardiomyocytes (Figures 4Aa and 4B). Reverse-mode NCX activity was decreased after anoxia for 2 h, and activity was reduced even more dramatically after anoxia for 14 h. After reoxygenation for 2 hours, however, there was rebound in NCX activity: much more Ca²⁺ transported into cells than controls (Figure 4Ab). Addition of levosimendan during reoxygenation suppressed heightened post-anoxic NCX activity to below control level (Figures 4Ab, 4Ac, 4B).

Levosimendan Decreased Plasma Membrane NCX Localization during Reoxygenation
To explain the decrease in NCX activity with levsimendan administration, we examined NCX localization, as NCX can only exert its action of Na⁺-Ca²⁺ exchange when localized to cellular membrane. Such localization of NCX increased after A/R (Figure 5A middle row and Figure 5B) compared to controls (Figure 5A upper row). When levsimendan was administered at reoxygenation, membrane localization of NCX was decreased (Figure 5A lower row and Figure 5B). In addition, cell size apparently increased after A/R, probably via osmosis due to greater intracellular concentration of Na⁺ and other solutes.

Levosimendan Abrogated GRP78 and GRP94 Expression Enhancement and Caspase 12 Activation in Anoxia-reoxygenation Induced ERS Response
Expression of GRP 78 and GRP94, ERS markers, significantly increased in CPC-derived cardiomyocytes in A/R (Figures 6A, 6B). Caspase 12 cleavage, which activates downstream apoptotic mechanism, also increased during A/R (Figure 6A). Yet levsimendan significantly reduced this GRP 78 and GRP94 expression and caspase 12 activation in A/R (Figures 6A, 6B).

NCX Knockdown by siRNA Achieved the Same Effect of ERS Response Mitigation during A/R as Levosimendan Administration
To test for increased NCX activity, with attendant increase in [Ca²⁺i] during A/R, as responsible for ERS response in A/R, we performed NCX knockdown experiment. NCX siRNA transfection alone could negate higher GRP 78 expression and caspase 12 activation in A/R (Figure 7), similar to levsimendan addition at reoxygenation (Figure 6). No further decrease in GRP78

Transient Transfection in vitro
CPC-derived cardiomyocytes were transiently transfected with NCX siRNA (Invitrogen, Carlsbad, CA) using HiPerFect Transfection Reagent (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer’s instructions. Meanwhile, cells were transfected with scrambled siRNA as control; these were used for experiments after transfection for 48 hours.

Subcellular Fractionation
Cytoplasmic and membrane fractions of human CPC-derived cardiomyocytes were prepared with a CNM compartment protein extraction kit (BioChain, Hayward, CA) according to manufacturer’s instructions. Briefly, transfected cells (3×10⁶ cells) were harvested, washed with PBS, and then centrifuged at 500×g for 5 min. Cell pellet was homogenized in 200 ul of buffer C plus protease inhibitors, rotated at 4°C for 20 min, then centrifuged at 4°C, 12000×g for 20 min. Cytoplasmic proteins in the supernatant were collected, pellet washed with 400 ul of buffer W and resuspended in 100 ul of buffer N at 4°C for 20 min. Nuclear proteins were extracted and recovered in the supernatant after centrifuge at 4°C, 12000×g for 20 min. Finally, to obtain membrane proteins, the cell pellet containing cell debris was extracted with 100 ul of buffer M and rotated at 4°C for 20 min. Supernatant was centrifuged at 4°C, 12000×g for 20 min. Membrane proteins in supernatant were collected, samples from these fractions denatured and subjected to SDS-PAGE.

Western Blot Analysis
Total proteins from CPC-derived cardiomyocytes were extracted by RIPA lysis buffer (Millipore, Billerica, MA), as per manufacturer’s instructions. Proteins (30 ug) were run on a 10% SDS-PAGE gel, transferred onto PVDF membranes, and blocked. Membranes were incubated with primary antibodies: anti-NCX (1:50000.), anti-GRP94 (1:5000, GeneTex, San Antonio, TX), anti-GRP78 (1:2000, Abcam, Cambridge, MA), anti-caspase-12 (1:2000, Abcam), anti-GAPDH (1:10000, GeneTex), anti-pan-cadherin (1:10000, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-β-actin (1:30000, Sigma, St. Louis, MO) overnight at 4°C. Membranes were extensively washed and incubated with horseradish peroxidase-labeled immunoglobulin G (Jackson Immunoresearch, West Grove, PA), immunoreactive bands detected by chemiluminescence methods (Millipore) and visualized on X-ray films (Kodak, Rochester, NY). Densitometric analysis was performed by Image J software (NIH, Bethesda, MD) [19].

Statistics
Statistical analyses used SPSS version 13.0 software (SPSS, Chicago, IL). Data were presented as mean ± standard deviation (S.D.), intergroup comparisons performed with Student’s t-test or one-way ANOVA followed by Tukey’s post-hoc test. p≤0.05 was considered statistically significant.
expression or caspase 12 cleavage appeared with levosimendan administered in addition to NCX knockdown by siRNA (Figure 7).

Discussion

As stated, myocardial I/R occurs frequently. Myocardial ischemia may be unpreventable (e.g., acute myocardial infarction), and attendant deleterious effects can only be reduced by keeping...
the duration of ischemia as short as possible. The start of reperfusion is more or less under the control of clinicians, but there is no effective countermeasure against reperfusion injury. Reperfusion induced cardiomyocyte death has become a significant limiting factor to the achievable result in treating patients with myocardial infarction [3]. Preserving viability of cardiomyocytes

Figure 4. Effect of levosimendan on NCX activity in cultured human cardiomyocyte progenitor cell (hCPC)-derived cardiomyocytes after anoxia/reoxygenation (A/R). (A) Reverse-mode NCX activity was measured with or without treatment of Levosimendan under control conditions (a), after anoxia for 2 hours (b), and after anoxia for 14 hours (c). Reverse-mode NCX activity 2 and 14 hours of anoxia were determined; higher expression of GRP78 and caspase 12 did not reach statistical significance until 14 hours of anoxia (Figure 7). (B) NCX activity was estimated by amplitude of increase in intracellular calcium concentration, \( \Delta [\text{Ca}^{2+}]_i = \text{peak} [\text{Ca}^{2+}]_i - \text{basal} [\text{Ca}^{2+}]_i \). (n=15 for each experimental group). Values are expressed as mean ± S.D. from three independent trials. *p<0.05 versus untreated control; **p<0.01 versus untreated control and anoxia for 2 h. Similar results emerged from three independent experiments. A: anoxia; Ctrl: control; Re: reoxygenation; Levo: Levosimendan.
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has also arisen as a key principle in managing patients with heart failure [11]. In short, there is an unmet demand for cardioprotection in care of cardiac patients; an applicable method to preserve cardiomyocytes is vigorously sought after. In addition, administration of an inotrope is frequently needed to treat manifestations of heart failure in setting of myocardial I/R. Levosimendan has been introduced into clinical usage as an unique Ca$^{2+}$-sensitizing inotrope, and reported to be protective against reperfusion injury in laboratory studies with animal cells. It raises cardiac contractility by increasing sensitivity of troponin C for Ca$^{2+}$ but not [Ca$^{2+}$]i or oxygen consumption [8]. The activation of mitochondrial ATP-sensitive potassium channels by levosimendan might produce pre- or post-conditioning effects and prevent cardiomyocyte necrosis and apoptosis in I/R [24,25]. It is also a vasodilator, enhancing coronary blood flow while reducing cardiac preload and afterload. These effects benefit myocardium recovering from I/R insult. Using an anti-ischemic inotrope thus remains a possible advantage over conventional β-adrenergic agonists in patients with myocardial I/R. Mechanisms of this levosimendan-conferred cardioprotection remain unclear. Given detrimental effects of

Figure 5. NCX expression on cellular membrane during A/R. (A) Double labeling of cultured human cardiomyocyte progenitor cell(hCPC)-derived cardiomyocytes with NCX (red) and Nuclei (blue). Upper row: untreated control (400X). Middle row: increased NCX expression on cellular membrane and cell swelling after A/R (800X). Lower row: leysosimendan administered at re-oxygenation and reduced cellular swelling (800X). (B) Western blot for NCX quantification on cellular membrane. Each point represents mean ± S.D. of three independent trials, *p<0.05 and **p<0.01. A/R: anoxia-reoxygenation; Levo: Levosimendan. doi:10.1371/journal.pone.0085909.g005

Figure 6. Increased GRP78 expression (6A), GRP94 expression (6B) and caspase 12 activation in anoxia-reoxygenation induced ERS response were abrogated by adding levosimendan, each result obtained from sample of 3×10^6 cells. Each point represents mean ± S.D. of three independent experiments. *p<0.05 and **p<0.01 versus untreated control and A/R+Levo. A/R: anoxia-reoxygenation; Levo: Levosimendan. doi:10.1371/journal.pone.0085909.g006

Figure 7. NCX knockdown with NCX siRNA attained effects similar to levosimendan administration in mitigating anoxia/reoxygenation-induced ERS response. Adding levosimendan did not enhance effects obtained by NCX siRNA alone. Results were obtained from samples of 3×10^6 cells each. Each point represents mean ± S.D. of three independent trials. *p<0.05 and **p<0.01 versus untreated control. Levo: Levosimendan. doi:10.1371/journal.pone.0085909.g007
intracellular Ca\textsuperscript{2+} overload in I/R, we set out to ascertain how levsosimendan affects this pathophysiology. We aimed to determine effects of levsosimendan on A/R in human cells, providing stronger and more direct evidence of its protective actions against reperfusion injury in clinical application. With no human cardiomycocyte cell line available, we obtained atrial tissue specimen from patients undergoing cardiac surgery, and followed reported methods to cultivate, propagate, and differentiate progenitor cells into cardiomyocytes for further tests. Such harvesting of endogenous cardiac stem cell from surgical waste has been utilized in a clinical trial recently [26].

Several groups have isolated cardiac stem cells relying on expression of certain markers (c-kit, Sca-1, or Isl1) or particular biological behavior of cells [Hoechst dye efflux or formation of cardiosphere] [27]. Little is known about the interrelationship between stem cell types isolated by diverse means, even less about their numbers and activities in various pathological conditions. It has been reported that number of stem cells rises significantly in hypertrophic myocardium [27]. All our patients show congenital heart malformations and right heart overload; we cannot comment on whether cells acquired from hearts with other diseases would behave differently.

NCX, working in reverse mode, is the main channel for Ca\textsuperscript{2+} influx during I/R. Inhibition of reverse mode NCX activity, with reduced cellular Ca\textsuperscript{2+} accumulation, proves cardioprotective and reduces infarct size [5]. Our results indicated NCX activity (reverse mode) initially reduced after hypoxic insult to CPC-derived cardiomycocytes, consistent with our previous report [4], and after reoxygenation NCX activity rebounded to above control levels. This should contribute significantly to aggravated intracellular Ca\textsuperscript{2+} overload during the initial phase of reoxygenation and ensuing changes causing cell death. Addition of levsosimendan during reoxygenation period abolished this heightened NCX activity and reduced [Ca\textsuperscript{2+}]\textsubscript{i}, with ensuant water influx and cell swelling. Reoxygenation aggravates cellular swelling due to rapid normalization (decrease) of extracellular osmolarity and pH (which intensifies intracellular Na\textsuperscript{+} overload through the action of Na\textsuperscript{+}/H\textsuperscript{+} exchanger). Cells after anoxia-reoxygenation appeared larger than controls in our experiments. Lighter-stained nuclei might result from dilutional effect, as these cells were swollen. Levsosimendan will contribute to [Na\textsuperscript{+}]\textsubscript{i} accrual in reoxyxygenation, as it inhibits outward Na\textsuperscript{+} transport via reverse mode NCX activity.

Intense ERS obviously emanates from A/R injury, with its attendant deprivation of substrate and energy source and perturbation of protein synthesis process. Greater expression of GRP78, GRP94 and activation of caspase 12 in results clearly indicated this reaction. Surprisingly, levsosimendan could return GRP78, GRP94 expression and caspase 12 activation to control levels, a mitigation of ERS response. NCX knockdown by siRNA could achieve a similar effect. Adding levsosimendan to NCX knockdown yielded no synergistic effects. This indicated increased [Ca\textsuperscript{2+}]\textsubscript{i} in cellular A/R plays a crucial role in occurrence of ERS, and NCX activity was responsible for this increase in [Ca\textsuperscript{2+}]\textsubscript{i}. Concomitant to this ERS response alleviation, there was also reduced cardiomyocyte apoptosis, as ascertained by FACS analysis. In short, results suggest levsosimendan inhibiting reverse-mode NCX activity to protect CPC-derived cardiomycocytes from A/R-induced ER stress and cell death. Further studies must confirm this result to enhance understanding of I/R injury mechanisms.

**Author Contributions**

Conceived and designed the experiments: YCY CYL. Analyzed the data: GYH LSK CYL. Contributed reagents/materials/analysis tools: GYH LSK CYL. Wrote the paper: PCL.
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