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Glutamine depletion impairs cellular stress response in human leucocytes

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During sepsis and major trauma the blood glutamine (Gln) level is reduced. The administration of Gln can improve the outcome of these patients. However, the mechanism of this beneficial effect of Gln is poorly understood. In the course of critical illness leucocytes are confronted with cytotoxic inflammatory mediators. To protect themselves against these factors, cells express heat shock proteins (HSP). Previous studies have shown that the expression of the major inducible HSP (HSP70) is improved by high Gln concentrations above 4 mM. In this study we investigated whether Gln depletion, such as observed during critical illness, has an effect on HSP70 expression. Human lymphocytes exposed for 2 h to 42°C showed a 3-fold increase in HSP70 expression ($P<0{\cdot}01$). A preceding Gln starvation period over 3 days had no influence on this increase. However, when Gln is reduced during the stress response, HSP70 expression is impaired. A reduction of Gln from 0·5 mM (physiological) to 0·125 mM (pathological) led to a 40 % lower HSP70 level ($P<0{\cdot}002$). In contrast, increasing Gln concentrations (up to 2 mM) had only minor stimulatory effects (about 15 %). This Gln-dependency of heat mediated HSP70 expression was observed in resting as well as proliferating lymphocytes. Our data indicate that during periods of reduced plasma Gln levels the stress response of human lymphocytes is impaired. Thus, Gln may be essential to minimize the susceptibility of leucocytes to cytotoxic inflammatory mediators. This is a new aspect of the protective effect of Gln supplementation in critically ill patients.

Lymphocytes: Heat shock proteins: HSP70: Glutamine

Introduction

Glutamine (Gln) is the most abundant free amino acid in the blood and in the amino acid pool of the body. During starvation and catabolic stress after trauma, surgical procedures, or during sepsis and certain cancers, plasma Gln levels are strongly reduced. Several studies have demonstrated that Gln supplementation can provide an approach for modulation of severity of these critical diseases (for review see Neu *et al.* 1996). The mechanisms of the beneficial effects of Gln supplementation in critical illness remain poorly understood. The availability of Gln has been found to be especially important for survival and function of leucocytes. Gln deprivation decreases mitogen-inducible proliferation in lymphocytes (Szondy & Newsholme, 1991) and increases susceptibility to apoptosis of premonocytic U937 cells (Petronini *et al.* 1996). In previous studies we could show that in the absence of Gln, expression of surface proteins responsible for antigen presentation and phagocytosis in human monocytes is down-regulated (Spittler *et al.* 1995) and differentiation of premonocytic U937 cells is induced (Spittler *et al.* 1997). Supplementation of Gln abolishes these effects.

In the course of critical illness leucocytes are exposed to huge amounts of cytotoxic inflammatory mediators such as reactive oxygen species. In order to protect themselves against these factors, leucocytes express a group of proteins which are essential to cellular survival under such stressful conditions, the heat shock proteins (HSPs) (Polla & Cossarizza, 1996; Welch, 1991). HSP can be divided into several families according to their molecular weight. The 70 kDa heat shock protein (HSP70) has attracted the most interest because it exhibits the strongest protective effect against environmental insults including heat, UV-irradiation, heavy metals and reactive oxygen species. Induction of HSP70 was found to increase survival of mice in an endotoxin-induced sepsis model (Ribeiro *et al.* 1994; Villar *et al.* 1994) and in a respiratory distress syndrome model (Villar *et al.* 1993). In addition, overexpression of

Abbreviations: Gln, glutamine; HSP, heat shock protein.

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HSP70 in a transgenic mouse model reduced the lethality after ischemia-reperfusion (Suzuki *et al.* 1997). Thus, high expression of HSP70 protects each cell against toxic agents as well as the whole body against pathological insults.

There are some reports in the literature describing that Gln can affect HSP70 expression in various cell types. Cai *et al.* (1991) observed that the addition of fresh medium to Chinese hamster ovary (CHO) cells induced HSP70 expression. They identified Gln as the stimulating compound. In the absence of any stress, addition of Gln to cells led to an increase of HSP70 expression. Similarly, HSP70 expression could also be induced in rat intestinal crypt IEC-18 cells simply by adding Gln (Musch *et al.* 1998; Wischmeyer *et al.* 1997). In contrast, Gln increases HSP70 expression in Opossum kidney OK cells (Nissim *et al.* 1993) and in rat intestinal IEC-6 cells (Ehrenfried *et al.* 1995) only after heat treatment of cells. In all these studies the Gln concentrations needed to yield the maximum increase in HSP70 expression were rather high (CHO cells: 5 mmol/l, IEC-18 cells: 20 mmol/l, OK cells: 4 mmol/l, IEC-6 cells: 8 mmol/l) and far beyond physiological plasma levels (0·5–0·7 mmol/l). In the present study we investigated the influence of Gln reduction, similar to what is observed during critical illness, on HSP70 expression. Using human lymphocytes as a model system we could show that Gln-depletion leads to a reduced HSP70 expression of heat stressed cells. This indicates that during periods of low plasma Gln levels, the stress response of leucocytes is impaired which makes these cells more susceptible to cytotoxic inflammatory mediators.

Materials and methods

Lymphocyte isolation and treatment

Lymphocytes were isolated from whole blood by density gradient centrifugation as has been previously described (Coligan *et al.* 1996). Briefly, heparinized whole blood was centrifuged through a FICOLL-PAQUE layer (from Amersham-Pharmacia, Buckinghamshire, UK). The resulting buffy coat, which consisted of lymphocytes and monocytes, was diluted in complete medium (RPMI-1640 supplemented with 10 % fetal calf serum (FCS) and 2 mM Gln) and placed in a plastic tissue culture flask for 3 h. During this time monocytes adhere to the plastic surface and lymphocytes remain in suspension. Then lymphocytes were washed, diluted in complete medium to 10^6 cells per ml and cultured for 72 h at 37°C. For heat treatment lymphocytes were washed in PBS, diluted in the indicated medium to 10^6 cell per ml and placed in a 42°C incubator. To allow HSP70 synthesis, lymphocytes were then placed into 37°C for 3 h. This treatment induced neither apoptosis nor necrosis as determined by annexin-V assay.

Flow cytometry

Intracellular staining of HSP70 was performed according to Bachelet *et al.* 1998 with modifications. Briefly, 100 µl heparinized whole blood was mixed with 5 ml lysis solution (155 mM NH_4Cl, 10 mM $KHCO_3$ pH = 7·4, and 0·1 mM EDTA) and incubated for 10 min at room temperature for elimination of erythrocytes. The remaining leucocytes were washed at 4°C in HBSS (Bio-Whittaker, Verviers, Belgium) containing 3 % FCS, suspended in 250 µl fixation solution containing paraformaldehyde (Cytofix/Cytoperm Plus Kit from BD-Pharmingen, San Jose, CA, USA) and incubated for 15 min at 4°C for fixation. Cells were then centrifuged for 5 minutes at 600 *g*, suspended in 100 µl of a 1:300 dilution of a HSP70 specific antibody (SPA810 from Stressgen, Victoria, Canada) in permeabilization solution containing saponin (Cytofix/Cytoperm Plus Kit) and incubated for 30 min at 4°C. After washing in permeabilization solution cells were incubated in a 1:250 dilution of FITC-labeled anti-mouse IgG1 antibody (BD-Pharmingen, San Jose, CA, USA) for 30 min at 4°C and washed again in permeabilization solution. Negative control staining was performed with appropriate fluorescence labeled isotype IgG antibodies (Immunotech, Marseilles, France). The results obtained with controls were subtracted from the specific signal. Stained cells were analyzed using a flow cytometer (Epics XL, Coulter, Miami, FL, USA). 10 000 events were acquired.

Results

Heat treatment of cultured lymphocytes induces HSP70 expression

HSP70 is induced by heat stress in almost all organisms, but the level of induction depends on the temperature as well as on the responding cell type. To investigate the influence of heat stress on HSP70 expression in human lymphocytes we exposed these cells to a heat shock (42°C for 2 h) and measured HSP70 expression by flow cytometry. The results are shown in Fig. 1A. Untreated cells showed a weak HSP70 expression. Heat treatment, however, led to a strong increase in fluorescence signal indicating enhanced HSP70 expression. The evaluation of the histograms, using the specific mean fluorescence intensity (MFI) as a measure for cellular HSP70 expression, revealed that in heat shocked lymphocytes HSP70 levels were about 200 % of untreated controls (see Fig. 1B).

Heat-mediated HSP70 expression depends on glutamine

HSP70 expression is improved by high Gln concentrations in various animal cell lines. However, the effects of Gln depletion are largely unknown. To investigate the effect of long-term Gln starvation on HSP70 expression we cultured isolated lymphocytes for 72 h in Gln-free medium. These Gln-depleted cells were suspended in complete medium, exposed to heat shock, and after recovery at 37°C HSP70 expression was measured. As shown in Fig. 2A, a Gln starvation period of 3 days neither had an effect on the constitutive HSP70 expression nor on the heat-induced HSP70 expression.

For further characterization we investigated the stress response to heat shock while cells were incubated continuously in reduced Gln concentrations. For this purpose, we suspended isolated lymphocytes cultured in complete medium for 72 h in medium with different Gln concentrations (0, 62·5, 125, 250, 500, 1000, or 2000 µM

Gln) and exposed them to heat shock. Then cells were allowed to recover for 3 h at 37°C, and HSP70 expression was analyzed. Cells treated with the same conditions but kept at 37°C were used as negative controls. We found that the varying Gln concentration in the medium had no effect on the constitutive HSP70 expression (data not shown).

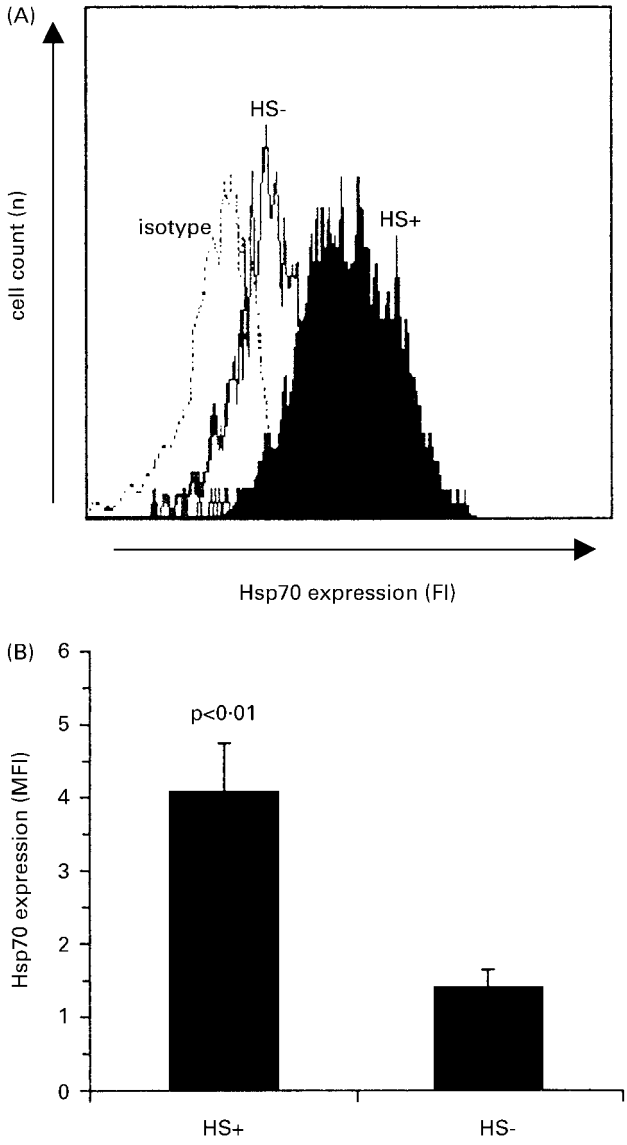

Fig. 1. HSP70 expression of heat-treated and untreated human lymphocytes. Lymphocytes were isolated from whole blood and cultured for 72 h at 37°C. Then cells were exposed for 2 h to 42°C HS+ or 37°C HS− and returned to 37°C for an additional 3 h. Intracellular HSP70 expression was determined by flow cytometry. (A) Histogram: HSP70 levels are expressed as fluorescence intensity [FI]. The dotted line represents the fluorescence of cells stained with isotype control antibodies. The other peaks represent the fluorescence of heat shocked HS+ and untreated HS− lymphocytes stained with HSP70 specific antibodies. (B) Statistical evaluation: the HSP70 expression was calculated by subtracting the mean fluorescence intensity of cells stained with isotype control antibodies from the mean fluorescence intensity of cells stained with HSP70 specific antibodies. The mean of four independent experiments is indicated. The error bars represent standard deviations. The *P*-value was calculated by Student's *t*-test.

After heat treatment, HSP70 levels increased at all Gln concentrations, but to a different extent (Fig. 2B). At Gln concentrations similar to the plasma levels of healthy subjects (500 μM) the HSP70 induction was slightly reduced (−15 %) in comparison to complete medium

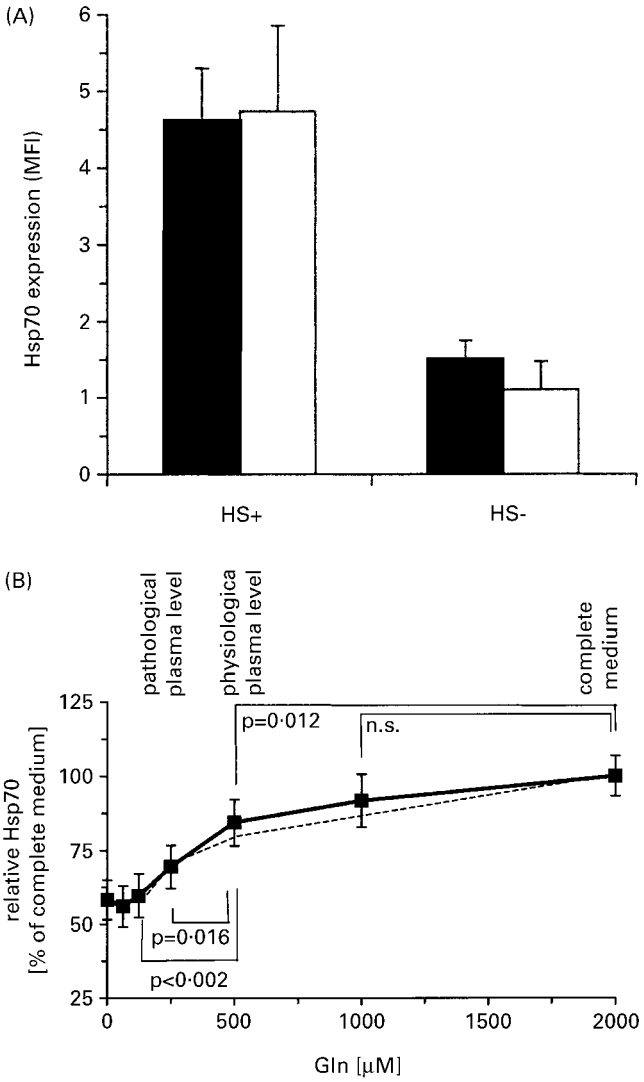

Fig. 2. Influence of Gln on HSP70 expression. (A) HSP70 expression after long-term Gln-depletion. Lymphocytes were isolated from whole blood and cultured for 72 h at 37°C in the absence of Gln, Gln depleted (■) or in complete medium non depleted (□). Then cells were suspended in complete medium, exposed to heat shock 2 h at 42°C and allowed to recover at 37°C for 3 h. HSP70 expression was determined by flow cytometry. The values shown represent the mean $n = 3$ and standard deviations are indicated by error bars. (B) HSP70 expression of lymphocytes exposed to various Gln concentrations during heat shock. Lymphocytes were isolated from whole blood and cultured for 72 h at 37°C in the absence of Gln dotted line or in complete medium traced line. Then cells were suspended in medium containing 0, 62·5, 125, 250, 500, or 1000 μM Gln or complete medium containing 2000 μM Gln, exposed to heat shock 42°C for 2 h and allowed to recover for 3 h at 37°C. HSP70 expression was determined by flow cytometry and expressed as the percentage of the HSP70 levels of cells grown and heat-treated in complete medium. The error bars represent standard deviations $n = 4$. The *P*-values refer to the non-starved cells and were calculated by Student's *t*-test.

(2000 μM). However, when Gln was reduced to levels seen in plasma of critically ill patients (250–125 μM) the HSP70 expression was greatly decreased (about −40 %). Repeating the same experiment with Gln-depleted lymphocytes (cultured for 72 h in the absence of Gln) gave essentially the same results (Fig. 2B dotted line).

Taken together these data clearly show that heat shock mediated HSP70 expression in human lymphocytes depends on the amount of Gln available during the stress response. The Gln concentration prior to heat shock, however, has no effect on HSP70 induction. Since the Gln-dependency of HSP70 induction occurs in the range between physiological and pathological plasma Gln concentrations this effect is of clinical relevance.

Glutamine effect on HSP70 expression induction is not dependent on the proliferative state of lymphocytes

In the course of an immune response lymphocytes become activated and start to proliferate for elimination of foreign antigens. The experiments described above show that Gln is essential for stress-mediated HSP70 expression in resting lymphocytes. To investigate the effect of Gln on the cellular stress response in activated lymphocytes we stimulated isolated cells with phytohemagglutinin (PHA). This stimulation resulted in a strong proliferation, as shown by [^{3}H]thymidine incorporation (Fig. 3A). We suspended activated lymphocytes grown in complete medium for 72 h in medium with varying Gln concentration (0, 62·5, 125, 250, 500, or 2000 μM Gln) and exposed them to heat shock. Then cells were allowed to recover for 3 h at 37°C and HSP70 expression was measured. Cells incubated under the same conditions but without PHA-stimulation were used as control. As shown in Fig. 3B, results were comparable in proliferating and non-proliferating lymphocytes. Lymphocytes heat-treated in the presence of 0–250 μM Gln showed a reduced HSP70 expression as compared to cells heat treated in medium containing 500 μM Gln or complete medium. Thus, the Gln-dependence of heat-mediated HSP70 induction occurs in activated as well as in resting human lymphocytes.

Discussion

This study shows that Gln depletion leads to an impaired stress response in human lymphocytes. When Gln is reduced from levels observed in plasma of healthy subjects to levels observed in plasma of critically ill patients, lymphocytes express lower amounts of HSP70 in response to heat stress. Gln is known to induce HSP70 expression in several animal cell lines including Chinese hamster ovary cells (Cai *et al.* 1991), rat intestinal crypt cells (Musch *et al.* 1998; Wischmeyer *et al.* 1997), opossum kidney cells (Nissim *et al.* 1993) and in rat intestinal IEC-6 cells (Ehrenfried *et al.* 1995). In these studies cells were incubated in medium with extremely high Gln concentrations (from 4 to 20 mM) which led either to an induction of HSP70 or to a further increase of HSP70 expression after heat stress. These Gln concentrations are far above the physiological plasma Gln concentrations (0·5–0·6 mmol/l). In contrast the present study investigates the effect of Gln depletion on HSP70 expression. We used human peripheral blood lymphocytes as a model system, because lymphocytes play a central role in the immune response and are exposed to all alterations of blood Gln levels. We found a rather low constitutive HSP70 expression in these cells. This is in accordance with mRNA data that show that HSP70 mRNA is barely detectable in unstressed human lymphocytes (Fincato *et al.* 1991). Heat treatment at 42°C, a temperature which is reached during a

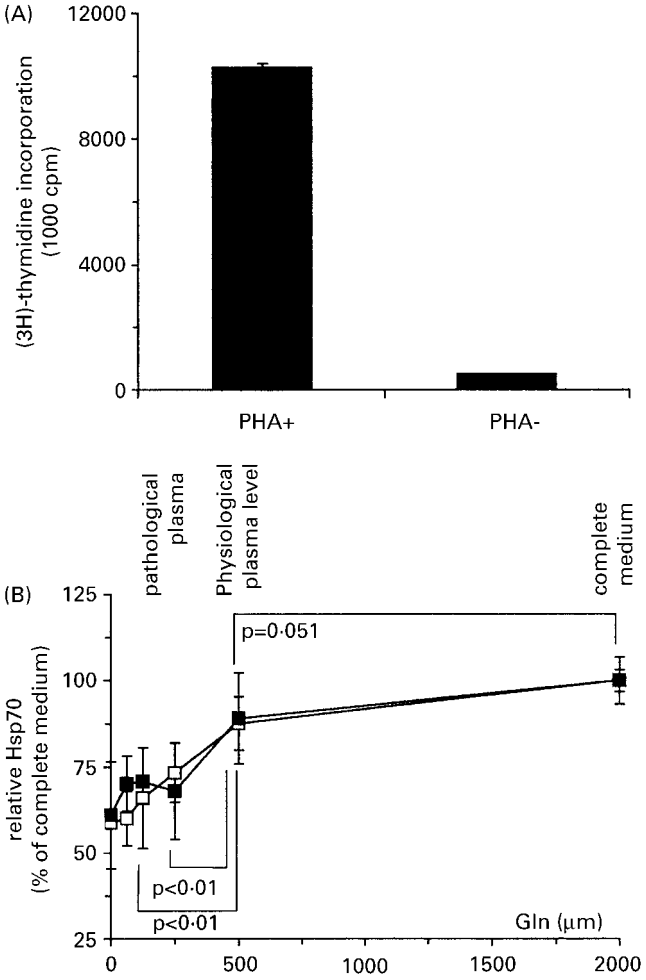

Fig. 3. Heat-induced HSP70 expression in proliferating and non-proliferating human lymphocytes. Lymphocytes were isolated from whole blood and cultured for 72 h at 37°C in complete medium containing 5 μg/ml phytohemagglutinin PHA+ or not PHA−. (A) Proliferation rate. 1 μCi [^{3}H]-thymidine was added to PHA treated and non-PHA treated lymphocytes and cells were cultured for an additional 6 h. [^{3}H]-thymidine incorporation was determined by scintillation counting and used as a measure for lymphocyte proliferation. Results are given as the mean of three independent experiments and the error bars indicate standard deviations. (B) Heat-induced HSP70 expression. PHA treated proliferating and non-PHA treated non proliferating cells were suspended in medium containing 0, 62·5, 125, 250, or 500 μM Gln or in complete medium containing 2000 μM Gln, exposed to heat shock 42°C for 2 h and allowed to recover for 3 h at 37°C. HSP70 expression was determined by flow cytometry and is expressed as percentage of the HSP70 level in cells grown and heat-treated in complete medium. Results are given as the mean of three independent experiments and the error bars indicate standard deviations. The *P*-values refer to the activated cells (■) and were calculated by Student's *t*-test. (□) refers to resting cells.

high fever, led to a 3-fold increase in HSP70 expression. A similar induction of HSP70 in heat stressed human lymphocytes was recently reported by others (Dressel & Gunther, 1999). We could show that this HSP70 induction is strongly impaired when the Gln concentration in the medium is reduced. The strongest effect of Gln deprivation was observed between 125 μM and 500 μM Gln (see Fig. 2B). At 125 μM HSP70 expression is essentially as low as without Gln, whereas at 500 μM Gln HSP70 levels are nearly the same as in complete medium. This indicates that the stress response in human lymphocytes is well adapted to the physiological plasma Gln levels (500–600 μM) and is impaired when Gln is reduced, as it happens during critical illness. Support for this view comes from a study previously published by our laboratory (Weingartmann *et al.* 1999). We investigated the HSP70 expression in leucocytes of polytrauma patients with reduced plasma Gln levels. Their leucocytes showed decreased HSP70 expression when compared to healthy controls. In the current study we show that the Gln concentration during the stress response is crucial for HSP70 expression. Gln-starvation prior to stress response has no influence. This suggests that Gln depletion interferes directly in the stress response cascade. The molecular mechanism of this Gln effect has to be investigated in further studies.

Our study shows that HSP70 expression is impaired at subphysiological Gln levels, similar to those observed in critically ill patients. Since HSP70 is a known cytoprotective protein, these results indicate that these immune cells are more susceptible to cytotoxic inflammatory mediators such as reactive oxygen species in the course of such diseases. Supplementation with Gln would help to overcome this effect and thus improve the immune response. This is a new aspect of the immunomodulatory effects of nutritional Gln supplementation.

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