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Expression of calcineurin, calpastatin and heat shock proteins during ischemia and reperfusion

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ABSTRACT

Objective: Calcineurin (CaN) interacts with calpains (Calpn) and causes cellular damage eventually leading to cell death. Calpastatin (Calp) is a specific Calpn inhibitor, along with CaN stimulation has been implicated in reduced cell death and self-repair. Molecular chaperones, heat shock proteins (Hsp70 and Hsp90) acts as regulators in Calpn signaling. This study aims to elucidate the role of CaN, Calp and Hsps during induced ischemia and reperfusion in primary cardiomyocyte cultures (murine).

Methods and results: Protein expression was analyzed concurrently with viability using flow cytometry (FACS) in ischemia- and reperfusion-induced murine cardiomyocyte cultures. The expression of Hsp70 and Hsp90, both being molecular chaperones, increased during ischemia with a concurrent increase in death of cells expressing these proteins. The relative expression of Hsp70 and Hsp90 during ischemia with respect to CaN was enhanced in comparison to Calp. Reperfusion slightly decreased the number of cells expressing these chaperones. There was no increase in death of cells co-expressing Hsp70 and Hsp90 along with CaN and Calp. CaN expression peaked during ischemia and subsequent reperfusion reduced its expression and cell death. Calp expression increased both during ischemia and subsequent reperfusion but cell death decreased during reperfusion.

Conclusion: The present study adds to the existing knowledge that Hsp70, Hsp90, CaN and Calp interact with each other and play significant role in cardio protection.

1. Introduction

The increase in Ca^{2+} concentration during ischemia causes activation of calpains (Calpn) [1]. Calpn activation results in protein degradation and cell death [2,3]. Calpn activation has been well studied in normal and ischemic cardiomyocytes [1,4]. Cells at the ischemic infarct edge, which have undergone partial ischemia are also vulnerable to remodeling [5]. Due to impaired intracellular Ca^{2+} homeostasis, such cells are predisposed to death following reperfusion [6]. Interestingly, cardiomyocyte proliferation and progenitor cell recruitment has been observed in the cardiac infarct border zone [7]. Calpastatin (Calp) is the most efficient and specific Calpn inhibitor present *in vivo* [8–10]. Calp along with its putative homolog high molecular weight calmodulin-binding protein (HMWCaMBP) regulate Calpn inhibition [11–15] and may reduce I/R injury in heart [16,17].

Among the proteins proteolysed by Calpn, calcineurin (CaN) is known to regulate cardiac hypertrophy and remodeling and has been implicated in both cell death and survival following reperfusion [18–20]. CaN is a heterodimer consisting of 19- and 57-59- kDa subunits which are referred to as CaNβ and CaNα, respectively [21–23]. The CaNα subunit has low endogenous phosphatase activity and requires Ca^{2+}, calmodulin (CaM) and CaNβ for full activity [24]. CaN activation during ischemia occurs due to elevated Calpn levels [13,25–27] which has been demonstrated through *in vitro* proteolytic degradation [28] or via the cleavage of the endogenous calcineurin inhibitor cain/cabin1 [29]. Recent studies propose that ischemia induced activation of CaN leads to further increase in cytosolic Ca^{2+} levels, which further activates Calpn during reperfusion [30]. This putative feedback mechanism can influence CaN–Calpn signaling in cardiomyocytes following ischemia and reperfusion (I/R) [1,26]. Interestingly, the CaM-dependent phosphatase activity of CaN is stimulated by the 70 kDa heat-shock protein (Hsp70) in cardiac muscle and thus provides an on/off switch for the regulation of CaN signaling by Hsp70 [31]. CaN–Hsp70 signaling results in the activation of NFAT which affects apoptosis, development and cellular adaptation in cardiac cells [31–33]. The importance of CaN–Hsp70 interaction lies with

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; FACS, flow cytometry; FITC, fluorescein isothiocyanate; PE, R-phycoerythrin

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downstream effectors such as NFAT and GATA-4, which are important in cardiac remodeling and regeneration [34–36].

Recently, Hsp90, another heat-shock protein, has generated attention due to its cardiac protective role in I/R induced injury [37–41]. In septic mice models, Calpn induces caspase-3 activation and apoptosis via the activation of the Hsp90/Akt pathway [42]; however, this activation can also promote CaN recruitment to prevent apoptosis [43,44]. Hsp90 also plays an important role in regulating Calpn-1 through specific interactions and associations at the functional sites. Nevertheless, Hsp90 can get degraded in concentrations higher than equimolar levels of Calpn [45]. Though both Hsp70 and Hsp90 are molecular chaperones [46] and appear to have cardioprotective properties, several differences exist especially at mRNA induction during I/R [47–51]. The interaction and the relevance of Hsp70 and Hsp90 in I/R with respect to Calpn-regulated proteins like CaN and Calp remains vague. The current study aims to reveal the underlying interplay of CaN, Calp, Hsp70 and Hsp90 during ischemia and subsequent reperfusion using flow cytometric analysis (FACS). The expression level of ubiquitous cardiac protein sarcomeric actin (SarcAct) has been also studied as a control.

2. Methodology

2.1. Cells

Neonatal murine cardiomyocyte culture (NMCC – primary cultures derived from isolated murine heart) was used for studying the induced I/R injury. CD-1 Swiss albino mice pups (2–6- day old) were sacrificed, in accordance to the norms provided by the Institutional Animal Ethics Committee, University of Saskatchewan. The hearts were instantly extracted, processed and cultured on 0.02% gelatin-precoated cell culture flasks, based on protocols previously described [52,53]. The primary cultures were sustained till the cultures attained ~80% and following which I/R injury was induced in cell cultures.

2.2. I/R injury induction

The media in NMCC cultures (~80% confluent) was replaced 24 h preceding induction. Ischemic conditions were induced by replacing the standard growth media with a nutrient deficient buffer (NDB). The NDB contains 136 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2 \cdot 7H_2O$ and 5.5 mM HEPES (pH 6.8) and therefore provides no nutrition and minimal buffering to the cells [54]. For inducing ischemia in NMCC, glucose and FCS were added to NDB to obtain a final concentration of 5 mM and 2%, respectively to provide basic minimal nutrition [55]. Consecutively, reperfusion was performed by switching NDB with standard growth media [54–56]. In addition, to emulate the oxidative stress in cardiomyocytes observed *in vivo* during reperfusion, hydrogen peroxide (H_2O_2) was added to the standard growth media (1 mM final concentration) [54,55]. The methodology was performed as per a previously published protocol [13,14].

2.3. Assessment of protein expression and viability

The concurrent assessment of protein expression in normal (untreated), ischemic and reperfused cardiomyocytes along with viability was performed by FACS based on a methodology carried out as per a previously published protocol [13,14]. Briefly, 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) [57]. 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. A tabulation of antibodies along with the dilutions used are provided in Supplementary Table 1.

2.4. Statistical analysis

Statistical analysis on the data obtained from the various assays was performed using ANOVA (Sigma Plot version 10 software package). The significance level of ≤ 0.05 is represented as * to indicate significant differences.

3. Results and discussion

The triple staining was performed by concurrently staining two proteins with specific antibodies tagged with fluorophore (FITC and PE, respectively) along with a live–dead assay of analyzed cells with 7-AAD. The analysis elucidated the expression of various cardiac proteins in both live and dead cells present in control and I/R treated cardiomyocyte cultures. This differentiation quantified cells which survived I/R injury and determined the important proteins expressed in cells [13,14]. In the present study, the interaction of Hsp70 and Hsp90 with respect to CaN (Figs. 1 and 2) was compared to Calp (Supplementary Figs. 1 and 2). The expression levels (as percentages and fold levels) were also compared in a control protein which is ubiquitously expressed (SarcAct) (Fig. 3, Supplementary Figs. 3 and 4).

3.1. Expression of Hsp70 and Hsp90 in CaN expressing cells

On comparing Hsp70 with CaN in normal, ischemia induced and reperfusion induced cells, we observed a global increase in expression of both Hsp70 and CaN following ischemia which significantly decreased following reperfusion (Figs. 1 and 3). Cardiomyocytes expressing both Hsp70 and CaN peaked during ischemia and then significantly decreased during reperfusion (Fig. 1D). A slight increase in global expression of Hsp90 with respect to CaN following ischemia and subsequent reperfusion was observed but not significant (Fig. 3). Conversely, the number of cells expressing Hsp90 alone decreased during both ischemia and reperfusion, whereas there was slight increase in cells co-expressing Hsp90 and CaN during ischemia and subsequent reperfusion (Fig. 2). There was simultaneous increase in number of dead cells expressing Hsp70 or Hsp90 during ischemia (Figs. 1B and 2B). The drastic increase in dead cells co-expressing Hsp70 or Hsp90 and CaN during ischemia was not observed during subsequent reperfusion (Figs. 1C and 2C).

The current study clearly shows that the stress induced by ischemic treatment simultaneously increased the expression of Hsp70 and Hsp90 since both being chaperone proteins [46]. The cells expressing Hsp70 and Hsp90 predominantly died during ischemia (Figs. 1B and 2B) [50]. Reperfusion did not enhance cell death indicating that the cells expressing Hsp70 or Hsp90 were protected against subsequent reperfusion induced injury (Figs. 1C and 2C). Activation of Heat Shock Transcription Factor 1 during ischemia stimulates Hsp70 mRNA expression whereas reperfusion stimulates Hsp90 mRNA [51]. The marked increase observed in Hsp70 expression in CaN expressing cells (Fig. 1B) suggests the