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Title: An *in vitro* model to consider the effect of 2mM glutamine and KNK437 on endotoxin stimulated release of HSP70 and inflammatory mediators

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Abstract

Objective: Glutamine has been shown to promote heat shock protein 70 (HSP70) release both within experimental *in vitro* models of sepsis and in adults with septic shock.

Methods: An *in vitro* whole blood endotoxin stimulation assay was used to investigate the effects of 2mM glutamine and an inhibitor of HSP70 (KNK437) on the release of HSP70 and inflammatory mediators in healthy adult volunteers.

Results: 2mM glutamine significantly increased HSP70 levels over time ($p<0.05$). HSP70 release had a positive correlation at 4 hours with IL-1 β ($r=0.51$, $p=0.03$), an inverse correlation with TNF- α ($r=-0.56$, $p=0.02$) and IL-8 levels ($r=-0.52$, $p=0.03$), and there were no significant correlations between HSP70 and IL6 or IL-10 or glutamine. Glutamine supplementation significantly ($p<0.05$) attenuated the release of IL-10 at 4 hours and IL-8 at 24 hours, compared to conditions without glutamine. In endotoxin stimulated blood there were no significant differences in the release of IL-6, TNF- α and IL-1 β with glutamine supplementation at 4 and 24 hours. However, glutamine supplementation (2mM) appeared to attenuate the release of inflammatory mediators (IL-1 β , IL-6, TNF- α), although this effect was not statistically significant. The addition of KNK 437, an HSP70 inhibitor significantly diminished HSP70 release which resulted in lower levels of inflammatory mediators ($p<0.05$).

Conclusion: Glutamine supplementation promotes HSP70 release in an experimental model of sepsis. Following the addition of KNK437 the effects of glutamine on HSP70 and inflammatory mediator release appear to be lost, suggesting the HSP70 in part orchestrates the inflammatory mediator response to sepsis. The clinical implications require further investigation.

Words: 250

Introduction

Heat shock protein 70 (HSP70) has been the focus of much interest, particularly during infection, with evidence suggesting a beneficial role in critical illness (1). During homeostasis, HSP70 acts as a cellular housekeeper and molecular chaperone facilitating the intracellular transport of proteins; folding and refolding of damaged proteins, as well as their removal from within the cell (2). During periods of homeostasis HSP70 levels are around 2% of the intracellular content, but during a physiological insult, cellular HSP70 levels can markedly increase to 20% (3).

Endotoxin has been used extensively to identify the pathophysiological response to sepsis/stress (4). In addition endotoxin has been used as a stimulant in both *in vivo* and *in vitro* models to further understand the effect of HSP70 on cytokine release (5-10). HSP70 can activate as well as deactivate the NF- κ B (6, 11) and MAPK signalling pathways (12, 13), mediating a pro- or anti-inflammatory response to stress (14). HSP70 plays an important role in the modulation of both immune and inflammatory response in sepsis, as it appears to prime immune cells to a potential “attack” from pathogenic organisms promoting the rapid release of bactericides such as nitric oxide and inflammatory mediators. If no “attack” ensues, the cell can quickly “stand down” with the rapid decline in extracellular HSP70 levels (15). However, the sequence in which this occurs is not clear i.e. does HSP70 potentiate the release of pro-inflammatory cytokines or do pro-inflammatory cytokines induce the release of extracellular HSP70 in response to stress (14).

Glutamine is known to effectively promote HSP70 release in experimental sepsis (*in vivo* and *in vitro*) (16-19) and during critical illness (1) and may, as a result, contribute to the inflammatory response seen in sepsis. In order to understand the effect of HSP70 on endotoxin stimulated release of inflammatory mediators, HSP70 inhibitors have been used (20-23). The use of KNK437 in an *in vitro* endotoxin stimulation model, using monocytes, inhibited the release of HSP70 attenuating the release of TNF- α , suggesting that HSP70 facilitates the upregulation of TNF- α via the NF- κ B signalling pathway (24). However, these studies have not considered the additional effect glutamine may have on HSP70 release following the addition of KNK437. We therefore investigated the effect of HSP70

inhibition and glutamine supplementation on HSP70 and inflammatory marker release using an *in vitro* endotoxin whole blood model of sepsis.

Methods

This study was approved by St. Mary's research ethics committee (reference number 09/H0712/98). Prior to these experiments a range of glutamine doses (2mM – 6.8mM) were evaluated in a whole blood endotoxin model from healthy volunteers (n=3). Our results suggested 2mM glutamine was the most effective dose, in addition to which it has previously been acknowledged as representing physiological concentrations of glutamine in the serum (25, 26).

Whole blood from healthy volunteers (n=18; age range 25 – 55 years; BMI range 19 – 29 kg/m²) was used in the *in vitro* endotoxin stimulation model. To optimise the LPS concentration required to assess the effects of 2mM glutamine on HSP70 and inflammatory mediator release, whole blood from healthy adult volunteers was initially diluted 1:1 with glutamine-free RPMI (SIGMA Aldrich, Germany), and 200µl of diluted blood was cultured in duplicate with LPS at 3 concentrations (0.01µg/ml, 0.1µg/ml or 1µg/ml). After incubation at 37°C, 5% CO₂ for 2 hours, 2mM glutamine was then added to selected wells. Blood was aspirated after 4 and 24 hours of culture and plasma recovered by centrifugation for 10 minutes at 1200g.

To investigate the effect of the HSP70 inhibitor, KNK437 (N-formyl-3,4-methylenedioxy-benzylidene-γ-butyrolactam (Sigma Aldrich, Germany), on glutamine induced HSP70 and inflammatory mediator release, whole blood was diluted as above, and cultured with 200µM KNK437 for 1 hour, after which 1µg/ml LPS was added. One hour later, 2mM glutamine was added to selected wells, and supernatant removed after blood culture as described above. To investigate differences between intracellular and extracellular release of HSP70 and inflammatory mediators, after plasma removal as described above, the cell pellet was resuspended in Dulbecco's Phosphate Buffered Saline, washed twice and centrifuged for 5 minutes at 450g. Only the cell pellet was lysed using CellLytic M and a protease inhibitor cocktail (both Sigma Aldrich Germany) according to the manufacturer's protocol. All samples were stored at -80°C for batch processing.

HSP70 was measured in duplicate using a high sensitivity ELISA (Enzo Life Sciences CA; USA) according to manufacturer's protocols. A multiplex ELISA kit (Mesoscale discovery, MD, USA) measured inflammatory mediators in duplicate (IL-6, IL-8, IL-1 β , TNF- α and IL-10).

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). One way ANOVA's were used on all data sets for each experiment with a Dunn's post-test comparison for each pairwise comparison for significant differences (27). Statistical significance was achieved with a p-value < 0.05.

Results

Serum HSP70 release in endotoxin-stimulated whole blood is upregulated by 2 mM glutamine

Serum HSP70 levels were significantly increased with the addition of 2mM glutamine in endotoxin stimulated blood, compared to controls without glutamine, for all LPS concentrations at 24 hours (Figure 1). Glutamine supplementation significantly attenuated the release of IL-10 at 4 hours (p<0.05) (Figure 2) and IL-8 at 24 hours (p<0.05) (Figure 3) compared to conditions without glutamine. In endotoxin stimulated blood (1 μ g/ml) there was no significant difference in the release of serum IL-6, TNF- α and IL-1 β with glutamine supplementation at 4 and 24 hours. However, 2mM glutamine supplementation appeared to attenuate the release of inflammatory mediators (IL-1 β , IL-6, TNF- α), although this effect was not statistically significant.

There was no significant relationship between serum HSP70 with IL-6 or IL-10 following glutamine stimulation at both 4 and 24 hours of culture regardless of LPS concentration. However, at 4 hours of culture serum IL-1 β was significantly positively correlated with serum HSP70 levels with 0.01 and 1 μ g/ml LPS stimulation (r=0.51, p=0.03, r=0.47, p=0.04 respectively) and serum HSP70 levels were significantly inversely correlated with serum TNF- α (r=-0.56, p=0.02), and serum IL-8 levels (r=-0.52, p=0.03) at 0.1 μ g/ml LPS after the addition of glutamine (data not shown).

The similarity in serum HSP70 release in endotoxin stimulated blood to glutamine is in contrast to results from the release of inflammatory mediators, which suggest that serum HSP70 release from glutamine stimulation is unlikely to be as a result of endotoxin contamination and is more likely due to a physiological response to the experimental conditions.

KNK437 reduces the glutamine mediated effects on HSP70 and cytokine release from endotoxin stimulated whole blood

The addition of KNK437 significantly inhibited extracellular HSP70 release at 24 hours regardless of glutamine supplementation (2mM) ($p<0.05$). LPS-induced intracellular HSP70 levels were greater when compared to extracellular levels, and the addition of KNK437 also significantly inhibited intracellular HSP70 release at 24 hours regardless of glutamine supplementation (2mM) ($p<0.005$) in the endotoxin stimulated group. These results suggest the release of HSP70 is inhibited following the addition of KNK437. The addition of glutamine did not increase the intracellular release of HSP70 (Figure 4 and 5).

We also examined the effect of KNK437 on the release of inflammatory mediators IL-6, IL-10 and TNF- α . Extracellular and intracellular release of IL-6 ($p<0.05$) (Figure 6 and 7), IL 10 ($p<0.05$) (Figure 8 and 9) and TNF- α ($p<0.0001$) (Figure 10 and 11) were significantly inhibited with the addition of KNK437. The addition of KNK437 to the endotoxin stimulation model, with and without glutamine, appeared to significantly inhibit the intracellular and extracellular release of HSP70 and inflammatory mediators (IL-6, IL-10, TNF- α). In the conditions using the inhibitor KNK437, glutamine supplementation had no effect on HSP70 or inflammatory mediator release.

Discussion

Our data sheds further insight into the effects of glutamine on the whole blood inflammatory response to endotoxin, suggesting much of it is mediated through the inflammatory modulating effects of HSP70. We observed that 2mM glutamine significantly upregulated HSP70 release in endotoxin stimulated whole blood at 24 hours compared to negative controls, in line with

other models of experimental sepsis (28). However, the effect of glutamine on inflammatory cytokine response in our model was less pronounced.

Hamiel *et al.* (29) showed glutamine supplementation (8mM) in lysed cells increased HSP70 release 10 fold following heat shock, although it should be noted within the experimental design there was no differentiation between intracellular and extracellular HSP70. Glutamine supplementation in our endotoxin model did not significantly increase the intracellular release of HSP70, compared to unsupplemented conditions and it may be the effect of glutamine on intracellular HSP70 differs from that of extracellular release.

In animal models of sepsis, glutamine (2 – 10mM) (19, 30) has been shown to attenuate the release of pro-inflammatory cytokines through the inhibition of IKK ($I\kappa B\alpha$) and the subsequent down-regulatory effect on the NF- κB pathway and MAPK pathways (31) attenuating the release of IL-6, TNF- α (19, 28, 32-34), IL-8 (35), IL-1 β (33) and IL-10 (32). The release of inflammatory mediators appears to be dependent on HSP70 release, since the effect of glutamine supplementation is lost in the absence of HSP70 gene upregulation, using HSP70.1/3 knock-out mice, with a subsequent uncontrolled release of TNF- α and IL-6, increasing the rate of mortality compared to normal wild type mice (36). However, some of the *in vitro* sepsis models use high doses of glutamine e.g. up to 10mM (19, 37, 38), far exceeding the dose of glutamine that would be used in clinical practice i.e. 0.35g/kg intravenously and 0.5g/kg parenterally (39). Where doses of glutamine were more moderate as used in septic rat models (0.5g/kg - 0.75g/kg), the effect on inflammatory mediators was less pronounced (31, 32, 34, 35, 40). Results from endotoxin stimulated blood in our study did not reveal significant differences in the release of IL-6, TNF- α and IL-1 β with glutamine supplementation at 4 and 24 hours. However, 2mM glutamine appeared to attenuate the release of inflammatory mediators (IL-1 β , IL-6, TNF- α), although this effect was not statistically significant.

Interestingly in an elegant study considering endotoxin stimulation in *ex vivo* and *in vivo* mouse model Cruzat VF *et al.* showed that glutamine supplementation attenuated the release of inflammatory mediators (41) an effect similar to one reported in our model. Although the effect

they reported on HSP70 release was dissimilar, as following glutamine supplementation HSP70 release was attenuated, compared to conditions with LPS alone (42). The different patterns of HSP70 release described may be arise from the site of release studied e.g. muscle versus serum, as within the muscle the addition of glutamine appears to re-establish muscle homeostasis, stabilising the translation of HSP70 e.g. mRNA expression of HSPA1A and HSPA1B, in addition to reducing mRNA expression of HSF-1 compared to the endotoxin group (42) .

Conversely, within a whole blood model the effect of 2mM glutamine on extracellular HSP70 release may be reflective of the effect of HSP70 within the *in vitro* cellular milieu. This local effect may subsequently influence the pattern of release of inflammatory mediators dependent on the type of immune cell with which HSP70 interacts and the co-presence of pro- or anti-inflammatory mediator and interactions between pathogenic organisms (14). The effect of glutamine supplementation on HSP70 release in our *in vitro* model was similar to the effect described in other *in vitro* septic models (17) and critically ill adults (1), although we were not able to discriminate between the cell mix in our *in vitro* model.

In addition to this, glutamine appears to exerts a complex regulatory activity with respect to the activation of inflammatory pathways mediating the release of cytokines independent of HSP70 (43, 44),.Glutamine supplementation may not always mediate an anti- inflammatory response as seen in our *in vitro* model of paediatric sepsis where glutamine supplementation appeared to promote the release of inflammatory mediators, which is in contrast the effect seen in adult volunteers (45).

To further examine the effect of glutamine and HSP70 on inflammatory mediator release, a HSP70 inhibitor (KNK437) was added to the *in vitro* model. KNK437 is effective at preventing HSP70 release *in vitro* (46) and *in vivo* models (47) by inhibiting HSF-1 transcription (12). The addition of KNK437 significantly inhibited the intracellular and extracellular release of HSP70 at 24 hours in all conditions regardless of glutamine supplementation. A similar finding was reported in an amphibian model (48). The release of inflammatory mediators (IL-6, IL-10, TNF- α) at 4 hours and 24 hours in all

conditions were also significantly diminished, although there was no significant difference in the release of inflammatory mediators between glutamine and unsupplemented conditions in endotoxin stimulated blood with KNK437. The use of KNK437 in an *in vitro* endotoxin model inhibited the release of HSP70, reducing levels of TNF- α in macrophages (49) and IL-6 from cultured mouse C2C12 myotubes (50). The inhibitory effect of KNK437 on HSP70 release, suggests that in part, HSP70 orchestrates the release of inflammatory mediators in response to stress, which is reported in other models (21).

Anti-sense oligomers have been successfully used in cell line work but not in whole blood. Transfection work shows that endotoxin induced HSP70 mediates inflammatory cytokine release. Conversely when HSP70 production is inhibited, mRNA expression of TNF- α , IL-10, IL-1 β and IL-12 is diminished (21). Inhibition of HSP70 only partially prevented cytokine release, as the accumulation of TNF- α , IL-10, IL-1 β , IL-6 and IL-12 was not completely abrogated in the presence of endotoxin stimulation, suggesting that there are other factors that mediate the release of inflammatory cytokines (21). These results suggest that HSP70 influences the cytokine milieu release, mediating an optimal inflammatory response dependent on the level of stress (21), an effect supported by others (51, 52).

The results from this work highlight the need to further explore the relationships of glutamine, HSP70 and inflammatory mediators, particularly as the use of glutamine supplementation in critically ill septic patients suggests that either hypo-glutamine < 430 μ mol/L (53) or hyper-glutamine levels >930 μ mol/L are associated with increased mortality (54, 55), although hyper-glutamine levels could be more a marker of organ failure and severe shock rather than a cause of harm (39). The effect of hyper- levels glutamine levels on inflammatory mediator and HSP70 release during sepsis is also unknown. In, *in vitro* models, high levels of glutamine supplementation (5Mm) resulted in respiratory uncoupling and energy wasting within the mitochondria and was associated with cell death (56). Therefore caution is required when translating results from experimental work particularly in septic shock models to inform clinical practice, especially as results from recent large

clinical trials questioned the benefits of glutamine supplementation in septic adults with multi organ failure (54). However, a recent editorial from Wischmeyer P, suggests that certain patient populations may benefit from glutamine supplementation e.g. adults with burns, malignancies, trauma or those requiring parenteral nutrition (39).

There are some limitations to this work, including the investigation of only one dose of glutamine (2mM), which although significantly upregulated HSP70 release, did not have the same effect on the release of inflammatory mediators. A much higher dose of glutamine (≥ 2 mM) may be required to mediate release of inflammatory mediators. Although a range of pro- and anti-inflammatory mediators were selected for study based on previous work in meningococcal disease, it was not possible to examine all those of interest (TNF- α , IL-8, IL-6, IL-10) within a single validated multiplex plate. Another limitation was the lack of discrimination between live and dead cells, which prevented the source of HSP70 release (e.g. passive release or as a result of necrosis) from being determined. Finally, although *in vitro* models of sepsis are well described, there are limitations to the use of such models in that they cannot completely replicate the individual response to infections as confounding factors also influence individual disease pathogenesis and the specific pathogen involved.

Conclusion

Our results suggest that in adult subjects, 2mM glutamine promoted the release of HSP70 in an *in vitro* endotoxin stimulation model. HSP70 production was partially inhibited through the use of KNK437 and the subsequent release of inflammatory mediators (IL-6, IL-10, TNF- α) was attenuated. Caution is required when translating results from experimental work into clinical practice and the results from this work highlight the need to further explore the relationships of glutamine, HSP70 and inflammatory mediators further in both adults and paediatric models.

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Figure 1: The effect of glutamine supplementation (2mM) on endotoxin stimulated serum HSP70 release in whole blood from healthy adult volunteers at 24 hours (n=18) Results are expressed as mean; $\pm$ SEM. Gln = glutamine, LPS = endotoxin **p<0.005 compared to control and when compared to control without LPS

Figure 2: The effect of glutamine supplementation (2mM) on LPS stimulated release of IL-10 at 4 hours and 24 hours in whole blood from healthy adult volunteers (n=18).

Results are expressed as mean; $\pm$ SEM. Gln = glutamine, LPS = endotoxin *p<0.05 compared to control, **p<0.005 compared to control

Figure 3:

The effect of glutamine supplementation (2mM) on LPS stimulated release of IL-8 at 4 hours and 24 hours in whole blood from healthy adult volunteers (n=12). Results are expressed as mean; $\pm$ SEM. Gln = glutamine. LPS = endotoxin *p<0.05 compared to control, **p<0.005 compared to control

Figure 4: The effect of KNK437 on extracellular HSP70 release at 4 hours and 24 hours in endotoxin stimulated whole blood with and without glutamine supplementation (2mM) (n=18). Results are expressed as mean; $\pm$ SEM. Gln = glutamine, LPS = endotoxin, *p<0.05 compared to control, **p<0.005 compared to control

Figure 5: The effect of KNK437 on intracellular HSP70 release at 24 hours in endotoxin stimulated whole blood with and without glutamine supplementation (2mM) (n=18). Results are expressed as mean; $\pm$ SEM. Gln = glutamine, LPS = endotoxin. *p<0.05 compared to control, **p<0.005 compared to control

Figure 6: The effect of KNK437 on extracellular IL-6 release at 4 hours and 24 hours in endotoxin stimulated whole blood with and without glutamine supplementation (2mM) (n=18). Results are expressed as mean; $\pm$ SEM. Gln = glutamine. *p<0.05 compared to control, **p<0.005 compared to control

Figure 7: The effect of KNK437 on intracellular IL-6 release at 4 hours and 24 hours in endotoxin stimulated whole blood with and without glutamine supplementation (2mM) (n=18). Results are expressed as mean; $\pm$ SEM. Gln = glutamine. * p <0.05 compared to control, ** p <0.005 compared to control

Figure 8: The effect of KNK437 on extracellular IL-10 release at 4 hours and 24 hours in endotoxin stimulated whole blood with and without glutamine supplementation (n=18). Results are expressed as mean; $\pm$ SEM. Gln = glutamine, LPS = endotoxin *p<0.05 compared to control, **p<0.005 compared to control

Figure 9: The effect of KNK437 on intracellular IL-10 release at 4 hours and 24 hours in endotoxin stimulated whole blood with and without glutamine supplementation (n=18). Results are expressed as mean; $\pm$ SEM. Gln = glutamine, LPS = endotoxin *p<0.05 compared to control, **p<0.005 compared to control

Figure 10: The effect of KNK437 on extracellular TNF-α release at 4 hours and 24 hours in endotoxin stimulated whole blood with and without glutamine supplementation (2mM) (n=18). Results are expressed as mean; ± SEM. Gln = glutamine, LPS = endotoxin * p<0.05 compared to control

Figure 11: The effect of KNK437 on intracellular TNF-α release at 4 hours and 24 hours in endotoxin stimulated whole blood with and without glutamine supplementation (2mM) (n=18). Results are expressed as mean; ± SEM. Gln = glutamine, LPS=endotoxin. **p<0.005 compared to control

Highlights**What is known:**

- Glutamine supplementation promotes HSP70 release in *in vitro* experimental models of sepsis.
- During times of infection, HSP70 signals via cell surface ligands TLR2/4 activating inflammatory pathway of NF- κ B promoting the release of inflammatory mediators.
- In an *in vitro* experimental model of sepsis, the HSP70 inhibitor KNK437 effectively blocks HSP70 release diminishing the release of inflammatory mediators.

What this study adds:

- The addition of 2mM of glutamine to a whole blood endotoxin model promotes the release of HSP70.
- The effect of 2mM glutamine on HSP70 release is lost following the addition of a HSP70 inhibitor (KNK437) to an *in vitro* model of sepsis.