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Original article

The effect of 2 mMol glutamine supplementation on HSP70 and TNF-α release by LPS stimulated blood from healthy children

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SUMMARY

Objective: Glutamine has been shown to promote heat shock protein 70 (HSP70) release both within experimental *in vitro* models of sepsis (2–10 mM) and in adults post trauma (0.5 g/kg), although the efficacy varies and is dependent on the model used. The effect of glutamine supplementation on HSP70 release in children is less clear. Therefore, the aim of this study was to investigate the effect of 2 mM glutamine added to incubation media on HSP70 and inflammatory mediator release in an *in vitro* model of paediatric sepsis using whole blood from healthy paediatric volunteers.

Methods: An *in vitro* whole blood endotoxin stimulation model using 1 μg/ml lipopolysaccharide (LPS) over a 24 h time period was used to investigate the effects of 2 mM glutamine on HSP70 and inflammatory mediator release in healthy children.

Results: The addition of 2 mM glutamine to the incubation media significantly increased HSP70 release over time ($p < 0.05$). This was associated with an early pro-inflammatory effect on TNF-α release at 4 h ($p < 0.005$) which was not seen at 24 h. There was a non significant trend towards higher levels of IL-6 and IL-10 following the addition of 2 mM glutamine, which appears to differ from the response reported in adult and animal models.

Conclusion: Glutamine supplementation of incubation media promotes HSP70 and early TNF- α release in an *in vitro* model using blood samples from healthy children.

What is known:

- The addition of glutamine to incubation media promotes HSP70 release in *in vitro* experimental models of sepsis.
- During times of infection, HSP70 release signals via cell surface ligands TLR2 and 4 activating inflammatory pathway of NF-κB promoting the release of inflammatory mediators.

What this study adds:

- The addition of 2 mM glutamine to incubation media in a paediatric *in vitro* model of sepsis significantly increased HSP70 levels over time.
- The addition of 2 mM glutamine to incubation media appeared to promote an early pro-inflammatory response in a paediatric model of sepsis.

1. Introduction

HSP70 forms part of the cellular response to stress [1]. Levels of extracellular HSP70 are increased, in response to infection, forming a network of molecules discharged by stressed or damaged cells [2,3]. HSP70 has powerful immunoregulatory effects [4–7], providing cellular protection [8] preventing apoptosis and cell death [9–13]. Under normal circumstances HSP70 is detectable in

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plasma of healthy individuals (who have no evidence of inflammation), suggesting that during times of homeostasis, HSP70 does not promote an inflammatory response and its immunoregulatory/inflammatory functions are tightly controlled [3]. However, during times of stress HSP70 is able to interact with antigen presenting cells (APC's) and activate both innate as well as adaptive immunity. The type of response elicited depends on whether HSP70 is within or external to the cell, the particular cell surface receptor sites it binds to [14] and the type of T cells stimulated. In experimental model of sepsis, *in vitro* cell culture models HSP70 has been shown to signal via the Toll like receptors (TLR) 2/4-MyD88-nuclear factor kappa B (NF-κB) pathway promoting the release of inflammatory mediators [15] influencing the mix of cytokines released [3]. As the stress response resolves, HSP70 acts to dampen down the inflammatory and immunoregulatory response, restoring cellular homeostasis [16,17].

As such the effects of HSP70 appear to be dependent of the model used (e.g. cell culture, animal, human). A recent analysis considering the effect of HSP70 in animal models compared to humans indicate that whilst HSP70 confers an almost entirely protective effect in animals (97.1%) the same may not be true in humans, with only a 50% protective effect demonstrated [1]. Therefore the clinical benefit of HSP70 upregulation during times of stress remains unclear, especially as both low and high levels in children and adults are associated with increased risk in children and adults mortality and infection risk [1,18]. In adults following trauma HSP70 levels $\leq$15 ng/ml is associated with increased mortality [19], conversely, levels $\geq$60 ng/ml is associated with increased mortality in traumatic brain injury [20] and severe sepsis in adults [21] and children [22]. Our own work in children with acute meningococcal disease, found significantly higher levels of HSP70 in the acute phase of illness, although this were not associated with increased mortality [23].

Glutamine, as a modulator of the heat shock response [24] is well described in experimental models of sepsis [25] and in adults following trauma [26]. Glutamine exerts a complex regulatory activity with respect to the activation of intracellular signalling pathways associated with inflammation and can influence the milieu of inflammatory mediator release in response to stress independently of HSP70 [27,28].

We have previously shown that glutamine depletion occurs in critically ill children and is correlated with length of stay and illness severity scores [23]. There is, however, limited information regarding the effect of glutamine supplementation on the HSP70 and inflammatory mediator release in children [29]. As such we sought to investigate the *in vitro* effects of endotoxin and glutamine on HSP70 release and its association with markers of inflammatory response, using an *in vitro* whole blood model in healthy paediatric volunteers. As HSP70 is produced by a large variety of cells including granulocytes and peripheral mononuclear cells [3] we chose to use an *in vitro* whole blood endotoxin stimulation model. Endotoxin has been used extensively to understand the pathophysiological response to sepsis/stress as well as for the study of cytokine [30] and HSP70 release [31], providing further insight into the host response [32].

The aim of this study was to investigate the effect of the addition of 2 mM glutamine to incubation media on HSP70 and inflammatory mediator release in an *in vitro* model of paediatric sepsis.

2. Materials and methods

This study was approved by the St Mary's Hospital Imperial College Foundation Trust (reference number EC3262). After obtaining informed parental consent, 25 healthy children were prospectively enrolled from a general paediatric outpatient clinic. Heparinised whole blood was diluted 1:1 with glutamine free RPMI (Sigma Aldrich), stimulated with ± LPS (1 μg/ml) in duplicate, and incubated at 37 °C, 5% CO_2. After 2 h of culture, 2 mM glutamine was added to half the conditions and incubated for another 2 h and for a further 22 h. At each time point (4 h and 24 h) supernatant was removed, centrifuged for 10 min at 1200 g and immediately stored at −80 °C. HSP70 and inflammatory mediators were measured as per manufacturer's instructions using High Sensitivity HSP70 (HSP72) ELISA (Enzo Life Sciences CA; USA) and MSD Human pro-inflammatory − 3 II ultra sensitive ELISA (Meso Scale; MY; USA) measuring inflammatory mediators IL-6, TNF-α and IL-10. As plasma from whole blood *in vitro* model was used, we were not able to discriminate between the source (e.g. granulocytes, lymphocytes or peripheral mononuclear cells) and subsequent role of the HSP70 released from the *in vitro* cell mix.

Statistical analysis of clinical variables, HSP70 and cytokine data was completed using statistical analysis package Graphpad Prism 4.0 for Windows (Graphpad Software, San Diego, CA) and Statistical Package for Social Sciences 19.0 (SPSS: An IBM Company, Chicago, IL).

3. Results

3.1. Effect of 2 mM glutamine on HSP70 release in endotoxin stimulated whole blood from healthy paediatric volunteers

In children, the addition of 2 mM glutamine to incubation media significantly upregulated HSP70 release at 24 h in endotoxin stimulated whole blood ($p < 0.005$) in comparison to conditions with no glutamine (Fig. 1). In unstimulated blood, the release of HPS70 was significantly upregulated at 24 h in conditions with/without glutamine compared to those at 4 h ($p < 0.005$) (Fig. 1).

The addition of 2 mM glutamine to incubation media with endotoxin significantly stimulated the release of TNF-α at 4 h ($p < 0.05$) but not at 24 h (Fig. 2). A similar effect was seen with IL-6, although this was not significant (Fig. 3). 2 mM glutamine appeared to have no effect on the release of IL-10 (Fig. 4). There was a positive correlations between HSP70 and IL-6 ($n = 25$, $r = 0.61$, $p = 0.004$) and TNF-α ($n = 25$, $r = 0.61$, $p = 0.005$) at 4 h following the addition of 2 mM glutamine to incubation media (Fig. 5a and b) but there was no relationship between HSP70 and IL-10 ($n = 25$, $r = 0.191$, $p = 0.382$).

4. Discussion

In an *in vitro* model of paediatric sepsis the addition of 2 mM glutamine to incubation media significantly increased HSP70 release at 24 h, concurring with other models of experimental sepsis [25,33] and critically ill adults [26]. However, in addition, to this we also found there was a significant release of HSP70 in unstimulated whole blood control wells with and without glutamine. The significant increase in HSP70 levels appears to be a physiological response to the experimental conditions, and has previously been described *in vitro* and *in vivo* studies [25,34–36] and is unlikely to be due to endotoxin contamination given that inflammatory mediator levels were low at each time point in control wells. The addition of 2 mM glutamine to incubation media in endotoxin stimulated blood from paediatric volunteers significantly increased the release of TNF-α at 4 h, although at 24 h the release of TNF-α in endotoxin stimulated blood was not significantly different. There was a similar but non significant trend for IL-6 release after the addition of 2 mM glutamine to incubation media. However, The addition of 2 mM glutamine had no discernible effect on IL-10 at 4 or 24 h following endotoxin stimulation though it may be that dosage greater than 2 mM glutamine is required to significantly influence the release of inflammatory mediators [37,38].