Biochemical and Biophysical Research Communications 443 (2014) 604–609

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Altered expression of calcineurin, calpain, calpastatin and HMWCaMBP in cardiac cells following ischemia and reperfusion

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ARTICLE INFO

Article history:
Received 28 November 2013
Available online 11 December 2013

Keywords:
Calpain
Calcineurin
Calpastatin
HMWCaMBP
Ischemia
Reperfusion
Primary cardiomyocyte culture

ABSTRACT

A rise in intracellular myocardial Ca^{2+} during cardiac ischemia activates calpain (Calpn) thereby causing damage to myocardial proteins, which leads to myocyte death and consequently to loss of myocardial structure and function. Calcineurin (CaN) interacts with Calpn and causes cellular damage eventually leading to cell death. Calpastatin (Calp) and high molecular weight calmodulin-binding protein (HMWCaMBP) (homolog of Calp), inhibit Calpn activity and thus prevent cell death. CaN stimulation can also result in self-repair of damaged cardiomyocytes. The present study attempts to elucidate the expression of these proteins in cells under pre-ischemic condition (control), following ischemia induction and also reperfusion subsequent to ischemia. For the first time, flow cytometric analysis (FACS) has been used for analyzing protein expression concurrently with viability. We induced ischemia and subsequently reperfusion in 80% confluent cultures of neonatal murine cardiomyocytes (NMCC). Viability following induction was assessed with 7-AAD staining and the cells were simultaneously checked for protein expression by FACS. We observed that ischemia induction results in increased expression of CaN, Calp and Calpn. HMWCaMBP expression was reduced in live cells following ischemia which suggests that there is a poor survival outcome of cells expressing HMWCaMBP thereby making it a potential biomarker for such cells. Most live cells following ischemia expressed CaN pointing towards self-repair and favorable survival outcomes.

1. Introduction

The onset of oxygen deprivation that leads to intracellular Ca^{2+} overload, has been shown to be a crucial event in myocardial cell injury and results in the activation of intracellular Ca^{2+}-dependent enzymes like calpains (Calpn) [1–4]. Calpn activation causes damage to cardiac proteins [5] leading to cardiomyocyte death and, subsequently the loss of heart structure and function [6]. Recent studies show that Calpn activation occurs exclusively during reperfusion [7,8]. Cardio-protective effects were observed following prevention of Calpn activation by either post-conditioning or by using pharmacological inhibitors applied at the onset of reperfusion [8]. Calpn activation has been well studied in normal and ischemic cardiomyocytes [9]. Calpain activity *in vivo* is tightly regulated by its natural endogenous inhibitor calpastatin (Calp) and its homolog namely the high molecular weight calmodulin-binding protein (HMWCaMBP) [10,11]. Elevated Calpn levels cause calcineurin (CaN) activation in ischemia [7,12–14] which has been demonstrated through *in vitro* proteolytic degradation [15]. Interestingly recent studies propose that the initial activation of Calpn by CaN initiates and influences CaN-Calpn signaling [16].

Furthermore, Calpn involvement in myocardial ischemia–reperfusion (I/R) injury, myocardial stunning, cardiac hypertrophy and ensuing cardiac remodeling has been suggested [3,7,17–20]. Reperfusion during acute myocardial infarction remains the best treatment for reducing infarct size [21]. Unfortunately, reperfusion is also responsible for additional myocardial damage which can be up to 50% of the final infarct size and lethal in nature [22,23]. It is known that post-conditioning applied at the onset of reperfusion reduces myocardial infarction both in animals and humans [24]. However, I/R injury still remains a challenge in the management of acute myocardial infarction patients and is a major cause of heart failure and mortality [23]. Interestingly, most of the studies to date have been performed on animal models and the results have been interpreted at the organ level [25]. The present study

Abbreviations: Calpn, calpain; CaN, calcineurin; Calp, calpastatin; HMWCaMBP, high molecular weight calmodulin-binding protein; NMCC, primary neonatal mouse cardiomyocyte culture; I/R, ischemia and reperfusion; NDB, nutrient deficient buffer; DPBS, dulbecco's phosphate buffered saline; FACS, flow cytometry; FITC, fluorescein isothiocyanate; PE, R-phycoerythrin.

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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.12.019

aims to elucidate the underlying interplay of various cardiac proteins during ischemia and subsequent reperfusion using flow cytometric analysis (FACS). The alteration in expression levels of cardiac proteins like Calp, Calpn, CaN and HMWCaMBP in relation to live-dead analysis can help us to predict which cells will be able to survive the I/R insult.

2. Methodology

2.1. Cells

Neonatal murine cardiomyocyte culture (NMCC – primary culture derived from murine heart) was used for studying induced I/R injury. 2–6 day old CD-1 Swiss albino mice pups were sacrificed in accordance to the norms provided by the Institutional Animal Ethics Committee, University of Saskatchewan. The hearts were immediately removed and cultured on 0.02% gelatin-precoated cell culture flasks as per the protocol described previously [26,27]. The primary cultures were maintained till the cultures attained ~80% and then the cells were induced with I/R injury.

2.2. I/R injury induction

The media in NMCC cultures (~80% confluent) was replenished 24 h before the induction. Ischemia was induced by replacing the media with a nutrient deficient buffer (NDB). The NDB contains 136 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2.7H_2O$ and 5.5 mM HEPES (pH-6.8) [28]. For inducing ischemia in NMCC, glucose and FCS were added to NDB to obtain a final concentration of 5 mM and 2%, respectively [29]. NDB was added to the cells for induction and incubated at various time periods for the initial optimization (1, 2 and 4 h). In order to enhance the oxidative stress in cells, hydrogen peroxide (H_2O_2) was added to NDB (1 mM final concentrations) [28]. Following induction, NDB was removed and the cells were immediately reperfused or were used for analysis. Reperfusion was carried out by replacing NDB with standard growth media [30]. The cells were incubated with this media for 2 h before they were used for further analysis. In control cultures (pre-ischemic conditions), the medium exchange was carried out with cardiomyocyte maintenance media for NMCC.

2.3. Assessment of protein expression

FACS was performed to quantify the protein expression in control, ischemic and reperfused cells. The methodology has been described in brief in the Supplementary data-methodology section.

2.4. Assessment of viability

The assay of live versus dead cells was used to assess viability following induction and compared to control cells. The assay was performed simultaneously with FACS analysis using 7-amino-actinomycin D (7-AAD) [31]. As suggested by the manufacturer, 7-AAD staining solution in DPBS (~0.25 μg/10^6 cells) was incubated with control, ischemia and reperfusion induced cells for 10 min at room temperature in the dark. The cells were washed twice with DPBS and dislodging for FACS. The ideal I/R injury induction was determined by inducing the cells at different parameters (ischemia induction – 1, 2 and 4 h; reperfusion induction following ischemia – 1 and 2 h). The induced cells along with control cells were stained with 7-AAD and dislodged by trypsinization. The cell suspension was immediately used for FACS to quantify live and dead cells in the control and induced population.

3. Results and discussion

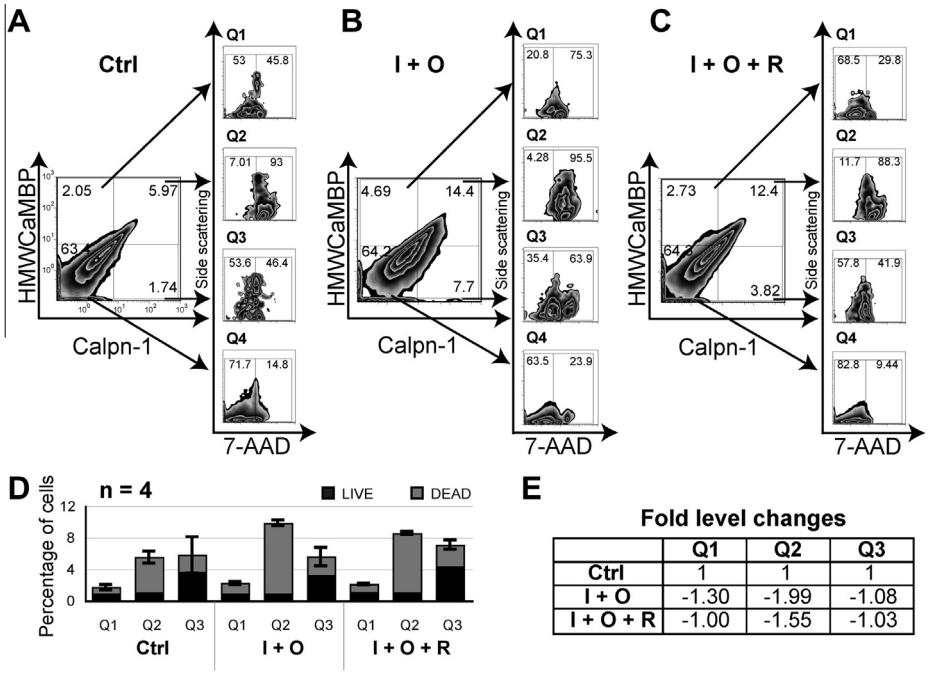
3.1. Optimization of ischemic and reperfusion induction

Ischemia followed by reperfusion induces stress in cardiomyocytes and often results in cell death [32,33]. The current work demonstrates the altered expression of various cardiac proteins in cardiomyocytes following I/R injury. The duration of ischemic induction and subsequent reperfusion was optimized such that most ischemic cells were able to revert back to pre-ischemic conditions. The results have been summarized in the Supplementary data. In Supplementary Fig. 1, the condition where ischemic induction and subsequent reperfusion gave the best results in NMCC was 2 h of ischemic induction followed by 2 h of reperfusion. With lesser ischemic induction (1 h), the shift of cells was predominantly towards cell death as observed by 7-AAD stain detection using FCAS. Similarly, there was no significant shift of cells towards survival when ischemic induction of 2 h and higher time (4 h) was followed by reperfusion for 1 h. Exposure of cells to ischemia over 4 h was too stressful as observed by the increased cell death (>50%) even after reperfusion for 2 h. Oxidative stress has been found to play a critical role in the death of cardiac tissue following ischemia and reperfusion [34]. H_2O_2 is a known reactive oxygen species generator; therefore cardiomyocytes were treated with H_2O_2 in this study. The data obtained from previous studies demonstrates that H_2O_2 treatment during ischemia increases cell death [34–36].

3.2. Assessment of protein expression by triple staining

Following standardisation of ischemia and subsequent reperfusion, expression of various cardiac proteins in live and dead cells were analyzed in control and induced NMCC. Triple staining was performed by simultaneously two proteins of interest with specific antibodies tagged with fluorophore (FITC and PE, respectively) along with a live-dead assay of analyzed cells with 7-AAD. The assay was devised to meet the specific aim of elucidating the expression of various cardiac proteins in cells following ischemia and subsequent reperfusion. This differentiation helps in determining cells which can survive I/R injury and most importantly the proteins responsible for the same. Previous studies showed that HMWCaMBP, Calp, CaNα and CaNβ interact with Calpn and regulates degradation of cellular proteins and resulting death of cardiac cells following ischemia and reperfusion [4,5,7–12,18]. In the present study HMWCaMBP, a homolog of Calp with calmodulin (CaM) binding property and the ability to inhibit Calpn [10,11,37–39] was prioritised and expression levels (as percentages) were compared to other CaM-binding proteins.

On comparing HMWCaMBP with Calpn-1 in normal, ischemia induced and reperfusion induced cells, we observed increased expression of both HMWCaMBP and Calpn-1 following ischemia which almost reverted back to normal levels following reperfusion (Fig. 1). The data was consistent with previous reports on the expression of both the proteins [7,18,38–40]. The live-dead assay using 7-AAD showed that the expression of HMWCaMBP in live cells decreased following ischemia and increased during subsequent reperfusion. Interestingly, the percentage of Calpn-1 expressing live cells decreased following ischemia; however subsequent to reperfusion, the percentages returned back to normal levels (pre-ischemia induction controls). Calpn-1 was used in this study since Calpn-1 activation required micromolar amounts of intercellular calcium ($[Ca^{2+}]i$) compared to millimolar amounts of $[Ca^{2+}]i$ for activating Calpn-2 [12]. Calpn-2 is subsequently activated during ischemia and subsequent reperfusion especially following chronic conditions [4]. Therefore, Calpn-2 could provide contradicting data due to the amounts of $[Ca^{2+}]i$ required for



	Q1	Q2	Q3
Ctrl	1	1	1
I + O	-1.30	-1.99	-1.08
I + O + R	-1.00	-1.55	-1.03

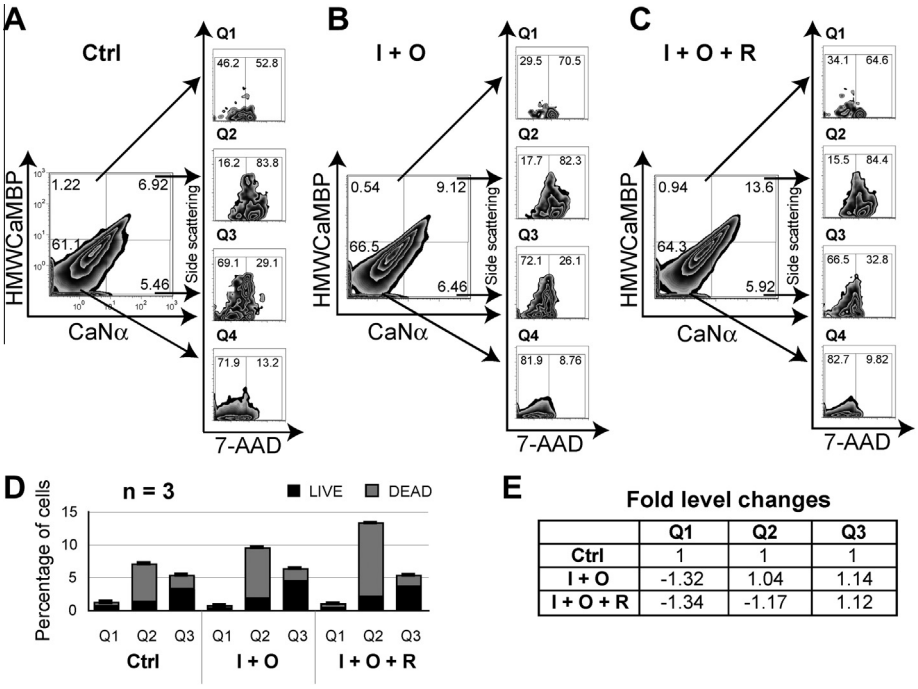
Fig. 1. (A–C) Representative FACS data of NMCC following I/R induction along with live-dead assay. In the vertical axis PE labeled antibodies against HMWCaMBP and for horizontal axis FITC labeled anti-Calpn-1 antibodies were detected. The remaining figures in the panel (Q1–Q4) are derived from the quadrants of the (Fig. 1A–C) (as denoted by the arrowheads) and have 7-AAD staining on horizontal axis and side scattering values on vertical axis. The conditions used were; normal untreated NMCC – Ctrl (A); NMCC treated with nutrient deficient buffer containing 1 mM H_2O_2 (ischemia induction) for 2 h – I + O (B); NMCC grown for 2 h in normal media following 2 h of ischemia induction (reperfusion induction) – I + O + R (C). (D) Combined data of performed experiments ($n = 4$) (quadrants with staining-Q1–Q3 only) represented as a histogram. The percentage of live and dead cells has been combined for each quadrant. (E) Tabulated representation of fold level changes of live cells in quadrants with staining (Q1–Q3) in comparison with normal control cells.

activation and lesser time of exposure used in the current study to generate data.

The expression levels of CaN (α and β subunits) in comparison with HMWCaMBP following ischemia and subsequent reperfusion are similar to those reported earlier [12,13,16,40,41]. The overall expression of HMWCaMBP decreased following ischemia and marginally increased following reperfusion (Figs. 2 and 3). The percentage of cells expressing in both HMWCaMBP and CaNα (Fig. 2) and HMWCaMBP and CaNβ (Fig. 3) increased following ischemia and again increased substantially (~1.5-fold) following reperfusion. CaNα and CaNβ expression especially in live cells increased following ischemia and reverted back to pre-ischemia (normal) levels following reperfusion. This increase in protein expressing live cells is in contrast to other cardiac proteins and suggests that CaN could be crucial in the survival of cells following ischemia and subsequent reperfusion. Interestingly, CaN is involved in self-repair through 70 kDa heat-shock protein (Hsp70) [42]. The interaction between Hsp70 and CaN is known to dephosphorylate NFAT resulting in transcriptional induction of various genes including GATA4, which is crucial in cardiomyocyte development and has a significant role in unassisted self-repair [43–45]. However, ischemia induced CaN dephosphorylates phospholamban which results in calcium overload during reperfusion and thus damage the cells [46].

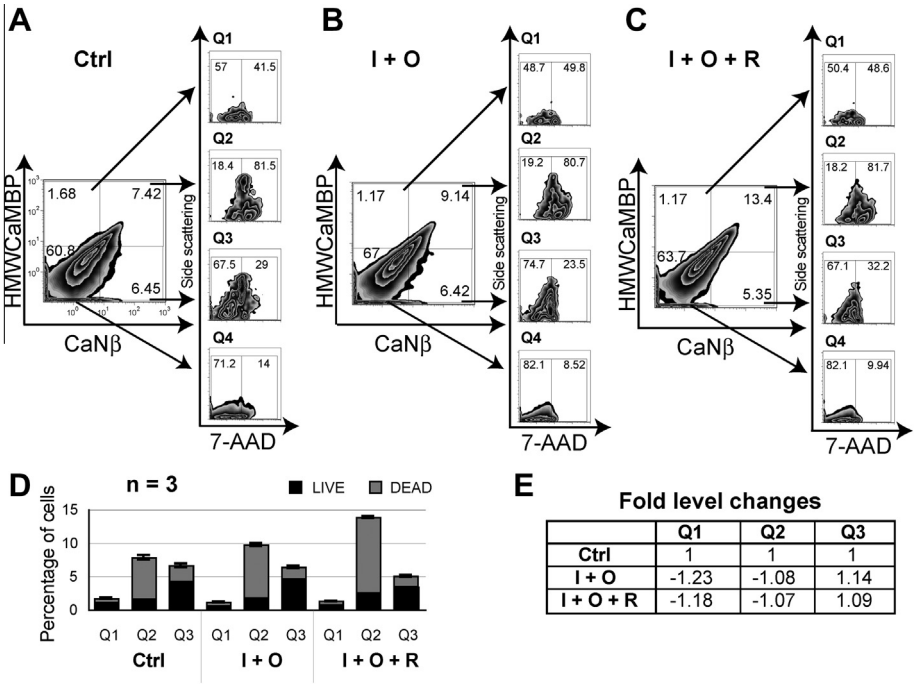
HMWCaMBP is a homolog of Calp and both these molecules are known endogenous inhibitors of Calpn [10,11,37]. Being a homolog, the antibodies raised against Calp can often react with HMWCaMBP and vice versa. Therefore, we used in-house raised rabbit polyclonal antibodies against HMWCaMBP and mouse monoclonal antibodies against Calp. Even then a certain amount of cross reactivity was observed (Fig. 4) where the percentage of cells expressing both HMWCaMBP and Calp was higher than the cells expressing HMWCaMBP and other proteins (Calpn-1, CaN α and CaN β) (Figs. 1–3). The overall percentage of HMWCaMBP expressing live cells decreased following ischemic induction but partially increased during subsequent reperfusion [10,11]. However, the percentage of live cells expressing Calp only did not vary much following ischemia and subsequent reperfusion. The percentage of cells expressing both Calp and HMWCaMBP increased following ischemia and reverted back to normal subsequent to reperfusion. However, there is a decrease in live cells expressing both Calp and HMWCaMBP following ischemia. Subsequent reperfusion only induced partial recovery in the cell number (Fig. 4). This difference in the percentage of live cells expressing both Calp and HMWCaMBP to live cells expressing either Calp or HMWCaMBP suggests that HMWCaMBP can be potentially used as a marker to determine cells which have a lesser chance of survival following ischemia and subsequent reperfusion.

A semi-quantitative estimation of levels of the above described proteins in cells was determined by Western blotting. The results obtained showed negligible amount of changes in the expression levels in proteins studied (data not shown). However, HMWCaMBP did show a reduced expression in ischemia similar to the previously reported data from our group [38,39]. Other than FACS, no currently used methodologies or technologies can determine the expression level of proteins simultaneously in live and dead cells. This hindrance affects the validation of the data obtained. The use of repeated experiments is the only evidence in this scenario. Another drawback is the potential loss of dead cells which often float and are often discarded during the washing steps. This retrieval of dead cells is important since the heart hardly loses any cell death from its 3-dimensional structure following ischemia and reperfusion. Any study on ischemia and reperfusion *in vitro* will require the conditions to be replicated as in the heart and hence dead cells have to be considered for the present study. To negate this issue, the supernatant of the media and the used washing buf-



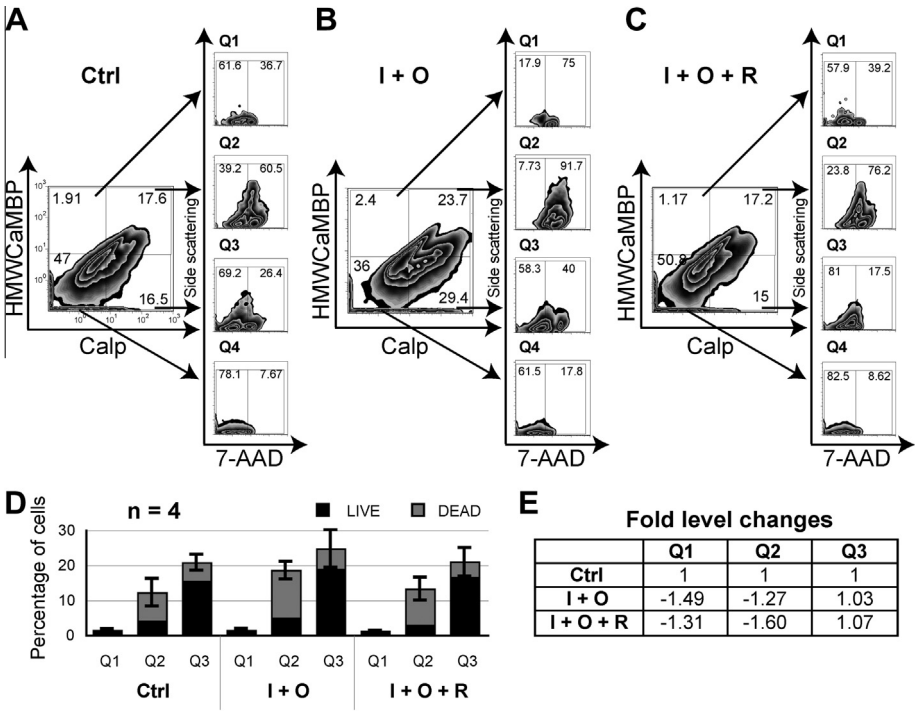
	Q1	Q2	Q3
Ctrl	1	1	1
I + O	-1.32	1.04	1.14
I + O + R	-1.34	-1.17	1.12

Fig. 2. (A–C) Representative FACS data of NMCC following I/R induction along with live-dead assay. In the vertical axis PE labeled antibodies against HMWCaMBP and for horizontal axis FITC labeled anti-CaNα antibodies were detected. The remaining figures in the panel (Q1–Q4) are derived from the quadrants of the (Fig. 2A–C) (as denoted by the arrowheads) and have 7-AAD staining on horizontal axis and side scattering values on vertical axis. The conditions used were; normal untreated NMCC – Ctrl (A); NMCC treated with nutrient deficient buffer containing 1 mM H_2O_2 (ischemia induction) for 2 h – I + O (B); NMCC grown for 2 h in normal media following 2 h of ischemia induction (reperfusion induction) – I + O + R (C). (D) Combined data of performed experiments (n = 3) (quadrants with staining-Q1–Q3 only) represented as a histogram. The percentage of live and dead cells has been combined for each quadrant. (E) Tabulated representation of fold level changes of live cells in quadrants with staining (Q1–Q3) in comparison with normal control cells.



	Q1	Q2	Q3
Ctrl	1	1	1
I + O	-1.23	-1.08	1.14
I + O + R	-1.18	-1.07	1.09

Fig. 3. (A–C) Representative FACS data of NMCC following I/R induction along with live-dead assay. In the vertical axis PE labeled antibodies against HMWCaMBP and for horizontal axis FITC labeled anti-CaNβ antibodies were detected. The remaining figures in the panel (Q1–Q4) are derived from the quadrants of the (Fig. 3A–C) (as denoted by the arrowheads) and have 7-AAD staining on horizontal axis and side scattering values on vertical axis. The conditions used were; normal untreated NMCC – Ctrl (A); NMCC treated with nutrient deficient buffer containing 1 mM H_2O_2 (ischemia induction) for 2 h – I + O (B); NMCC grown for 2 h in normal media following 2 h of ischemia induction (reperfusion induction) – I + O + R (C). (D) Combined data of performed experiments (n = 3) (quadrants with staining-Q1–Q3 only) represented as a histogram. The percentage of live and dead cells has been combined for each quadrant. (E) Tabulated representation of fold level changes of live cells in quadrants with staining (Q1–Q3) in comparison with normal control cells.



	Q1	Q2	Q3
Ctrl	1	1	1
I + O	-1.49	-1.27	1.03
I + O + R	-1.31	-1.60	1.07

Fig. 4. (A–C) Representative FACS data of NMCC following I/R induction along with live-dead assay. In the vertical axis PE labeled antibodies against HMWCaMBP and for horizontal axis FITC labeled anti-Calp antibodies were detected. The remaining figures in the panel (Q1–Q4) are derived from the quadrants of the (Fig. 4A–C) (as denoted by the arrowheads) and have 7-AAD staining on horizontal axis and side scattering values on vertical axis. The conditions used were; normal untreated NMCC – Ctrl (A); NMCC treated with nutrient deficient buffer containing 1 mM H_2O_2 (ischemia induction) for 2 h – I + O (B); NMCC grown for 2 h in normal media following 2 h of ischemia induction (reperfusion induction) – I + O + R (C). (D) Combined data of performed experiments ($n = 4$) (quadrants with staining-Q1–Q3 only) represented as a histogram. The percentage of live and dead cells has been combined for each quadrant. (E) Tabulated representation of fold level changes of live cells in quadrants with staining (Q1–Q3) in comparison with normal control cells.

fers were carefully pooled and then added with the sample before 7-AAD staining in this study.

In brief, the present study describes the use of triple staining for comparative protein expression analysis in normal, ischemia-induced and reperfused cardiomyocytes by FACS. As in previous reports, the expression of HMWCaMBP decreased following ischemia and partially reverted back to the pre-ischemia levels. The overall percentage of Calpn-1 expressing cells increased with ischemic induction and reduced following reperfusion; however the number of live cells expressing Calpn-1 decreased during ischemia and reverted to normalcy after reperfusion. Interestingly, the expression of CaN α and CaN β increased overall and in live cells following ischemia reverted back to normal levels following reperfusion. Though the expression of Calp increased following ischemia and then decreased following reperfusion much similar to CaN α and CaN β, the percentage of live cells expressing Calp and its homolog HMWCaMBP decreased significantly during ischemia and partially reverted back to normal levels following reperfusion. The above studies suggest that HMWCaMBP can be potentially used as a marker to detect cells predestined towards cell death. The study also shows that live cells expressing CaN have a higher chance to survive ischemia and subsequent reperfusion. Though validation of the present data is not possible through currently available methodologies and techniques including microscopy and immunoblotting, attempts will be made to validate these results by the use of knockout animal models. This definitely merits further in-depth studies.

Acknowledgments

This work was supported by the Heart and Stroke Foundation of Saskatchewan, Canada. R.K.S. and S.P. were involved in conceptualisation and preparation of manuscript and S.P. was involved in the designing and performing of the experiments. The authors are grateful to Dr. J. R. Dimmock, College of Pharmacy and Nutrition, University of Saskatchewan for the reading and helpful discussion regarding this manuscript, Dr. K. A. Ahmed for the helpful discussion regarding the experiments and Mr. Mark Boyd for the technical help rendered.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.019.

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