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The extracellular stress response to pediatric cardiopulmonary bypass

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Abstract. The heat shock response, also frequently referred to as the stress response, is an ancient, highly conserved, endogenous cellular defense mechanism characterized by the rapid upregulation of a specific class of proteins known collectively as heat shock proteins, or stress proteins. The 70 kDa family of heat shock proteins are highly inducible and have been shown to possess important immunomodulatory effects in both the intracellular and extracellular compartments. In the current prospective translational study, we measured extracellular (i.e. plasma) levels of heat shock protein 72 (Hsp72) in 49 children undergoing cardiopulmonary bypass (CPB) for either palliation or repair of congenital heart disease. There was a significant and transient increase (less than 24 h) in extracellular Hsp72 levels following CPB. Extracellular Hsp72 levels significantly correlated with levels of the pro-inflammatory cytokines interleukin (IL)-6 and IL-8, as well as the anti-inflammatory cytokine, IL-10. In addition, plasma Hsp72 levels correlated with troponin-I levels, a marker of myocardial injury. Increased extracellular Hsp72 levels at 6 h following CPB were independently associated with increased length of stay in the cardiac intensive care unit. Importantly, the source of extracellular Hsp72 does not appear to be cardiomyocytes. However, the mechanism of release and clinical relevance of the increase in extracellular Hsp72 need to be further delineated.

Keywords: Heat shock proteins, danger signals, stress proteins, alarmins, cardiopulmonary bypass, congenital heart disease, extracellular Hsp72

1. Introduction

Consistent with their function as molecular chaperones, heat shock proteins (also known as stress proteins) have traditionally been considered to be exclusively intracellular proteins. However, there is a growing body of literature suggesting that extracellular stress proteins may be released outside of the cell, either through passive release via cellular necrosis or in some cases, active secretion [1–7]. We have been particularly interested in the immunomodulatory effects of a highly inducible stress protein, known as heat shock

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protein 72 (Hsp72). Increased intracellular Hsp72 expression appears to downregulate pro-inflammatory gene expression, primarily through inhibition of the pluripotent transcription factor, nuclear factor κB [8, 9]. In stark contrast, extracellular Hsp72 appears to increase pro-inflammatory gene expression, at least partly via the Toll-like receptor (TLR)-2 and TLR-4 pathways [5, 7, 10–15]. As such, the release of Hsp72 into the extracellular environment may serve to signal an impending danger signal to neighboring cells [16]. In this context, extracellular Hsp72 is now included in a growing list of so-called alarmins, a group of endogenous proteins that are released by necrotic cells or secreted via non-classical pathways in order to convey a danger signal to surrounding cells [17]. Endogenous alarmins, such as Hsp72, and exogenous pathogen-associated molecular patterns, such as lipopolysaccharide, both convey a similar message of danger that results in a patterned response. Together, alarmins and pathogen-associated molecular patterns comprise the so-called danger-associated molecular patterns [18].

Myocardial expression of Hsp72 is consistently observed following cardiopulmonary bypass (CPB) in both animals [19–21] and humans [22–24]. Importantly, increased myocardial expression of Hsp72 appears to be protective [22, 25–29]. However, to our knowledge the effect of CPB on extracellular Hsp72 expression in children has not been previously shown. Accordingly, we hypothesized that extracellular Hsp72 levels would increase in children following CPB for surgical correction or palliation of congenital heart disease.

2. Materials and methods

2.1. Patient population

The study was approved by the Cincinnati Children's Hospital Medical Center Institutional Review Board. Our cohort has been previously described [30]. Briefly, forty-nine consecutive children under the age of 17 yr undergoing surgical correction or palliation of congenital heart disease requiring CPB were enrolled in the study following written informed consent. Exclusion criteria included the presence of sepsis, acute or chronic lung disease, immunodeficiency, corticosteroid use within 1 wk preceding surgery, or cardiac arrest within 1 wk preceding surgery. Patient demographics, including age at surgery, diagnosis, risk adjustment for congenital heart surgery (RACHS)-1 score [31], duration of CPB, aortic cross-clamp time, and cardiac intensive care unit (CICU) length of stay (LOS) were collected.

2.2. Sampling

Arterial blood samples were obtained at baseline (prior to the initiation of CPB, at hour 0 (after the termination of CPB), and at hours 6 and 24 following cessation of CPB. Samples were collected in tubes containing sodium citrate and were centrifuged immediately at 4,000× *g* for 10 min in order to separate plasma from the cellular components. Samples were stored in 50 μL aliquots in order to avoid multiple freeze-thaw cycles at −70°C until later analysis. Atrial tissue was obtained via biopsy immediately before initiation of CPB and immediately following termination of CPB. Atrial tissue was collected in sterile specimen containers, immediately snap frozen in liquid nitrogen, and stored at −70°C until later analysis

2.3. Cell culture

Cultured neonatal cardiomyoblasts (H9C2 cells (American Type Culture Collection, Manassas, VA) were maintained in a room air/5% CO_2 incubator at 37°C using Dulbecco's Modified Eagle Medium (DMEM) media (Gibco, Gaithersburg, MD) containing sodium pyruvate, sodium bicarbonate, 10% fetal bovine serum, and penicillin/streptomycin. Cells in passage 3–5 were subjected to classic heat shock (43°C for 1 h) and allowed to recover at 37°C without changing the culture media. Media were collected at 24 h following heat shock and centrifuged at 1,200 rpm for 10 min at 4°C. The supernatant was stored in 50 μL aliquots in order to avoid multiple freeze-thaw cycles at −70°C until later analysis. Intracellular proteins were isolated for Western immunoblot analysis using a previously published protocol [5].

2.4. Hsp72 enzyme-linked immunosorbent assay (ELISA)

The presence of Hsp72 in plasma was measured using a commercially available sandwich ELISA (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

2.5. Multiplex cytokine assay

A multiplex cytokine kit (Beadlyte® Human Multi-Cytokine Detection System 2; Upstate Cell Signaling Solutions, Lake Placid, NY) was used to measure interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12, and tumor necrosis factor-α according to the manufacturer's instructions. Briefly, the appropriate cytokine standards and plasma samples (50 μL) were diluted in plasma dilution buffer and added to each well of a 96-well plate. The samples were incubated with 25 μL of the antibody-coupled cytokine microspheres at room temperature for 2 h, after which time 25 μL of biotin reporter solution was added. After 1.5 h incubation at room temperature, 25 μL of diluted streptavidin-phycoerythrin solution was added to the wells and incubated at room temperature for 30 min. Twenty-five μL of stop solution was added and the plate was analyzed on a Luminex® 100 instrument (Luminex, Austin, TX) according to the manufacturer's instructions. Data analyses of all assays were performed with BeadView Multiplex Data Analysis Software package (Upstate Cell Signaling, Lake Placid, NY). Cytokine detection using multiplex bead assays has a high degree of intra-assay (<10% variation) and inter-assay (10–20% variation) precision and is comparable to ELISA (correlation coefficients from $r = 0.75$ to $r = 0.99$) [32–34].

2.6. Troponin-I ELISA

Plasma cardiac-specific troponin-I enzyme was measured using a commercially available ELISA according to the protocol recommended by the manufacturer (TriChem Resources, Inc.; West Chester, PA).

2.7. Analysis of atrial tissue samples

Atrial tissue samples were collected, processed, and analyzed for subcellular fractionation and nuclear protein extraction using previously published methods. Western blot immunoassay and heat shock transcription factor (HSF)-1 electrophoretic mobility shift assay were performed as previously described [5, 7, 13].

2.8. Statistical analysis

Data was analyzed using SigmaStat for Windows Version 3.11 software (Systat Software, Inc, San Jose, CA). Continuous data are expressed as median and interquartile ranges and were compared using Kruskal-Wallis ANOVA with Dunn's post hoc test, as indicated. To detect correlations between continuous data, we used the Spearman correlation coefficient. Univariate analyses were performed to determine which factors were associated with prolonged postoperative CICU LOS. For purposes of model building, variables, which were associated with postoperative CICU LOS at a P value ≤ 0.20 were then included in a list of potential independent risk factors in stepwise linear regression analysis, with postoperative CICU LOS the dependent variable. A P value <0.05 was considered statistically significant.

3. Results

3.1. CPB with myocardial ischemia and surgical stress elicits a transient systemic inflammatory response in children

Arterial blood samples were obtained at four time-points (baseline, immediately upon completion of CPB, 6 h after completion of CPB, and 24 h after completion of CPB from a cohort of 49 infants and children undergoing CPB for surgical correction or palliation of congenital heart disease (Tables 1 and 2). These infants and children were in RACHS-1 risk category 1 ($n = 7$, 15%), 2 ($n = 17$, 36%), 3 ($n = 12$, 26%), 4 ($n = 3$, 6%), and 5/6 ($n = 8$, 17%). Our cohort was therefore relatively evenly balanced with regards to the severity of congenital heart disease and case mix. Consistent with previous studies [35–37], CPB elicited a transient increase in the expression of the pro-inflammatory cytokines IL-6 and IL-8, as well as the anti-inflammatory cytokine, IL-10 (Table 3). The pro-inflammatory cytokines IL-1β and tumor necrosis

Table 1
Patient demographics

Characteristics	Results ($n = 49$)
Age (mo), median (IQR)	5.1 (1.7–34.2)
Gender, female: male	26: 23
Weight* (kg), median (IQR)	5.95 (4.9–12.2)
Cardiopulmonary bypass time (min), median (IQR)	114 (76–150.5)
Aortic cross-clamp time (min), median (IQR)	69.5 (43.5–92)
Length of stay in cardiac intensive care unit (d), median (IQR)	4 d (2–35)

IQR = Interquartile range. *The 24-h post-operative fluid balance is indexed to patient weight (kg).

Table 2
Procedures performed and corresponding complexity of surgery

Procedure	n* (%)
Ventricular septal defect repair	10 (21)
Atrioventricular canal repair	8 (17)
Norwood procedure	7 (15)
Atrial septal defect repair	7 (15)
Tetralogy of Fallot repair	6 (13)
Ross procedure	2 (4)
Arterial switch	2 (4)
Interrupted aortic arch repair	1 (2)
Aortic coarctation repair	1 (2)
Aortic stenosis repair	1 (2)
Truncus arteriosus repair	1 (2)
Rastelli procedure	1 (2)
Anomalous origin of the coronary artery repair	1 (2)
Slide tracheoplasty	1 (2)

*Percentages do not add up to 100% as some patients underwent multiple procedures.

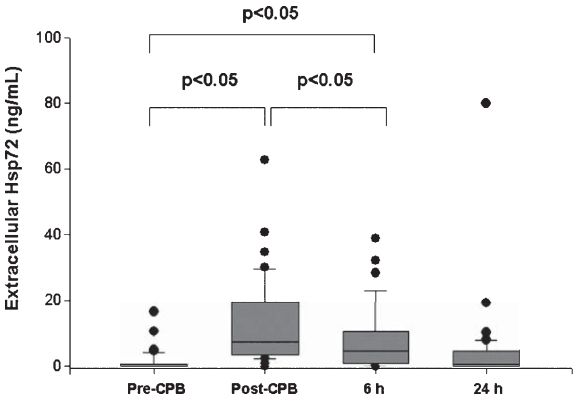

Fig. 1. Box-and-whisker plot of plasma Hsp72 concentrations in children following cardiopulmonary bypass (CPB). Arterial blood samples were obtained at four time-points (pre-CPB, immediately upon completion of CPB, 6 h after completion of CPB, and 24 h after completion of CPB) from a cohort of 49 infants and children undergoing CPB for surgical correction or palliation of congenital heart disease. The box represents the 25th and 75th percentiles, respectively. The median is shown as a solid line across the box.

factor-α were not detected at baseline or at any time-point following CPB. CPB also elicited a transient increase in troponin-I levels, a marker of myocardial injury [38].

3.2. *CPB with myocardial ischemia and surgical stress elicits a transient increase in extracellular Hsp72*

CPB with myocardial ischemia and surgical stress produced a transient increase in extracellular Hsp72 levels (Fig. 1). There was a significant correlation between the plasma concentration of extracellular Hsp72 immediately following CPB and the plasma concentration of IL-6 (Spearman rho = 0.42, $P = 0.03$), IL-8 (Spearman rho = 0.45, $P = 0.02$), and IL-10 (Spearman rho = 0.37, $P = 0.05$). The correlation between extracellular Hsp72 and IL-8 remained significant at 6 h following CPB (Spearman rho = 0.52, $P = 0.01$), while the correlation between extracellular Hsp72 and IL-10 remained significant at 6 h (Spearman rho = 0.47, $P = 0.02$) and 24 h (Spearman rho = 0.56, $P < 0.001$) following CPB. Finally, there was a significant correlation between extracellular Hsp72 and plasma troponin-I immediately after (Spearman rho = 0.67, $p < 0.001$), at 6 h (Spearman rho = 0.70, $P < 0.001$), and at 24 h (Spearman rho = 0.49, $P = 0.01$) following CPB. These results are consistent with the data derived from our porcine model of CPB showing that CPB results in a transient increase in extracellular Hsp72 expression, which is strongly associated with the inflammatory response to CPB, myocardial ischemia, and surgical stress.

3.3. *Extracellular Hsp72 levels following CPB are independently associated with prolonged post-operative CICU LOS*

Using univariate regression analysis, deep hypothermic circulatory arrest time ($p < 0.001$), aortic cross-clamp time ($P = 0.19$), age ($P < 0.001$), RACHS-1 score ($P = 0.006$), baseline plasma Hsp72 ($P < 0.001$),

Table 3
Cardiopulmonary bypass elicits a transient inflammatory response in children

Cytokine	Pre-CPB	Post-CPB	6 h post-CPB	24 h post-CPB
Tumor necrosis factor-α	ND	ND	ND	ND
Interleukin-1β	ND	ND	ND	ND
Interleukin-6	0 (0–12.9)	54.7 (19.8–108)*	106 (60.1–199.8)*	64.7 (30.9–154)*
Interleukin-8	3.8 (0–9.2)	31.2 (13.3–78.3)*	32.5 (16.2–61.5)*	27.3 (5.9–45.2)*
Interleukin-10	0.6 (0–3.8)	296 (146.8–562.8)*	19.6 (9.7–42.4)*	3.5 (2.0–8.0)
Cardiac troponin I	0.6 (0–3.8)	4.1 (1.4–15.2)*	7.4 (4.0–25.0)*	6.8 (1.8–20.6)*

CPB = Cardiopulmonary bypass; ND = Not detected. *$P < 0.05$ compared to pre-CPB.

and plasma Hsp72 immediately after CPB ($P = 0.006$), 6 h after CPB ($P = 0.02$), and 24 h after CPB ($P < 0.001$) were all associated with prolonged postoperative CICU LOS. These variables were then included in a list of potential independent risk factors in stepwise linear regression analysis, with postoperative CICU LOS as the dependent variable. Aortic cross-clamp time ($P = 0.02$), deep hypothermic circulatory arrest time ($P < 0.001$), and the plasma Hsp72 obtained 6 h after CPB ($P = 0.03$) remained significantly associated with postoperative CICU LOS.

3.4. CPB does not increase atrial levels of intracellular Hsp72

In order to further define the stress response following CPB, we next analyzed intracellular expression of Hsp72 in atrial samples obtained immediately before and immediately after CPB. There were no significant differences in activation of HSF1, the key transcription factor necessary for induction of intracellular Hsp72 expression, as detected by electrophoretic mobility shift assay or in the levels of intracellular Hsp72 as detected by Western immunoassay in the atrial samples either before or after CPB in our cohort (*data not shown*). Collectively, these results suggest that myocardial Hsp72 is not likely to be a significant source of extracellular Hsp72 following CPB.

3.5. Rat neonatal cardiomyocytes do not release extracellular Hsp72 in response to stress

The results above strongly suggest that the myocardium is not likely to be the major source of extracellular Hsp72. In order to further define the potential source of extracellular Hsp72, we subjected cultured, neonatal rat cardiomyoblasts (H9C2 cells) to classic heat shock and measured both intracellular and extracellular Hsp72 expression. Consistent with the results above, we were unable to detect an appreciable increase in extracellular Hsp72 from baseline in response to various cell stressors (*data not shown*).

4. Discussion

Herein we show that CPB produces a significant and relatively time-limited increase in extracellular (i.e. plasma) Hsp72 levels in children with congenital heart disease. Extracellular Hsp72 levels significantly correlated with levels of the pro-inflammatory cytokines IL-6 and IL-8, as well as the anti-inflammatory cytokine, IL-10. In addition, plasma Hsp72 levels correlated with troponin-I levels, a marker of myocardial injury. Increased extracellular Hsp72 levels at 6 h following CPB was independently associated with increased CICU LOS, which has been associated with poor cognitive outcome [39]. We were unable to demonstrate any significant difference in either myocardial Hsp72 (neither intracellular Hsp72 no extracellular Hsp72) or HSF1 activation pre- or post-CPB. Collectively, these data suggest that; (i) extracellular Hsp72 is released (though the mechanism remains speculative at this point) either through active secretion or passive release from dying cells in response to CPB, (ii) the major source of extracellular Hsp72 is not likely to be cardiomyocytes, and (iii) extracellular Hsp72 likely has downstream immunomodulatory effects on the host response to CPB. The mechanism by which extracellular Hsp72 impacts the host response and the manner in which it is associated with worse outcomes remains speculation at this point.

CPB elicits a complex host response characterized by activation of the vascular endothelium, complement, and activation of leukocytes, resulting in the release of a cascade of both pro- and anti-inflammatory mediators. While ischemia-reperfusion injury, leukocyte activation [37], complement activation by the CPB circuit [40], and release of endotoxin from the gastrointestinal tract have been implicated [35, 36, 41], other factors play an important role as well. In the current study, we showed that CPB results in a transient and significant (<24 h) increase in the pro-inflammatory cytokines IL-6 and IL-8, as well as the anti-inflammatory cytokine IL-10. Similarly, CPB results in a transient and significant increase in extracellular Hsp72. The mechanism and source of extracellular Hsp72 is mostly speculative at this point. Several studies have suggested that Hsp72 is secreted through non-classical pathways [1–5], though passive release of all of the intracellular contents of dying cells could certainly be at play as well [42]. To this point, there was a significant correlation between extracellular Hsp72 and troponin-I, a marker of cardiomyocyte cellular injury and/or death [38, 43] following CPB.

We have previously reported that white blood cells [5] and airway epithelial cells [7, 13] release extracellular Hsp72 following cell stress. Other investigators have demonstrated the release of extracellular Hsp72

in neuronal cells [1, 2], hepatocytes [6], and tumor cells [3, 4, 42]. To our knowledge, release of extracellular Hsp72 from stressed cardiomyocytes has not been previously reported. Several lines of evidence in our study would suggest that cardiomyocytes are not a major source of extracellular Hsp72. First, we were unable to show a significant increase in intracellular Hsp72 in atrial tissue following CPB. The available literature is conflicting on this matter. Storti et al. [44] were unable to show variation in Hsp72 mRNA levels in cardiac tissue samples pre- and post-cardiopulmonary bypass. Nakamura and colleagues [45] showed significant increases in Hsp72 protein following cardiopulmonary bypass. However, in this study, expression was only measured following surgery. Second, there was no significant difference in the activation of HSF1, the transcription factor that regulates production of Hsp72 in the atrial tissue before or after CPB. Finally, we were unable to show any significant increase in extracellular Hsp72 in a rat cardiomyocyte cell line following application of heat stress a common *in vitro* method shown to elicit extracellular Hsp72 release in several different cell lines. Regardless, at this point, we can only speculate and further studies will be necessary to determine the source and mechanism of extracellular Hsp72 release in this clinical setting.

Recent work by our group and others suggest that release of Hsp72 may act as an endogenous danger signal [1–4, 8]. Extracellular Hsp72, through its ability to act as a "chaperokine" by inducing pro-inflammatory gene expression via TLR-4 [6, 10–12, 46], could therefore play an important role in the host response to CPB. To this end, the results of our regression model showed that extracellular Hsp72 levels at 6 h following CPB was independently and significantly associated with prolonged CICU LOS. Of interest, extracellular Hsp72 has been shown to induce cardiomyocyte inflammation and contractile dysfunction *in vitro* [47].

In conclusion, we show that CPB is associated with a transient increase in extracellular Hsp72 in children undergoing surgery for palliation or repair of congenital heart disease. The source and mechanism of this extracellular Hsp72 is not known and will require further study. However, our preliminary studies suggest that cardiomyocytes may not be the major source of extracellular Hsp72. Finally, extracellular Hsp72 likely has downstream immunomodulatory effects on the host response to CPB, as there was a significant correlation between extracellular Hsp72 levels at 6 h following CPB and prolonged CICU LOS. Again, further studies will be necessary to further delineate the clinical relevance of these findings.

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