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ORIGINAL ARTICLE

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Reduced stress tolerance of glutamine-deprived human monocytic cells is associated with selective down-regulation of Hsp70 by decreased mRNA stability

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Abstract In critically ill patients, clinicians observe a reverse correlation of survival and a decreased plasma concentration of the most abundant free amino acid, glutamine (Gln). However, in this context, the role of Gln remains largely elusive. Gln is used as an energy substrate by monocytes. Gln deprivation of these cells results in an increased susceptibility to cell stress and apoptosis, as well as in a reduced responsiveness to pro-inflammatory stimuli. We performed a systematic study to elucidate the molecular mechanism by which Gln depletion affects the heat stress response of the monocytic cell line U937. Proteomic analysis revealed that Gln depletion was associated with specific changes in the protein expression pattern. However, the overall level of tRNA-bound Gln remained unaffected. The stress protein heat shock protein (Hsp) 70 showed the highest reduction in protein synthesis. This was due to enhanced mRNA decay during Gln starvation while the transcriptional and the translational control of Hsp70 expression remained unchanged. A physiological Gln concentration and above was found to be necessary for maximum Hsp70 accumulation upon heat shock. Thus, the study shows a specific link between Gln metabolism and the regulation of heat shock proteins.

Keywords Glutamine · Amino acid starvation · Heat shock protein 70 · Thermotolerance

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Introduction

Biological mechanisms sensing for amino acid levels play a key role in coupling the changes in amino acid metabolism to the appropriate physiological responses. In mammalian cells, it has been shown that the concentration

of amino acids determines the expression of numerous genes. The identified levels of regulation include transcriptional control, as well as mRNA stability and translational control. However, amino acid metabolism depends strongly on the diet and varies therefore between mammalian species. The regulatory capacities of the different amino acids in humans are still poorly understood. A strong interest has been taken in glutamine (Gln) especially in the medical context since it plays a versatile role in cell homeostasis and inter-organ metabolism. With a concentration of 0.5–0.6 mM, Gln is the most abundant free amino acid in human plasma, and Gln homeostasis is rigidly controlled. It plays a central role in inter-organ nitrogen exchange by transporting excess nitrogen from peripheral tissues especially from skeletal muscle to liver and kidney, while the carbohydrate backbone is recycled via gluconeogenesis (for review see [1]). Due to its high plasma concentration, Gln is a ready source of energy and is used as an energy substrate for the intestine and in particular for macrophages [2]. However, during critical illness, the Gln consumption rate exceeds the supply, and plasma as well as skeletal muscle pools of free Gln are severely reduced. The plasma Gln levels can drop to 0.4 mM, and this 'Gln-deficient' state is associated with a reduced responsiveness of immune cells [3]. However, it has not been demonstrated that Gln reduction is the sole cause for the observed immune deficiency. Several years ago, our laboratory demonstrated that the survival of critically ill patients correlates with the concentration of Gln in both plasma and skeletal muscle [4] and that a decrease in Gln levels is associated with a reduced cellular hydration state [5]. However, a patient benefit of Gln supplementation in clinical trials remains controversial [6]. Gln or Gln-dipeptides reportedly improve the postoperative nitrogen balance [7] and T-cell response [8]. Furthermore, the gastrointestinal function is preserved [9, 10]. Goeters et al. showed in an open randomised trial with 144 patients an improved 6-month outcome of critically ill patients who received parenteral nutrition supplemented with the dipeptide alanyl-Gln for more than 9 days [11]. These findings are in contrast to a report by Powell-Tuck and his co-workers, who replaced a part of the available nitrogen in the parenteral formulation with Gln [12]. They found that Gln supplementation did not influence the incidence and extent of infective complications nor the mortality rate or the duration of hospitalisation, with the exception of surgical patients who recovered faster upon Gln supplementation. A meta-analysis on the usefulness of Gln in intensive care units has sparked further controversy, but came to the conclusion that Gln supplementation may decrease the rate of infectious complications, shorten the hospital stay and reduce the mortality of critically ill patients when given at high doses [13]. A recent double-blind, randomised clinical trial demonstrated that enteral Gln supplementation in burn patients reduces infections and may be associated with a decreased mortality rate. In addition, ex vivo assessment of neutrophil functions showed an increased capacity for prevention of bacteraemia [14].

Further in vitro studies with isolated cells revealed a close correlation between Gln utilisation by leucocytes and their immunological functions. In monocytes, Gln deficiency leads to a reduced major histocompatibility complex (MHC) class II expression, a decreased antigen presentation and a diminished phagocytosis rate [15]. In a recent study with primary monocytes and monocytic U937 cells, we found that Gln depletion resulted in a decrease in proteasome-mediated proteolysis and also in a reduced overall protein synthesis rate. This was accompanied by an accumulation of ubiquitin–protein conjugates and a reduction in intracellular adenosine 5c-triphosphate (ATP) [16]. Furthermore, we found that Gln-starved monocytic cells are more susceptible to the apoptotic triggers Fas ligand and tumour necrosis factor (TNF)-alpha [17]. Comparably, studies in leukaemia-derived CEM and HL-60 cells revealed that Gln deprivation induces ligand-independent CD95 receptor signalling and apoptosis [18]. In addition, there is evidence for a Gln-dependent anti-apoptotic interaction of glutaminyl-tRNA synthetase with the apoptosis signal-regulating kinase 1 [19].

Here we report that Gln starvation directly and specifically affects the cellular stress response in human monocytic U937 cells. We demonstrate that Gln starvation induces alterations in the proteome, with a strong effect on the stress protein heat shock protein (Hsp) 70. We could show that Gln starvation has no effect on the transcriptional or the translational control of Hsp70 expression but enhances the decay of Hsp70 mRNA. Thus, a sufficient supply of monocytic cells with Gln is a prerequisite for an adequate cellular stress response.

Materials and methods

Chemicals

Radiochemicals and the DNA 5′-end-labelling kit were from Amersham Bioscience (Uppsala, Sweden); *o*-phtaldehyde, potassium borate solution (pH=10.4), and β-mercaptoethanol were from Pierce (Rockford, IL, USA). Unless otherwise specified, all other chemicals used were obtained from Sigma (St. Louis, MO, USA).

Cell culture and treatment

Human premonocytic cells U937 (CRL-1593.2, ATCC, Rockville, MD, USA) were cultured in RPMI-1640 medium (Gibco, Paisley, UK) supplemented with 10% foetal calf serum (FCS) and 2 mM Gln at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For Gln starvation, the same medium but without Gln supplementation was used. This still contained 0.05 mM Gln derived from FCS. Cells incubated in the Gln-supplemented medium are referred to as 'Gln+ cells' and cells incubated in the Gln-reduced medium as 'Gln− cells'. If not otherwise indicated, heat shock (HS) was performed for 10 min at 44°C by re-

suspending cells in pre-warmed medium. To determine viability, the reduction of tetrazolium salt WST-1 to formazan by living cells was measured photometrically as described by the manufacturer (Roche Diagnostics, Mannheim, Germany).

Analysis of intracellular amino acids

Cells were washed in PBS and counted; 5×10^6 cells were suspended in 100 µl 3% 5-sulfosalicylic acid containing 100 µM β2-thienylalanine, and the suspension was shock-frozen and defrosted. After centrifugation, the amino acids in the supernatant were analysed by high-pressure liquid chromatography (HPLC): 1 vol of OPA-reagent (13 mg *o*-phtalaldehyde, 200 µl methanol, 800 µl 1 M potassium borate, pH=10.4, 25 µl β-mercaptoethanol and 9 ml water) was added, and 60 s later, the sample was loaded onto a 150×3-mm reversed-phase Hypersil-C_{18} HPLC column (Maisch, Ammerbuch, Germany) on a Beckmann Gold HPLC-system (Beckmann, Palo Alto, CA, USA) fitted with a JASCO FP-920 fluorometer (JASCO, Osaka, Japan). Binding to and elution from the column were performed with a programmed gradient elution using 13 mM sodium acetate, pH=6.8, in 0.28% tetrahydrofurane as buffer A and 50% acetonitrile in 1.8% tetrahydrofurane as buffer B (gradient: 0.1 min 0% B, 0.3 min increase to 17.5% B, 2.6 min 17.5% B, 0.4 min increase to 19.0% B, 4.6 min 19.0% B, 0.5 min increase to 25.0% B, 4 min 25.0% B, 1.0 min increase to 32.0% B, 0.5 min 32.0% B, 7 min increase to 43% B, 3.6 min 43% B, 1.4 min increase to 63.0% B, 1 min increase to 100% B with a flow rate of 0.485 ml/min). Fluorometer output was quantified by comparison to the internal standard (β2-thienylalanine) as well as to data obtained for a standard mixture of amino acids. The intracellular concentration in millimoles per litre was calculated using a volume of 1.09 pl per U937 cell according to our previous study [20].

Protein extraction, Western blot and enzyme immuno assay

Whole cell lysates were obtained by hypotonic treatment (10 mM Tris–HCl, pH=7.8, 1 mM EDTA, 10 mM KCl, 0.3% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml leupeptin and 1 µg/ml aprotinin) combined with 15 min sonification at 0°C. After centrifugation (10 min, 15,000×*g*, 4°C) the supernatant was stored in aliquots at −70°C. For Western blot analysis, 10-µg samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) transferred to a nitrocellulose membrane by electroblotting, and Hsp70 expression was revealed with an Hsp70-specific antibody (SPA-810 from Stressgen, Victoria, Canada). The analysis of Hsp70 expression by enzyme immuno assay (EIA) was performed according to the instructions of the manufacturer (Stressgen). Results are given as intracellular Hsp70 concentration normalised to total amount of protein.

Analysis of protein synthesis and two-dimensional gel electrophoresis

Metabolic labelling was performed in complete medium: 200 µCi/ml L-[^{35}S]methionine was added, and cells were incubated for 2 h at 37°C, then protein was collected as described above. One dimensional gel electrophoresis was carried out at standard SDS-PAGE conditions: 10 µg whole protein extract was equilibrated with SDS and reduced with β-mercaptoethanol, followed by electrophoresis (10% T, 2.67% C) at room temperature with Laemmli buffer. The gel was stained with Coomassie dye and dried before autoradiogram with ECL-filmsheets (Amersham Bioscience). For two-dimensional gel electrophoresis, equal amounts of proteins were dissolved in sample buffer (10 M urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propranesulfonate [CHAPS], 0.5% SDS, 100 mM dithiothreitol) supplemented with 2% (w/v) ampholyte, pH 7–9 (BDH Laboratories Supply, Poole, UK). To optimise solubilisation of proteins, the protein solution was saturated with urea by addition of solid urea. Isoelectric focusing of equal amounts of protein samples was performed at 15.5 kV/h in a stepwise fashion (2 h at 200 V, 3 h at 500 V, 17 h at 800 V) at an acrylamide concentration of 4% T (Gerbu, Gaiberg, Germany)/0.1% C (piperazine diacrylamide; Bio-Rad, Hercules, CA, USA) in 1.5-mm× 16-cm tube gels (Protean II xi electrophoresis system; Bio-Rad)). The gel buffer contained 0.035% Nonidet P-40, 0.1% CHAPS and 2% ampholytes (Merck, Darmstadt, Germany) (1 vol pH 3.5–10 / 1 vol pH 4–8 / 2 vol pH 5–7). Degassed 20 mM NaOH served as catholyte, and 6 mM H3PO4 served as an anolyte. For SDS-PAGE, the extruded tube gels were placed on top of 1.5-mm 12% T, 2.6% C polyacrylamide slab gels. Tube gels were overlaid with equilibration buffer (2.9% SDS, 70 mM Tris–HCl, pH 6.8, 0.001% bromphenol blue), and after 3 min, the gels were run at 15°C in electrode buffer (0.1% SDS, 25 mM Tris base, 192 mM glycine). The gels were dried before autoradiogram as described above. Comparative spot pattern analysis was accomplished with Melanie 3.0 software (GeneBio S.A., Geneva, Switzerland).

Analysis of glutaminyl-tRNA

Cells were lysed in TRIZOL (Gibco, Paisley, UK) after accurate washing in ice-cold PBS (three times). RNA was isolated according to the instructions provided by the manufacturer with the following exceptions: The RNA precipitate was washed twice in 70% EtOH to remove excess free amino acids and then dissolved in 100-µl water containing 1 µM β2-thienylalanine. tRNA-bound amino acids were then separated from tRNA under alkaline con-

ditions by adding 4 µl 2 M $NaHCO_3$/100 µl and incubation at room temperature for 1 h. After addition of 4 µl 10 mM HCl for neutralisation, samples were prepared for HPLC analysis of amino acid content as described above.

Apoptosis assay

Apoptosis was analysed with the use of an Annexin V kit (Trevigen, Gaithersburg, MD, USA) and additional propidium iodide staining according to the enclosed instruction provided by the manufacturer. Briefly, flow cytometer analysis was performed with Coulter Epics XL from Beckman Coulter (Fullerton, CA, USA).

Electrophoretic mobility shift assay

Nuclear extracts were prepared as described previously [21]. Binding reactions were performed with a pair of complementary oligonucleotides that contain four consensus heat shock elements (HSEs) when annealed (5′-CTA GAA GCT TCT AGA AGC TTC TAG-3′). The oligonucleotides were subjected to 5`phosphorylation with [γ^{32}P]ATP (6,000 Ci/mmol) prior to annealing according to a standard protocol. Binding reactions were carried out at room temperature for 30 min and contained 3 µg of nuclear extracts, 100,000 cpm of radio-labelled DNA-probe, 2 µg poly(dI-dC) (Pharmacia) and 1 µl HSF1 monoclonal antibody (clone10H8; Labvision, Fremont, CA, USA) in 50 mM NaCl, 20 mM HEPES-KOH (pH=7.9), 1 mM EDTA, 1 mM β-mercaptoethanol, 0.1% TWEEN-20 and 5% glycerol in a total volume of 25 µl. Free and bound DNA was separated on a non-denaturing 5% polyacrylamide gel (acrylamide:bisacrylamide 80:1, in 380 mM glycine, 50 mM Tris, 2.7 mM EDTA and 2.5% glycerol at pH=8.5). Formed DNA/protein complexes were visualised by autoradiography.

Northern blot

Cells were resuspended in 1 ml TRIZOL (Gibco, Paisley, UK), and total RNA was isolated as described by the manufacturer. RNA was separated in 1.5% agarose gels and transferred to a positively charged nylon membrane. Hybridization was carried out overnight at 65°C in binding buffer (500 mM Na-phosphate pH=6.8, 7% SDS) containing denatured [^{32}P]-dCTP-labelled, random primed cDNA probe covering the coding region of the *hsp70* gene (bases: 419–2411 of GenBank M59828) or β-actin (bases: 74–1195 of GenBank NM001101). Autoradiography was performed with Imaging Screen-K (Bio-Rad).

Polysome gradient

Harvesting, cell lysis (5×10^7 cells) and the preparation of sucrose step gradients were performed as described for polysome gradients [22]. After phenol-chloroform extraction and ethanol precipitation, samples were dissolved in 50 µl H_20. An aliquot of 15 µl was analysed by Northern blot as described above.

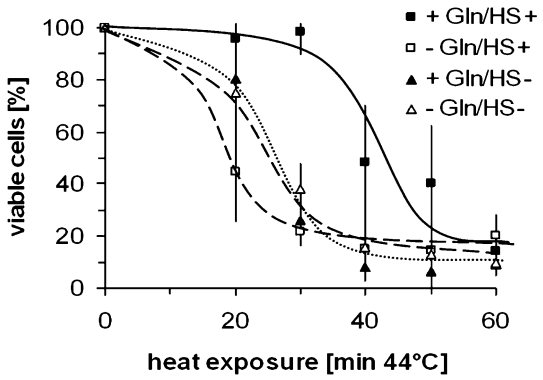

Fig. 1 Influence of Gln-starvation on HS-induced thermotolerance. Gln+ and Gln− cells were exposed to a non-lethal HS (10 min, 44°C) 6 h before thermotolerance was tested (*HS*+) or as control cells incubated at 37°C for 6 h (*HS*−) in medium with Gln (*Gln*+) or without Gln (*Gln*−). To test the thermotolerance, cells were resubmitted in medium with Gln and exposed to 44°C for up to 60 min. Twenty-four hours later, cell viability was determined. Values are the mean±SD (n=8) in percentage to control (t=0) in each group

mRNA stability analysis

mRNA synthesis was inhibited in HS-treated cells after 2 h of recovery by addition of actinomycin D (5 µg/ml). At

Table 1 Four hours of Gln starvation reduces the intracellular pool of Gln and Glu

Gln	Gln_i (µM)	Glu_i (µM)
a. Without heat shock[a]		
+	4220.2±1564.7	8106.0±580.9
−	91.7±51.5*	1966.6±518.1*
b. With heat shock[b]		
+	4311.9±1062.6	6220.2±724.7
−	158.3±85.2*	2323.5±523.7*
c. Without heat shock + DON[c]		
+	6709.4±402.3	8693.2±870.8
−	255.2±14.0*,‡	726.7±18.8*,‡
d. With heat shock + DON[d]		
+	5688.0±409.3	7527.3±115.0
−	157.6±102.0*	1675.8±618.6*

*P value<0.05; ANOVA analysis between Gln+ and Gln− cells
‡P value<0.05; ANOVA analysis between 200 µM DON and no supplementation
[a]Cells suspended in complete medium (Gln+) or Gln-deficient medium (Gln−) were incubated for 4 h at 37°C and the intracellular levels of free glutamine (Gln_i) and glutamate (Glu_i) was measured
[b]Cells were exposed to a heat shock (10 min, 44°C) followed by 4 h cultivation at 37°C
[c]Cells were suspended in the presence of 200 µM DON and cultured for 4 h at 37°C
[d]As in c, but the cells were exposed to a heat shock (10 min, 44°C) at the beginning of the treatment

time points indicated, total cellular RNA was isolated and Hsp70 mRNA was quantified by Northern blot analysis as described above. The mRNA half-life ($t_{1/2}$) was determined by plotting the natural logarithm of the fractional response (Fr) vs time: $Fr=(R_t-R_0)/(R_b-R_0)$, where R_t is the relative amount of mRNA at time point t, R_0 is the amount of mRNA detected before HS and R_b is the mRNA in the cells at the beginning of the actinomycin D chase (2 h upon HS). The slope values obtained from linear regression were used to calculate $t_{1/2}$.

Results

Stress response in Gln-starved cells

When exposed to a non-lethal environmental stress, cells activate a specific stress response, which increases their resistance against a secondary insult. In order to determine whether Gln-starvation influences this activation, we tried to increase the thermotolerance of monocytic U937 cells by a mild hyperthermic treatment in the absence of Gln in the medium. Cells were exposed to a non-lethal HS for 10 min at 44°C followed by incubation at 37°C for 6 h. We then analysed the thermotolerance of these cells by determining the lethal heat-dose. For this, cells were transferred to complete medium containing 2 mM Gln and exposed to 44°C for up to 60 min. Then they were returned to 37°C for 24 h for recovery, and cell viability was measured. Fig. 1 shows a dose–response curve describing the thermotolerance of Gln+ and Gln− cells. A heat-shock-induced increase in thermotolerance could only be observed in Gln+ cells, which indicates that the stress response is Gln-dependent in monocytic cells.

Influence of Gln starvation on Gln metabolism and protein synthesis rates

To investigate why Gln is essential for the cellular stress response, we analysed the Gln metabolism and protein synthesis in Gln-starved cells. The intracellular pool of free Gln (Gln_i) is the source for incorporation of Gln into proteins via glutaminyl tRNA as well as for the intracellular free glutamate pool (Glu_i), which in turn enters the TCA cycle to yield ATP or is used as a substrate for glutathione synthesis. Our analysis reveals that removal of extracellular Gln supply induces substantial reduction in both Gln_i and Glu_i within 4 h (Table 1a), which is not

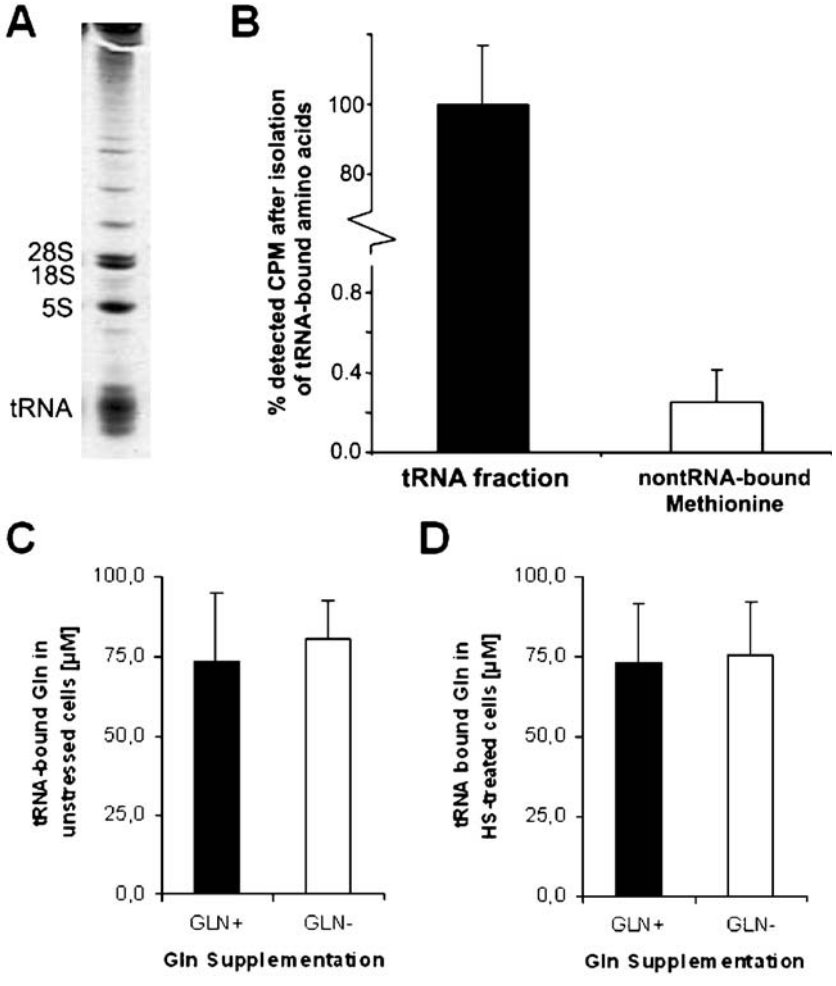

Fig. 2 Analysis of tRNA-bound Gln in Gln− cells with and without HS. **a** Sybro-green-stained polyacrylamide gel (6% T, 1% C) loaded with RNA isolated with $CHCl_3$. The tRNAs form a broad band at the *lower part* of the gel. **b** Validation of the contamination level of free amino acids in the tRNA-bound amino acid fraction. *Left column*: cells were labelled with [^{35}S]-methionine for incorporation in Met-tRNA. Then total RNA was extracted, and the radioactivity was measured. *Right column*: [^{35}S]-methionine was added immediately before RNA extraction. The radioactivity after RNA extraction represents the contamination of the extract with free amino acids. Values are mean±SD (n=3); CPMs detected in the tRNA-bound fraction is set to 100%. **c** Comparison of the level of glutaminyl-tRNA in Gln+ and Gln− cells without HS treatment, n=5, and the ANOVA analysis revealed no statistical difference. **d** As (**c**), but in HS+ cells

influenced by a mild HS at the onset of the starvation period (Table 1b). The concentration of intracellular Gln drops very fast upon withdrawal of extracellular Gln. Already within 10 min, it sinks to 2.1±0.6% of normal value in unstressed Gln− cells and to 4.0±0.6% in HS+ cells (P=0.002 and 0.004, respectively). This reduction also perturbs intracellular Glu content within 10 min in HS− cells (76.6±8.4%, P=0.011), but not in HS+ cells (89.0± 14.9%, P=0.9). We analysed the level of tRNA-bound Gln to investigate whether the protein synthesis is stalled due to lack of substrate in Gln− cells. For this, we isolated complete cellular RNA by the acidic phenol method. This method extracted substantial amounts of tRNAs (Fig. 2a) and reduced the content of free amino acids to less than 0.3% (Fig. 2b). The tRNA-bound amino acids were cleaved from the tRNA and quantitated by HPLC. The levels of tRNA-bound Gln in Gln− cells compared to Gln+ cells are shown in Fig. 2c,d. Glutaminyl-tRNA does not differentiate between Gln+ and Gln− cells both in HS− and in HS+ cells.

To investigate whether Gln starvation induces specific changes in the protein expression profile, we performed a proteomic analysis of metabolically [^{35}S]-labelled Gln+ and Gln− cells. Equal amounts of protein were separated by SDS-PAGE, and the [^{35}S]-methionine incorporation was visualised by autoradiography. As shown in Fig. 3a, heat shock induces the synthesis of different proteins (lane 3). This induction is reduced in the absence of Gln (lane 4). The most prominently changed protein was found at ≈70 kDa. For a more detailed analysis, we performed a two-dimensional gel electrophoresis. Equal amounts of proteins were separated, and the spots on the autoradiogram of the two-dimensional gels were evaluated. The intensity of each spot reflects the synthesis rate of the respective protein, and this value was set in relation to the sum of the intensities of all spots. We detected 235 protein spots, and the relative intensity of each was compared. These relative intensities of most spots were identical between Gln− and Gln+ cells, but for five protein spots (indicated in the figure: p1, p2, p3, ER-60 and Hsp70).

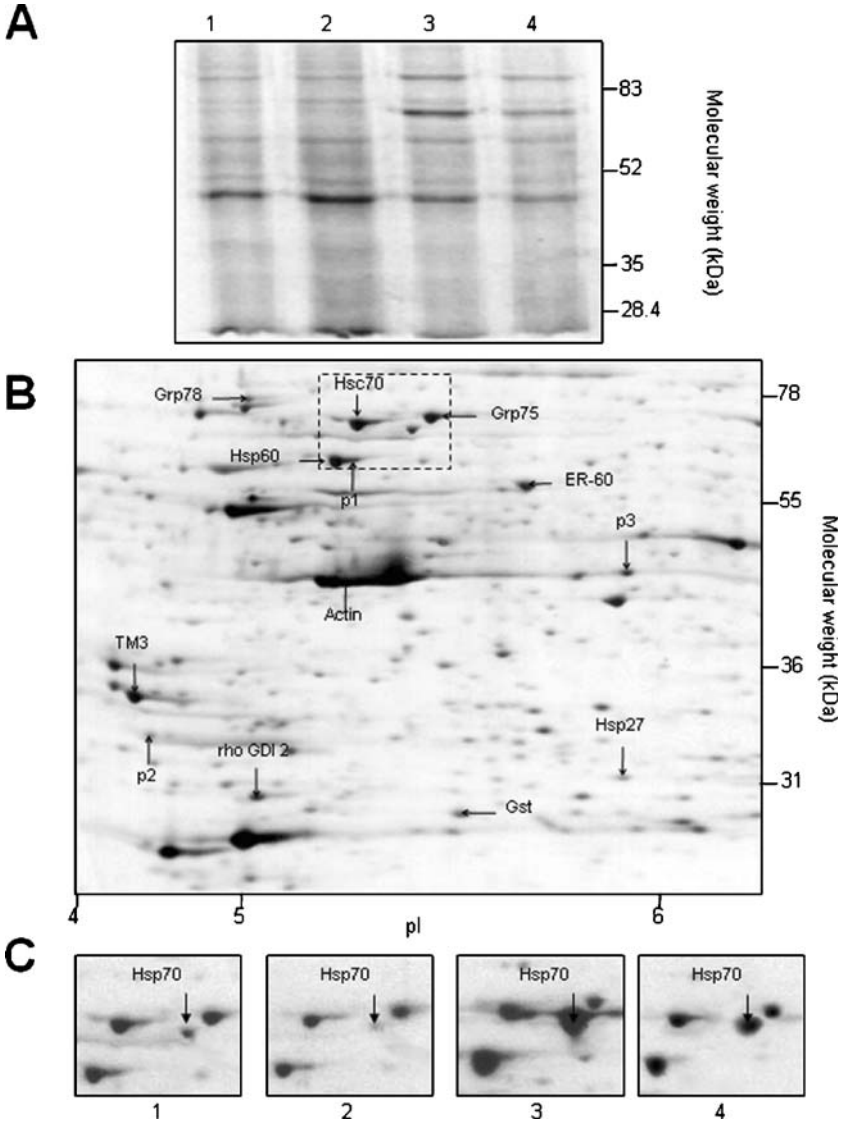

Fig. 3 Proteome analysis of [^{35}S]-methionine incorporation in Gln+ and Gln− cells. **a** Cells were cultured for 5 h in Gln+ and Gln− medium. [^{35}S]-methionine was added after the third hour. Then proteins were extracted and separated by SDS-PAGE. Samples are: *1* Gln+/HS−, *2* Gln−/HS−, *3* Gln+/HS+, *4* Gln−/HS+. **b** Resolving two-dimensional gel electrophoresis of [^{35}S]-methionine-labelled protein yielded 235 proteins, exemplified as Gln+/HS−. Indicated on the gel are: *Grp78* glucose-regulated 78-kDa protein, *actin*, *TM30* tropomyosin, *rho GDI 2* rho GDP-dissociation inhibitor 2, *ER-60* disulfide isomerase ER-60, *Hsp27* heat shock protein 27, *Gst* glutathione S-transferase, as well as three non-identified proteins, *p1*, *p2* and *p3*. The experiment was repeated once with same results, except for β-actin, which is of such abundance that reproducible electrophoresis is difficult. **c** *Enlarged area* from the gels shows the sensitivity of the heat-inducible Hsp70 to Gln starvation in the four samples, annotated as in (**a**)

Among these proteins, the cytoprotective stress protein Hsp70 was the most impaired in heat-shocked as well as in not-heat-shocked cells. These data show that the negative effect of Gln-starvation on protein synthesis rates occurs not only globally but also protein-specifically and reduces the constitutive and heat-inducible expression of Hsp70.

Stress-mediated Hsp70 induction in Gln-starved cells

Hsp70 belongs to the family of stress proteins (HSPs) and shows a high constitutive expression in monocytic cells [23]. This expression is strongly increased in response to stress factors including HS. For further investigation of the effect of Gln starvation on the stress-induced expression of Hsp70, we compared the Hsp70 induction between Gln− and Gln+ cells in response to increasing hyperthermic stress. Cells were exposed to 44°C for up to 15 min then placed to 37°C for 6 h, and Hsp70 expression was assayed. In Gln+ cells, the amount of Hsp70 increased linearly with the duration of HS (0–10 min at 44°C) and showed a maximum induction (508±20%) at 10 min. In contrast, in Gln− cells, Hsp70 induction already reached a plateau of 255±20% after 5 min. Longer HS (7.5–10 min) failed to increase Hsp70 expression further (P<0.009 between Gln+ and Gln− cells). HS at 44°C for up to 10 min did not induce apoptosis in neither Gln− nor Gln+ cells, but longer exposure to 44°C (≥15 min) resulted in substantial apoptosis (data not shown). Heat treatment of cells at different Gln concentrations revealed that the HS-mediated Hsp70 induction is clearly Gln-dependent. A minimum concentration of 0.5 mM was required to induce maximum

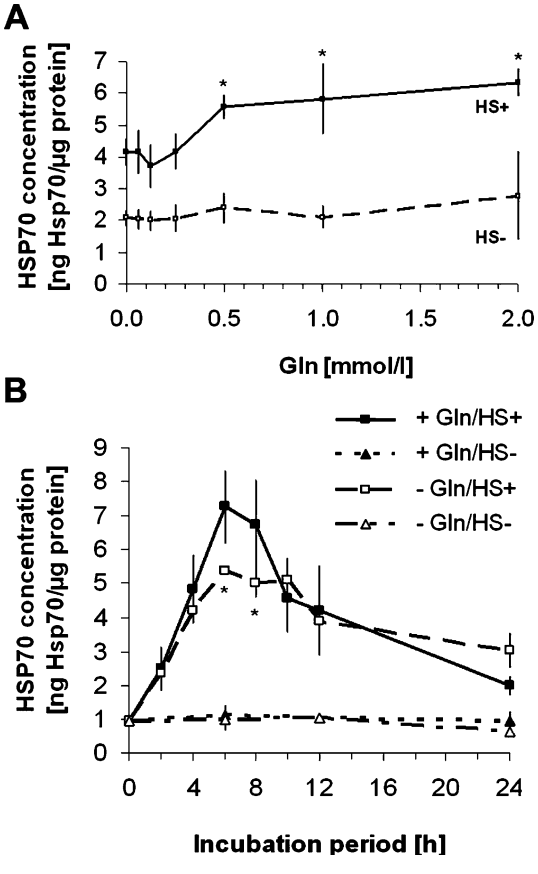

Fig. 4 HS-induced increase in Hsp70 in Gln+ and Gln− cells. **a** Relationship between Gln supplementation and Hsp70 induction by HS (10 min, 44°C). The Hsp70 concentration was analysed by ELISA 6 h after HS. Values are the mean±SD (n=4; *P<0.05 in comparison to 0 mmol/l Gln). **b** Time-course of HS-induced Hsp70 accumulation. HS was induced as described in Fig. 3a, and the Hsp70 concentration was determined by ELISA at the indicated times post HS. Values are the mean±SD (n=4; *P<0.05, ANOVA analysis between Gln+ and Gln− in the HS+ groups)

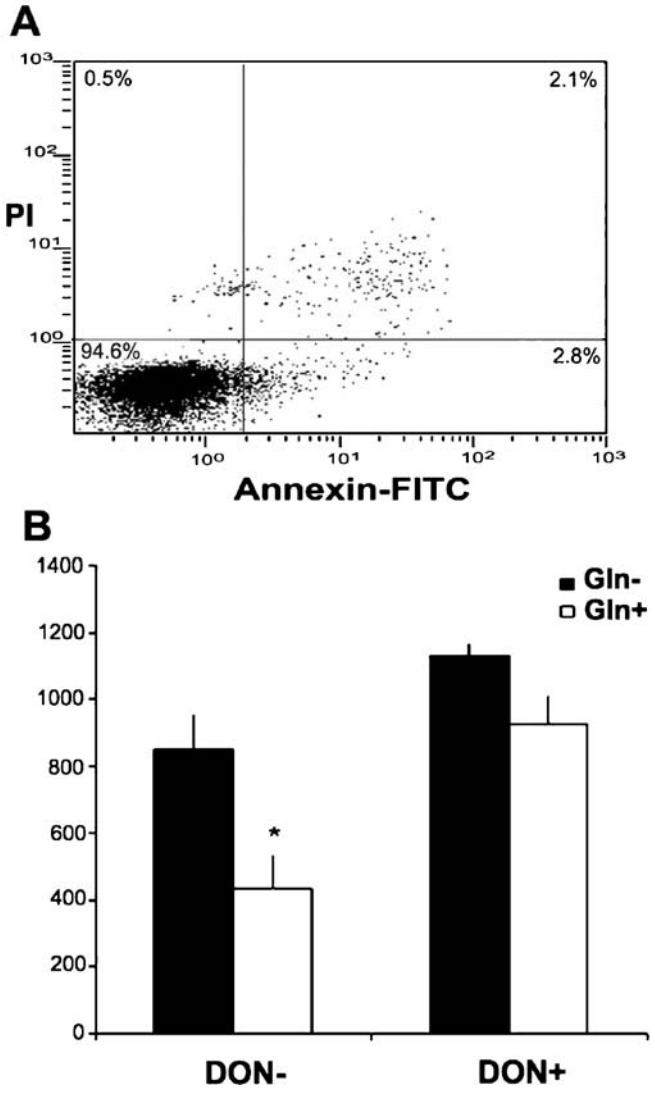

Fig. 5 Effect of glutaminase inhibitor 6-diazo-5-oxo-L-norleucine (DON) on cell survival and Hsp70 expression in HS-treated U937 cells. **a** Typical result of flow cytometer analysis of HS-treated Gln− cells cultured in the presence of 200 µM DON for 24 h. The cells were stained with Annexin-V conjugated with fluorescein isothiocyanate (FITC) (*x*-axis) and propidium iodide (*PI*; *y*-axis) to analyse apoptosis and/or necrosis. *Lower left quadrant* represents viable cells, which are negative-stained for both Annexin V and PI. *Lower right quadrant* represents apoptotic cells, which are Annexin-V-positive but PI-negative, and the *upper right quadrant* represents late apoptotic or necrotic cells. **b** Gln+ and Gln− cells were heat-treated in the presence of DON, and 6 h post HS, Hsp70 content was determined by Western blot. Values are mean±SD (n=3; *P<0.05, ANOVA analysis between Gln+ and Gln−)

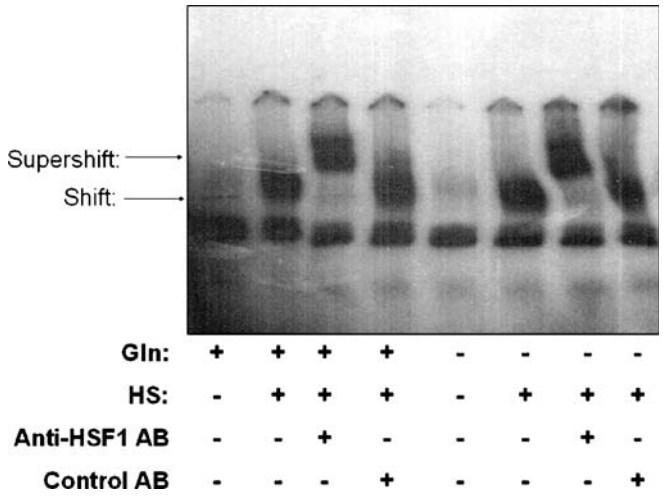

Fig. 6 EMSA autoradiogram of HSF1 binding to HSE 1 h upon HS. Gel positions of shift (HSF1–oligonucleotide complex) and supershift (binding of HSF1–oligonucleotide complex to antibody) are indicated with *arrows*. Gln supplementation, HS, as well as co-incubation with anti-HSF1 or control antibody are annotated in the figure. The experiment was repeated two times with comparable results

Hsp70 expression (Fig. 4a). This corresponds to the physiological plasma concentration.

The impaired Hsp70 induction in Gln− cells might be due to a delay rather than an actual decrease in Hsp70 synthesis. To clarify this, we determined Hsp70 expression at different time points after HS. As shown in Fig. 4b, the HS-induced accumulation of Hsp70 peaked after 6 h of recovery both in Gln+ and in Gln− cells. However, in Gln− cells, the maximum concentration of Hsp70 was significantly reduced. In addition, non-heat-treated Gln− cells exhibited a slow decrease in constitutive Hsp70 levels within 24 h. Taken together, these data clearly show an impact of Gln on constitutive as well as HS-induced expression of Hsp70.

Regulatory mechanism of Gln

Gln is not only a substrate for protein synthesis, but is also metabolised largely. Therefore, the observed down-regulation of Hsp70 expression in Gln− cells may be mediated by an altered concentration of a metabolite along the glutaminolysis pathway. To investigate this, we used 6-diazo-5-oxo-L-norleucine (DON), which is a Gln analogue that cannot be metabolised to Glu by glutaminase. Gln+ and Gln− cells were incubated in medium with 200 µmol/l DON. This concentration was sufficient to abolish the conversion of Gln into Glu in mouse macrophages [24]. However, DON is also a potent inhibitor of aminotransferases and is therefore toxic for cells at high concentrations. We found that supplementation of 200 µmol/l DON is sufficient to preserve Gln_i at the expense of Glu_i in Gln− cells (Table 1c,d) but does not induce apoptosis or necrosis within 24 h (Fig. 5a). This indicates that glutaminolysis is blocked in DON-treated U937 cells without reducing their viability. Fig. 5b shows that despite of this inhibitory effect on glutaminolysis, DON completely abolishes the negative effect of Gln− on HS-mediated Hsp70 induction. Furthermore, it does not reduce but seems to enhance the HS-mediated Hsp70 induction in Gln+ cells. These data exclude that Gln starvation reduces Hsp70 expression via a diminished glutaminolysis. In contrast, they suggest that besides Gln itself, the Gln analogue DON is also able to promote a full-scale Hsp70 induction in stressed cells.

To determine the molecular mechanism by which Gln regulates Hsp70 expression, we assessed the binding capacity of stress-inducible transcription factor HSF1 to HSEs in the promoter region of the *hsp70* gene, the formation of Hsp70 mRNA as well as its translation efficacy in Gln− cells. The binding capacity of HSF1 to HSE was measured 1 h after HS by electrophoretic mobility shift assay (EMSA) (Fig. 6). The subsequent accumulation of Hsp70 mRNA within the first 2 h after HS was detected by Northern blot (Fig. 7a). In both cases, Gln deprivation did not show any adverse effects. However, the decay of Hsp70 mRNA in the later phase of recovery was markedly enhanced in Gln−. Half-life analysis revealed a lower Hsp70 mRNA stability in Gln− cells (Fig. 7b). In addition, we

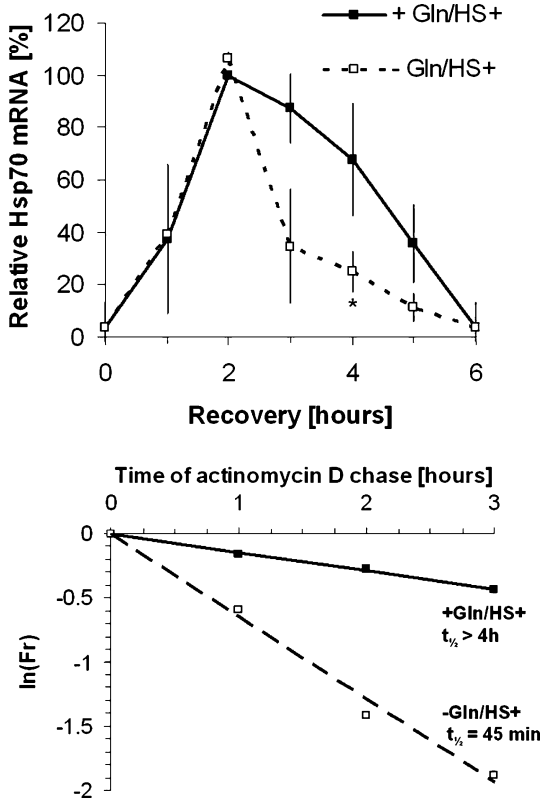

Fig. 7 Influence of Gln-starvation on HS-induced Hsp70 mRNA content. **a** Time-course of the cellular Hsp70 mRNA content relative to β-actin mRNA as analysed by Northern blot. Values are the mean ±SD in percentage to the maximum induction in Gln+ cells at 2 h post HS (n=3; *P<0.05, ANOVA). **b** Half-life analysis of Hsp70 mRNA turnover. The decay rate of Hsp70 mRNA in heat-treated cells was analysed by inhibition of transcription with actinomycin D starting 2 h after HS. Decay constants were deducted from the slopes of a linear regression analysis of ln(Fr) vs time

Table 2 Distribution of Hsp70 mRNA between non-polysomal and polysomal pool

	Recovery from HS (h)			
	2		4	
	Non-polysomes	Polysomes	Non-polysomes	Polysomes
Gln+ cells	38.4±12.8	61.6±20.2	40.8±7.4	59.2±8.8
Gln− cells	32.5±6.3	67.5±14.4	45.6±3.2	54.4±5.0
P value*	0.191	0.575	0.720	0.297

Gln− cells and Gln+cells were exposed to HS (10 min, 44°C) and allowed to recover at 37°C for either 2 or 4 h before separation of polysome- and non-polysome-bound Hsp70 mRNA. Values are mean±SD of n=3 in percent. The sum of detected mRNA (polysome-bound + non-polysome fraction) is 100%
*P value was calculated by ANOVA

investigated the influence of Gln starvation on the initiation of translation. We quantified the functional state of Hsp70 mRNA based on its distribution on polysomes illustrated in Table 2. We found that the portion of translationally active Hsp70 mRNA was not dependent on Gln. These results indicate that the reduced expression of Hsp70 in Gln− cells is due to a faster decay of Hsp70 mRNA after heat treatment, whereas the transcriptional control and the translational control remain unaffected.

Discussion

The present study shows that a reduced Gln supply of human monocytic U937 cells affects intracellular Gln metabolism and attenuates the constitutive as well as the stress-induced Hsp70 expression. In addition, Gln depletion inhibits heat-shock-induced thermotolerance, while it has no effect on thermotolerance of non-heated cells.

Gln catabolism is initiated by the deamidation of the molecule to form Glu, which can be further metabolised to CO_2, ammonia and alanine. The immediate drop of intracellular Gln and Glu content was observed after withdrawal of extracellular Gln points to a high-Gln turnover in human monocytic cells. Similar data were obtained for other Gln-consuming cell types [25, 26]. Both Gln and Glu can be utilised for the formation of Gln-tRNA^{Gln} in eukaryotic cells [27]. In our study, the level of tRNA-bound Gln remained unaffected by Gln starvation. Thus, we propose that the previously reported effect of Gln starvation on the protein synthesis in U937 cells is not due to simple limitation of tRNA substrate for protein synthesis. However, our data do not elucidate whether Gln starvation increases the amount of uncharged tRNA^{Gln}. Under physiological conditions, however, i.e. with adequate amino acid supply, all available tRNA molecules are charged with the respective amino acid [28]. Since Gln-depleted cells show no reduction in the level of Gln-tRNA^{Gln}, we consider an increase in uncharged tRNA^{Gln} as unlikely. There is, however, an example where uncharged tRNA was caused by a limited supply of amino acids and reduced the global protein synthesis rates in perfused rat liver cells [29]. This effect was associated with enhanced phosphorylation of the eukaryotic initiation factor 2-alpha (eIF-2α), which converts it to an inhibitor of the eukaryotic initiation factor 2B (eIF2B), finally leading to the shut down of translational initiation. Although a decrease in the overall protein synthesis was observed in Gln-starved U937 cells [16], the SDS-PAGE analysis that we present herein reveals that the majority of proteins are unaffected. Therefore it seems unlikely that unloaded tRNA^{Gln} accumulates in Gln− cells. Comparably, in serum-starved Chinese hamster ovary (CHO).K1 cells, complete withdrawal of all amino acids for up to 60 min does not enhance eIF-2α phosphorylation [32]. Furthermore, so far, only GCN4 in *Saccharomyces cerevisiae* has been identified to overcome the inhibition of translational initiation by phosphorylated eIF-2α [30]. Since inhibition of eIF2B through amino acid deprivation can also occur through a yet unknown mechanism that is independent of eIF-2α phosphorylation [31], we cannot completely exclude that eIF2B activity is affected by Gln-starvation in U937 cells. Yet a separate mechanism withdrawal of amino acids from the nutrient medium is known to cause inactivation of the mammalian target of rapamycin (mTOR), which in turn prevents phosphorylation of eIF-4E-binding protein and reduces the activity of p70-S6K, which finally inhibits the initiation of translation [32, 33]. However, in our cellular system we found no influence of Gln starvation on the initiation of translation of the Hsp70 or β-actin mRNA. This is in accordance with observations in CHO-IR cells, where the ability of each single amino acid to regulate p70-S6K activity was analysed [34]: Depletion of Gln showed only a minor effect.

Here we show that Gln starvation for 5 h is already associated with specific alterations of the monocytic proteome: the relative synthesis rate was reduced for four proteins (p1, p2, ER-60 and Hsp70) and increased for one protein (p3) as demonstrated by two-dimensional gel electrophoresis. Similarly, in a recent study on TSE-human breast cancer cells, specific effects of Gln starvation on angiogenic and mitogenic factors were described [35]. Gln depletion for 8 h induced an increase or decrease in particular mRNA species, including IL-8, VEGF and GAPDH. Unfortunately, Hsp70 and ER-60 were not investigated in this study. However, the analysis of TSE cells and our data clearly show that Gln starvation induces a selective cellular response rather than an overall metabolic shut-down. In the present study, a strong reduction in Hsp70 synthesis rate was observed in Gln− cells, which resulted in a decrease in the constitutive Hsp70 protein content within 24 h. Furthermore, the stress-induced accumulation of Hsp70 in response to HS was also found to be attenuated in Gln− cells. At physiological Gln levels, heat shock led to a strong increase in Hsp70 and Hsp60 expression in comparison to the non-inducible Hsc70 reflecting a classical heat shock response (see Fig. 3c). In the absence of Gln, however, the increase in ratio between Hsp70 and Hsc70 was less pronounced, suggesting an impaired heat shock response under these conditions.

Induction of Hsp70 by HS is mediated by the activation of the stress-inducible transcription factor HSF1, followed by an increased transcription rate of the *hsp70* gene and accumulation of Hsp70 protein. In addition, the stability of Hsp70 mRNA is increased, allowing for a rapid accumulation [36, 37]. In the present study, the binding capacity of HSF1 to HSE elements and the subsequent rapid increase in Hsp70 mRNA levels were both unaffected by Gln starvation. However, a half-life analysis of Hsp70 mRNA showed a lower stability in Gln− cells as compared to Gln+ cells following HS. Stress-induced stabilisation of Hsp70 mRNA depends on multiple factors such as PKR [38], ubiquitin-mediated proteolysis [39] and protein synthesis [40]. The half-life of Hsp70 mRNA is reported to be 54 min in unstressed 293 embryonic kidney cells and to increase dramatically in response to HS [37]. Our mRNA turnover studies showed that HS of Gln+ cells resulted in an Hsp70 mRNA half-life ($t_{1/2}$) of more than 4 h, whereas it was only 45 min in Gln− cells. This suggests that Gln is essential for efficient 'thermal stabilisation' of Hsp70 mRNA.

In our experiments, Gln-starved cells show only a partial reduction in Hsp70 expression due to decreased Hsp70 mRNA levels. Nevertheless, Gln-starved cells do not develop thermotolerance 6 h after mild HS. Thus, the resistance of U937 cells to hyperthermia does not linearly correlate with Hsp70 expression. The central role of Hsp70 in thermotolerance has been shown in numerous studies for various cell types and is well established. Therefore, it is likely that the decrease in Hsp70 is responsible for the reduction in thermotolerance in Gln− cells. The involvement of Hsp70 in the cellular thermotolerance of U937 cells was verified by the workgroup of Michael Sherman [41]. Inhibition of JNK upon increased levels of Hsp70 appeared to be the major mechanism of acquired thermotolerance. However, the effects of Gln starvation are not limited to Hsp70 (see Fig. 3), and we cannot exclude that an additional Gln-dependent factor may be involved in the reduction in thermotolerance.

Treatment of cells with DON, a Gln analogue and inhibitor of glutaminase, has no effect on the Gln turnover in Gln+ cells, but significantly enhances the intracellular pool of free Gln in Gln− cells at the expense of Glu formation. Analysis of the heat-induced Hsp70 expression in DON-supplemented cells showed that DON can fully overcome the detrimental effect of Gln starvation. This suggests that either Gln itself or a non-glutaminase-dependent metabolite exerts the effect on Hsp70 expression. In IEC-18 cells, a similar link between the impact of Gln and DON on Hsp70 induction was reported by Wischmeyer et al. [42]. Cai and co-workers revealed more than a decade ago that Gln is the 'nutrient' for a normal constitutive Hsp70 expression [43]. Hsp70 expression decreased in CHO cells when cultured for 40 h or longer in complete medium. Refeeding with fresh Gln restored the constitutive expression levels. The present study identifies the molecular mechanism of this effect. There are other reports describing that the addition of Gln leads to an increase in Hsp70 expression in different mammalian cell types [42, 44]. However, in these studies, Gln was added to the complete cell culture medium, and its concentration was increased from 2 up to 5 or even 20 mM. The effect of Gln starvation was not investigated, and it is therefore difficult to compare the results of our study with these reports. Furthermore, the present study is the first comprehensive investigation of the functional mechanism involved in Gln-dependent Hsp70 control.

Here we show that Gln is indeed crucial for HS-induced thermotolerance in U937 cells. Similarly, Gln was found to protect against thermal killing, freeze-thaw damage, radiation killing and chromosomal damage by doxorubicin in U937 cells and mouse B lymphocyte hybridoma cells [1]. The exact mechanisms of these protective effects of Gln are unknown. Interestingly, high expression of Hsp70 is also known to be cytoprotective against these stress agents [45, 46]. The beneficial effect of Gln on the constitutive Hsp70 expression, as described in this study, indicates that the cytoprotective effects of Gln described above might be mediated by Hsp70. In a previous study, we observed a reduction in the constitutive Hsp70 expression in neutrophils of critically ill patients with low plasma Gln levels [47]. Furthermore, Gln supplementation to these patients was found to improve their clinical outcome. Comparably, induction of HSPs in rats by hyperthermia enhanced survival under sepsis [48] and protected them from the lethal effect of endotoxin shock [49]. The dependency of Hsp70 expression on the availability of Gln, as described in this study, may contribute to this effect. Further evidence for the physiological importance of Hsp70 control by Gln comes from our recently published study [50]. We could show that Gln depletion of primary monocytes leads (similar to the U937 cell line) to reduced thermotolerance as well as to reduced Hsp70 expression. However, it has to be considered that the present experiments investigating the underlying molecular regulation mechanisms were conducted with the cell line and that the situation may be different in vivo. Therefore, additional studies are needed to clarify the role of Hsp70 in the beneficial effect of Gln supplementation for critically ill patients.

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